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DISTRIBUTION OF *NEOTYPHODIUM LOLII*-ENDOPHYTE METABOLIC ACTIVITY IN PERENNIAL RYEGRASS (*LOLIUM PERENNE*, L.) AND ITS IMPLICATIONS FOR ALKALOID DISTRIBUTION AND PHOTOSYNTHESIS

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Martin Jürgen Spiering

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Abstract

Neotyphodium lolii is a fungal endophyte of perennial ryegrass (*Lolium perenne*). In this symbiosis, alkaloids are produced that significantly impact on the performance of farmed animals. Little was previously known about the physiological conditions for the endophyte in the plant leading to their production. A strain of *N. lolii,* previously transformed with the ß-D-glucuronidase (GUS) gene of *E. coli* under the control of a constitutive fungal promoter, was used for investigations into the *in planta* metabolic activity of the endophyte; distribution of the alkaloids ergovaline, peramine, and lolitrem B; and photosynthesis.

In vitro studies with this transformed *N. lolii* strain demonstrated the utility of constitutive GUS expression for assessing the metabolic state of the endophyte. By using constitutive GUS expression and a method for quantitation of the *in planta* biomass of the endophyte, the endophyte metabolic state (EMS) in the grass plant was determined. The EMS was high and uniform in plant tissues and genotypes differing in endophyte concentration, indicating that proliferation of the endophyte in the plant is not controlled *via* the EMS.

Ergovaline, peramine, and lolitrem B exhibited each a characteristic within-tiller distribution maintained across different plant genotypes. None of the alkaloids was distributed in exact proportion to the distribution of metabolically active endophyte mycelium. Differences in the accumulation of the alkaloids per mycelium were observed between plant tissues and plant genotypes, suggesting differential rates of synthesis and/or degradation of the alkaloids in the mycelium and translocation within the grass tiller.

Rates of net photosynthesis at high light intensities were lower in plants infected by *N. lolii*, indicating for an effect on photosynthetic capacity. However this effect was plant-age dependent. Plant growth was not strongly affected by the endophyte, but infected plants had consistently lower leaf elongation rates. The changes in leaf elongation and photosynthetic capacity in infected plants might signal for an effect of *N. lolii* on the nitrogen metabolism of its host.

The experiments concerning the *in planta* EMS, alkaloid distribution, plant growth, and photosynthesis were conducted in a controlled environment the establishment of which was part of this study. In addition, for the detailed mapping of ergovaline within the grass tiller a method for quantitative extraction of this alkaloid was developed and optimised.

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Table of Contents

Abstract............ii

Acknowledgements............iv

Table of Contents............vi

List of Tables............xii

List of Figures............xiv

Related Publications............xvii

1.0 INTRODUCTION............1

1.1 The Fungi - An Important Group of Organisms............1

1.2 Fungal Endophytes of Grasses............2

1.2.1 Endophyte Growth in the Grass Plant and Plant-Endophyte Interactions at the Cellular Level............4

1.2.2 Effects of Endophyte-Infection on Plant Growth and Photosynthesis............7

1.2.3 The Production of Secondary Metabolites by Grass Endophytes and Their Effects on Animal Health and Plant Persistence............8

1.3 Biosynthesis, Analysis, *In Planta* Distribution, and Physiology of Alkaloid Production............12

1.3.1 The Biosynthesis of Ergovaline, Lolitrem B and Peramine............12

1.3.2 Analytical Methods for the Quantitative Determination of the Alkaloids............15

1.3.3 Alkaloid Distribution within the Plant............18

1.3.4 Endophyte Concentration and Alkaloid Production as Influenced by the Environment, and Putative Roles of Physiological Parameters for Alkaloid Synthesis............19

1.4 The GUS Reporter Gene: A Tool for Assessing the Metabolic State of Endophyte Mycelium............21

1.5 Aims and Objectives of this Study............23

2.0 MATERIALS AND METHODS............26

2.1 Fungal Strains and Plant Lines............26

2.2 Media............26

2.2.1 Potato Dextrose Agar and Potato Dextrose Broth (PDA and PDB)............26

2.2.2 Yeast Extract Glucose Medium (YEG Medium) 26
2.3 Buffers and Solutions 26
2.3.1 GUS Extraction Buffer 28
2.3.2 GUS Assay Buffer 28
2.3.3 GUS Stop Buffer 28
2.3.4 GUS Calibration Solution 28
2.3.5 Saline Solution 28
2.3.6 Lacto-Glycerol Solution 28
2.3.7 Decolorisation Solution 29
2.3.8 Bleaching Solution 29
2.3.9 Mounting Solution 29
2.3.10 Aniline Blue Solution 29
2.3.11 10x Fungal Nuclear Staining Solution 29
2.3.12 10x Fungal Septa Staining Solution 29
2.3.13 Chlorine Bleach 29
2.4 Growth and Maintenance of Fungal Cultures and Plants 30
2.4.1 Fungal Isolation from Plant Tissues 30
2.4.2 Preparation of Inoculum and Growth in Liquid Cultures 30
2.4.3 Determination of Fungal Biomass as Dry Weight in Liquid Cultures 31
2.4.4 Plant Culture and Maintenance of Symbioses 32
2.4.5 Inoculation of Plants with Endophyte Mycelium 35
2.4.6 Generating of Endophyte-Free Clonal Plants by Fungicide Treatment 35
2.5 Harvesting of Plants, Isolation of Endophyte and Plant Tissue Extractions ... 36
2.5.1 Plant and Tiller Dissection for Analyses of GUS Activity and Alkaloids 36
2.5.1.1 Plant Dissection 36
2.5.1.2 Tiller Dissection 36
2.5.2 Simultaneous Assessment of GUS Activity and Alkaloids in Plant Tissues . 38
2.5.3 Extraction of Endophyte-Linked GUS Activity from Fungal Mycelium and Plant Tissue 38
2.6 ß-Glucuronidase (GUS) Assays 39
2.6.1 Quantitative Extractive Assay 39
2.6.2 Semi-quantitative Microtitre Plate GUS Assay 41
2.6.3 Qualitative Microtitre Plate GUS Assay 42

2.7 Determination of Hyphal Biomass Concentration in Plant Tissues 42
2.8 Analyses of Alkaloids in Plant Tissues 46
2.8.1 Extraction and Quantitation of Peramine 46
2.8.1.1 Extraction with Isopropanol-Lactic Acid 46
2.8.1.2 Separation and Quantitation of Peramine by HPLC 47
2.8.2 Extraction and Quantitation of Lolitrem B 48
2.8.2.1 Extraction with Chloroform-Methanol 48
2.8.2.2 Extraction with 1,2-Dichloroethane, 10% Methanol 48
2.8.2.3 Separation and Quantitation of Lolitrem B 49
2.8.3 Extraction and Quantitation of Ergovaline 49
2.8.3.1 Chloroform-Methanol-Ammonia Extraction 49
2.8.3.2 Acetic Acid Extraction 50
2.8.3.3 Isopropanol-Lactic Acid Extraction 50
2.8.3.4 Separation and Quantitation of Ergovaline 50
2.9 Fluorescent Microscopy of Nuclei and Septa in Fungal Mycelium 51
2.10 Observations during Plant Growth 52
2.10.1 Tiller Number and Tillering Rate 52
2.10.2 Leaf Extension Rates 52
2.11 Assessment of Leaf Net-Photosynthesis 52
2.11.1 Principle of the Open Infrared Gas Analysis System 52
2.11.2 Configuration of the Analysis System for Measurements of Leaf Net Photosynthesis 53
2.11.3 Measurements of Leaf Net-Photosynthesis 54
2.11.3.1 Determination of Light Response 54
2.11.3.2 Determination of Temperature Response 55
3.0 RESULTS 56
3.1 Method Development 56
3.1.1 Development of a Method for Quantitative Extraction of GUS Activity from Fungal Mycelium 57

3.1.2 Development of a Selection System for Obtaining Genetically Homogeneous Endophyte Isolates from Non-Sporulating Mycelium 59
3.1.3 Establishment of a Controlled Environment for Studies of Endophyte-Plant Interactions 63
3.1.3.1 Plant Growth in the Growth Cabinet 63
3.1.3.2 Assessment of the Impact of Fungicide and Insecticide Application on *In Planta* GUS Activity 64
3.1.4 Development and Optimisation of a Method for Extraction of Ergovaline from Small Quantities of Endophyte-Infected Plant Material 66
3.1.4.1 Sample Material for Method Development 67
3.1.4.2 Extraction with Solvents Containing Isopropanol and Lactic Acid 70
3.1.4.3 Extraction and Recovery of Ergovaline and Internal Standard with an Acetic Acid Solvent 74
3.1.4.4 Time Course of Ergovaline Extraction and of Recovery of Internal Standard 76
3.1.4.5 The Influence of Sample Dry Weight in Extraction of Ergovaline and Recovery of Internal Standard 78
3.1.4.6 Summary and Conclusions 80
3.1.5 Comparison of the Quantitative Extractive GUS Assay with a Semi-Quantitative Microtitre Plate Assay 81
3.1.6 Determination of *In Planta*-Endophyte Mycelial Concentration by Microscopy 83
3.1.7 Assessment of Fresh Weight-Dry Weight Coefficients for Plant Tissues 86
3.1.8 Positive Selection for GUS Reporter-Gene Activity in Endophyte Mycelium - Segregation of GUS-Expressing and Non-Expressing Mycelium into Grass Tillers 88
3.1.9 Estimation of Inhibition of GUS Activity in Plant Tissue Extracts 91
3.2 Endophyte Metabolic Activity and Alkaloid Synthesis in *N. lolii* Endophyte-Infected Perennial Ryegrass 93
3.2.1 Assessment of the Correlation of GUS Activity with Endophyte Metabolic Activity in Fungal Transformants 93

3.2.1.1 GUS Activity in the Endophyte Correlates with Active Growth of Mycelium 93
3.2.1.2 Determination of the Maximum GUS Activity Level in Metabolically Active Mycelium........ 97
3.2.1.3 GUS Activity Declines in the Presence of an Inhibitor of Endophyte Metabolism 99
3.2.1.4 Summary and Conclusions........ 101
3.2.2 Selection of Different Plant Genotypes Infected by a GUS-Transformed Endophyte Strain 102
3.2.3 The Endophyte Metabolic State is Uniformly High in Different Plant Tissues and Plant Genotypes........ 105
3.2.4 *In Planta* Alkaloid Distributions and Their Relation to Endophyte Metabolic Activity – None of the Alkaloids is distributed in Exact Proportion to Metabolically Active Endophyte Mycelium........ 111
3.2.4.1 Within-Tiller Distribution of Endophyte Metabolic Activity in Three Plant Genotypes 112
3.2.4.2 Levels of Endophyte Metabolic Activity in Leaf Meristematic Regions Provide Evidence for Synchronous Growth of Endophyte and Plant........ 114
3.2.4.3 Distribution of the Alkaloid Ergovaline in Plant Tissues, and Its Relationship to Endophyte Metabolic Activity........ 116
3.2.4.4 Distribution of the Alkaloid Peramine in Plant Tissues, and Its Relationship to Endophyte Metabolic Activity........ 120
3.2.4.5 Distribution of the Alkaloid Lolitrem B in Plant Tissues and Its Relationship to Endophyte Metabolic Activity........ 123
3.2.4.6 Levels of the Alkaloid Lolitrem B are Affected by Plant Age........ 126
3.2.4.7 Summary and Conclusions........ 128
3.3 Effects of Endophyte-Infection on Plant Growth and Photosynthesis... 129
3.3.1 Some Effects of Endophyte-Infection on Plant Growth........ 129
3.3.2 Leaf-Net Photosynthesis in Response to Light Intensity and Temperature in Endophyte-Infected and Endophyte-Free Plants........ 132
3.3.2.1 Endophyte-Infection Reduces Net Photosynthesis Under High Light Intensities and Moderate Leaf-Temperature in Young, Actively Growing Plants........ 132
3.3.2.2 Net Photosynthesis Assessed at Different Leaf-Temperatures is not affected by Endophyte-Infection in Older Plants........ 135

3.3.3 Summary and Conclusions 138

4.0 DISCUSSION 139

4.1 GUS Reporter Gene Expression in Culture – Constitutive Expression Indicates for the Metabolic State of Endophyte Mycelium 142

4.2 Plant Growth in a Controlled Environment – A Necessary Pre-Requisite for Studying Alkaloid Production in Endophyte-Grass Associations 144

4.3 Endophyte Distribution and Its Metabolic State *in Planta* 146

4.3.1 Evidence that the Endophyte remains in a Highly Active Metabolic State throughout the Plant 146

4.3.2 Endophyte Growth and Proliferation in the Plant is not Controlled *via* Regulation of the EMS 149

4.3.3 The *In Planta* Distribution of the Endophyte Suggests Plant-Regulated Proliferation of Mycelium 154

4.5 Alkaloid Accumulation and Its Relationship to Endophyte Metabolic Activity 159

4.5.1 An Optimised Extraction Method for the Alkaloid Ergovaline Allows for Quantitation from Small Sample Quantities 159

4.5.2 Each Alkaloid Exhibits a Characteristic Distribution in Grass Tillers 162

4.5.3 The *in Planta* Concentrations of the Alkaloids are influenced by Plant Genotype 167

4.6 Some Evidence for Defined Segregation of GUS-expressing and Non-expressing Hyphae into Developing Tillers 170

4.7 Photosynthesis and Plant Growth in Perennial Ryegrass are affected by Infection with *N. lolii* 176

4.7.1 Rates of Leaf Photosynthesis and Elongation could indicate for an Effect of the Endophyte on the Nitrogen Metabolism of Its Host 176

4.7.2 Altered Regulation of Nitrogen Allocation in Endophyte-Infected Plants may improve Plant Persistence 180

5.0 SUMMARY AND CONCLUSIONS 182

APPENDIX 185

REFERENCES 189

List of Tables

page

Table 1 Fungal strains and plant genotypes used in this study 27

Table 2 GUS activity concentration in fungicide-treated plants and untreated controls 65

Table 3 Ergovaline concentrations in pseudostem fractions of four clonal endophyte-infected plants extracted with chloroform-methanol-ammonia 69

Table 4 Estimated ergovaline concentrations and peak area of internal standard and ergovaline after extraction with isopropanol and lactic acid 71

Table 5 Estimated ergovaline concentrations and peak areas of ergovaline and internal standard after extraction with five concentrations of aqueous isopropanol, 1% lactic acid, and recoveries of ergovaline and internal standard after re-extraction overnight 73

Table 6 Estimated concentrations of ergovaline and peak areas of internal standard from plant tissue extractions with aqueous acetic acid; isopropanol, and peak areas in re-extractions with the same solvents 75

Table 7 Estimation of ergovaline concentrations from samples varying in dry weight.... 79

Table 8 Hyphal diameters of the endophyte strain Lp 19 in leaf sheaths and leaf blades of two plant genotypes 85

Table 9 GUS activity concentration in exponentially growing endophyte mycelium grown in serial liquid cultures 98

Table 10 Averages for endophyte biovolume concentration, GUS activity concentration and endophyte metabolic state in the plant genotypes Nui D, UIII, and UIV 107

Table 11 Averages for endophyte biovolume concentration, GUS activity concentration and endophyte metabolic state in leaf blades of Nui UIII and UIV 107

Table 12 Averages for endophyte biovolume concentration, GUS activity concentration and endophyte metabolic state in different-aged leaves of Nui UIII 107

Table 13 The endophyte metabolic state in three different leaf tissues calculated with endophyte biovolume and with estimated endophyte biomass 108

Table 14 Ratios of endophyte metabolic activity concentration between blade and sheath in meristematic and mature leaves 115

Table 15 Means of endophyte metabolic activity concentration, ergovaline, peramine, lolitrem B, and of ratios alkaloid : endophyte metabolic activity for each Nui D, UIII, and UIV 117

Table 16 Concentration of the alkaloid lolitrem B in leaf tissues of plants varying in age 127

Table 17 Leaf extension rates of plants infected by the endophyte strain Lp 19 and uninfected 130

Table 18 Rank numbers for the three plant genotypes used in this study, depending on the concentration of endophyte metabolic activity, each of the alkaloids, and ratios alkaloid to endophyte metabolic activity 169

List of Figures

page

Figure 1 Structures of the three major alkaloids produced in the symbiosis of *Lolium perenne* with *Neotyphodium lolii* ... 10
Figure 2 Putative biosynthetic pathways of the alkaloids lolitrem B and ergovaline ... 13
Figure 3 Grass plants grown and maintained in an environmentally controlled plant growth chamber ... 34
Figure 4 Dissection scheme for a grass tiller ... 37
Figure 5 Light microscopy of a cross-section through a leaf section of an endophyte-infected plant ... 44
Figure 6 GUS activity released from endophyte mycelium *versus* inverse number of sonication cycles applied ... 58
Figure 7 Scenarios of nuclear segregation after a mutation had occurred, for two known types of nuclear and cell division ... 60
Figure 8 Fluorescent microscopy of mycelium of the endophyte strain Lp19 grown in liquid culture ... 61
Figure 9 Time course of extraction of ergovaline and recovery of internal standard from grass tissue extracts ... 77
Figure 10 Comparison of the extractive method with the microtitre assay method for determination of GUS activity ... 82
Figure 11 The relationship of fresh weight to dry weight in three different leaf tissues ... 87
Figure 12 GUS expression in two endophyte-infected tillers, grown from one plant ... 89
Figure 13 Maintenance of the GUS phenotype in endophyte mycelium ... 89
Figure 14 GUS activity in assays with different dry weight amounts of extracted plant tissue ... 92

Figure 15 GUS activity concentrations in mycelium and mycelial concentration per culture volume of the GUS-transformant KS1 during growth in YEG medium ... 95

Figure 16 GUS activity concentration in mycelium and mycelial concentration per culture volume of the GUS-transformant KS1 in PDB medium ... 96

Figure 17 GUS activity concentration in endophyte mycelium in PDB and PDB + 10 mM KCN ... 100

Figure 18 GUS activity (endophyte metabolic activity) concentration and concentrations of the three alkaloids ergovaline, peramine, and lolitrem B in 5 (4) genotypes of the three cultivars KLM, Nui, and Greenstone ... 104

Figure 19 GUS activity (endophyte metabolic activity) concentration in 19 sections of a tiller of the three plant genotypes Nui D, UIII, and UIV infected by the strain Lp19 (GUS-transformant KS1) ... 113

Figure 20 Distribution of the alkaloid ergovaline within a tiller of the three plant genotypes Nui D, UIII, and UIV infected by the strain Lp19 (GUS-transformant KS1) ... 118

Figure 21 Ergovaline-GUS ratios in 19 stem and leaf sections of a tiller of the plant genotypes Nui D, UIII, and UIV infected by Lp19 (GUS-transformant KS1) ... 119

Figure 22 Distribution of the alkaloid peramine within a tiller of the three plant genotypes Nui D, UIII, and UIV infected by the strain Lp19 (GUS-transformant KS1) ... 121

Figure 23 Peramine-endophyte metabolic activity ratios in 19 stem and leaf sections of a tiller of the plant genotypes Nui D, UIII, and UIV infected by Lp19 (GUS-transformant KS1) ... 122

Figure 24 Distribution of the alkaloid lolitrem B within a tiller of the three plant genotypes Nui D, UIII, and UIV infected by the strain Lp19 (GUS-transformant KS1) ... 124

Figure 25 Lolitrem B-endophyte metabolic activity ratios in 19 stem and leaf sections of a tiller of the plant genotypes Nui D, UIII, and UIV infected by Lp19 (GUS-transformant KS1) ... 125

Figure 26 Growth assessed as number of tillers (per plant) of plants infected by the endophyte strain Lp 19 and endophyte-free controls ... 131

Figure 27 Net photosynthesis in endophyte-infected and endophyte-free perennial ryegrass leaves in response to light intensity ... 134

Figure 28 Net photosynthesis in endophyte-infected and endophyte-free plants of the genotypes Nui D and Nui UIV in response to leaf temperature ... 136

Figure 29 Model outlining a hypothetical mechanism by which regulated colonisation of tillers by clonal hyphae could occur ... 175

Related Publications

Some of the material presented in this thesis has been published:

Schmid, J., Spiering, M. J. & Christensen, M. J. (2000). Metabolic activity, Distribution, and Propagation of Grass Endophytes *in Planta*: Investigations Using the GUS Reporter System. In *Microbial Endophytes* (White, J. F. W., Jr. & Bacon, C. W., eds.), pp. 295-322. Marcel Dekker, New York.

1.0 INTRODUCTION

1.1 THE FUNGI - AN IMPORTANT GROUP OF ORGANISMS

The fungi are a highly diverse group of heterotrophic organisms that are distinct from plants as well as animals (Jensen & Salisbury, 1972; Raven *et al*., 1992). Many species of the fungi have been extremely successful in colonising different niches and environments often intolerable for other organisms (Dix & Webster, 1995). It has been estimated that there are as many as 1.5 million species in this group, ranking them the second largest group of eukaryotic organisms after the insects (Hawksworth, 1991). Thus, the fungi probably surpass the animal kingdom and vascular plants in species diversity (Rayner, 1992). As saprotrophs, parasites, and mutualistic symbionts the fungi are profoundly involved in the regulation of energy and nutrient fluxes in natural ecosystems (Dix & Webster, 1995; Rayner, 1992). As symbionts of algae and cyanobacteria they form mutualistic associations collectively known as lichens, and these can colonise environments that are characterised by extreme differences in temperature and humidity. The ability of lichens to pioneer these adverse environments, which are otherwise inaccessible to other living organisms, means that lichens possess a pivotal role in the initiation of biological succession (Raven *et al.*, 1992; Rayner, 1992). Another mutualistic plant-fungus symbiosis, the mycorrhiza, or fungal infection of plant roots has been implicated in the successful colonisation of the land by early vascular plants (Dix & Webster, 1995). On the other hand, fungi are considered the single most important cause of plant diseases (Raven *et al.*, 1992), and are also pathogenic to animals and other fungi. Hence, they regulate the population dynamics of organisms within many ecosystems (Rayner, 1992).

A unique plant-fungus relationship is the association of endophytic fungi with plants. These symbiotic relationships are characterised by symptomless fungal growth in aerial plant tissues of an extremely wide variety of plants, including mosses, ferns, and trees (Petrini, 1991). Because of their inconspicuous lifestyle within healthy plants, they have been assigned the name endophytes (Carroll, 1988). An important group of these endophytic fungi, having recently received increased attention are those that infect grasses.

These grass endophytes often have profound effects on the physiology, ecology, and reproductive biology of their host plant (Clay, 1990). Moreover, in many of these fungus-grass associations toxic alkaloids are produced that defend the grass against a wide range of insect and mammalian herbivores (Clay, 1990).

1.2 FUNGAL ENDOPHYTES OF GRASSES

Fungal endophytes in the family Clavicipitaceae (Ascomycota, Hawksworth *et al.*, 1995) form close associations with many grass species. At least 80 grass genera and several hundred species within the family of the Poaceae, including important grain crops, are infected by endophytes (Clay, 1990), and an important feature of most of these associations is that the infection is stable and long-term (Clay, 1990); once infection has occurred, the endophyte propagates vegetatively together with the grass plant.

Of particular interest to this study are the fungal species of the genera *Neotyphodium* (formerly classified *Acremonium* sect. Albo-lanosa, Glenn *et al.*, 1996) and *Epichloë* that infect grasses in the subfamily Pooideae. These fungi are collectively known as *Epichloë* endophytes, and are hereafter referred to as endophytes. In the grass-endophyte associations, the fungus colonises the intercellular spaces (apoplast) of leaves, stems, and often seeds of the grass plant (Clay, 1988; Clay, 1990, White *et al.*, 1993; Clay & Leuchtmann, 1989). The associations between endophytes and their host grasses span the range from antagonistic and highly mutualistic (Clay, 1988; Siegel & Schardl, 1991; White, 1988; White *et al.*, 1993). In antagonistic associations, the sexual reproduction of the grass host is suppressed by the formation of epiphytic fungal stromata on developing inflorescences. This condition is known as "choke" disease and enables horizontal transmission of the endophyte to new plants by the release of sexual reproductive (asco-) spores (Kirby, 1961; Schardl, 1996; White *et al.*, 1993). In mutualistic associations, no choking of the plant occurs. In this situation, the endophyte grows into the developing ovule within an inflorescence and becomes incorporated into the seed, which eventually gives rise to an infected seedling (Schardl, 1996, White *et al.*, 1993). Associations that are intermediate to antagonistic and mutualistic types are common, where stromata are formed

on some tillers (= single grass plants), while on others healthy inflorescences develop that produce endophyte-infected seeds. This pleiotropic association is considered to mark the transition from antagonism to mutualism (White *et al.*, 1993). The ability to form a stroma and undergo the sexual life cycle is characteristic of endophytes of the genus *Epichloë*. Endophytes of the genus *Neotyphodium* do not form stroma and are strictly seed transmitted, hence, they are asexual fungi that have evolved from *Epichloë* species (Schardl, 1996; Tsai *et al.*, 1994).

Infection of grasses by endophytic fungi has been recognised many decades ago (McClennan, 1920), however, the ecological significance of this symbiosis has come to light only recently. Particularly the role of the mutualistic *Neotyphodium* endophytes for the survival and persistence of infected grasses is indeed profound. In this type of symbiosis, a number of secondary metabolites are produced that belong to the alkaloid-chemical class. For these alkaloids it is now known that they help protecting the grass against many herbivores and pathogens (Clay, 1988; Clay, 1990; Funk *et al.*, 1993; Schardl & Phillips, 1997; Siegel & Schardl, 1991). In addition, the presence of the endophyte conveys greater persistence against adverse environmental conditions, and generally enhances plant vigour and growth (Bacon, 1993; Clay, 1990; Joost, 1995). Many effects on the growth of the grass hosts are derived from the alkaloid-mediated higher resistance of endophyte-infected plants to pests and pathogens, such as nematodes (Pedersen *et al.*, 1988), insects (Dymock *et al.*, 1989; Prestidge *et al.*, 1982), and other fungi (Gwinn & Gavin, 1992). In addition to greater resistance to herbivores, endophyte-infected plants often have improved survival after drought (Arachevaleta *et al.*, 1989), probably due to bettered osmotic adjustment in infected plant tissues (Elmi & West, 1995). Infected plants outperform uninfected plants when planted in mixtures (Malinowski *et al.*, 1997), have higher rates of photosynthesis under high temperature and water stress (Marks & Clay, 1996; Richardson *et al.*, 1993), and stronger root growth (De Battista *et al.*, 1990b). As a consequence of the significant amelioration of plant growth, in many natural environments incidence of endophyte-infection of grasses is high, occasionally reaching 100 per cent (Clay, 1993).

The desirable qualities of higher yield and persistence of endophyte-infected grasses notwithstanding, the infection also poses a serious problem in agriculture. This is due to the production of alkaloids that are toxic to grazing mammals, such as cattle and sheep. Toxicity of endophyte-infected grasses is particularly problematic in the agronomically important symbioses of tall fescue (*Festuca arundinaceae*, Schreb.) with *Neotyphodium coenophialum* (Morgan-Jones and Gams) Glenn, Bacon & Hanlin, and perennial ryegrass (*Lolium perenne*, L.) with *N. lolii* (Latch, Christensen & Samuel) Glenn, Bacon & Hanlin.

The toxicity of endophyte-infected pastures causes substantial economic losses. In the United States, the negative impact of the endophyte-associated toxins on animal farming has been estimated to exceed 600 million dollars annually (Hoveland, 1993). However, the eradication of the fungal component, in order to overcome the problem of endophyte-associated toxicity concomitantly compromises plant viability and, hence, reduces the agronomical value of grasses (Prestidge, 1993; Read & Camp, 1986). Consequently, research is now aimed at understanding the biology of this symbiosis, with the aim to improve its cost to benefit ratio for agriculture.

1.2.1 Endophyte Growth in the Grass Plant and Plant-Endophyte Interactions at the Cellular Level

The endophytes colonise stems, meristems, leaves, seeds, and occasionally the roots of grass plants (White *et al.*, 1993). In leaves, the basal region (= leaf sheath) is more extensively colonised than the leaf blade (Christensen *et al.*, 1998; Hinton & Bacon, 1985; Keogh *et al.*, 1996; Musgrave, 1984). Endophyte hyphae appear to be confined to the intercellular spaces in all plant tissues (Clay, 1990; Hinton & Bacon, 1985; Koga *et al.*, 1993; White *et al.*, 1993). Growth of the endophyte into the leaves and shoots of single grass plants (tillers) is thought to occur *via* the meristems that give rise to these tissues (White *et al.*, 1993).

However, much remains to be learned about how and when hyphae grow and proliferate within the grass plant. Despite its importance for determining levels of the endophyte in the plant, the general understanding of physiological regulation of endophyte growth in the various tissues of the grass plant is still very much in its infancy.

Notwithstanding the lack of knowledge concerning physiological interactions between the symbiotic partners, some general features of the grass-endophyte associations have been established. The infection of grasses by endophytes in natural and compatible associations is relatively uneventful compared to other fungal-plant associations, where remarkable changes at the cellular and ultrastructural level can be observed (reviewed by Manners & Gay, 1983). In artificially produced endophyte-infections of foreign grass hosts, variation has been observed in the ability to form stable symbiotic relationships (Christensen, 1995). Adverse effects on growth of plant and endophyte have also been reported in these artificial associations (Koga *et al.*, 1993). These findings indicate that in some situations, plant and/or endophyte can affect each other negatively, hence, in compatible relationships these reactions are not elicited and/or may be suppressed in the symbiosis. In some natural associations, the presence of the endophyte may bring about host responses such as higher levels of chitinases (Roberts *et al.*, 1992), which are known inhibitors of fungal growth (Schlumbaum *et al.*, 1986). However, at present little more information exists whether or how the plant may actively respond to the infection, but regulation of endophyte growth by the plant is probable. It is known, for example, that strong growth of *Epichloë* endophytes required for the formation of the stroma (see above, previous section) is induced at a very defined stage in the development of the grass inflorescence (Kirby, 1961). Contrasting to its vigorous growth on the inflorescence, mycelium of the fungus appears only relatively sparsely in leaf tissues, which is suggestive of inhibition or lack of induction of endophyte growth in these tissues.

Specialised structures for nutrient uptake have not been found for clavicipitaceous grass endophytes. However, there is indirect evidence, which suggests that endophytes possess mechanisms for active nutrient uptake. *Neotyphodium* endophytes express a proteinase that is similar to the proteolytic enzymes produced by pathogenic fungi (Reddy *et al.*, 1996). These may serve to break down proteins to amino acids for uptake by the

fungus. The activities of invertases in infected plant tissues have also been implicated in the active uptake of apoplastic sucrose by the endophyte (Lam *et al.*, 1994; Lam *et al.*, 1995).

It has been suggested previously that nutrient distribution within leaves is a determinant for the characteristic basal-apical distribution of the endophyte within the grass plant (Hinton & Bacon, 1985). Reports on greater concentrations of sugars in the leaf sheath (Smith, 1973), the location of higher concentrations of the endophyte, were however not much supported by other studies (Belesky & Fedders, 1996; Belesky *et al.*, 1989; Richardson *et al.*, 1992). More importantly, very little is known about the availability of these nutrients in the intercellular spaces of the different tissues. It appears that significant amounts of nutrients are present in/on the internal cell wall surfaces (Juniper, 1991), and it has been proposed that nutrient supply in the intercellular spaces is high enough to allow for fungal growth (Hancock & Huisman, 1981). Moreover, storage and mobilisation of carbohydrates within the grass plant is known to be variable, depending on a number of factors, such as leaf age, annual season and nutrients available to the plant (Parsons, 1988; Robson *et al.*, 1988). Because of the reported variability in nutrient distribution, other, but yet unknown factor(s) probably bring about the consistent pattern of *in planta* endophyte distribution found for different endophyte-grass associations (see above, this section). Therefore, more detailed investigations seem necessary to identify the factor(s) involved in determining the endophyte concentration in the different plant tissues. The elucidation of the regulation of endophyte growth in the grass plant is critical for the aetiology of the endophyte-associated toxicoses, since concentration levels of endophyte mycelium in the plant may determine levels of the alkaloids that impact on the health of farmed animals. Investigations in the study presented were therefore aimed at a more solid understanding of endophyte growth and the relationship between levels of endophyte mycelium and alkaloids in the grass plant.

At the ultrastructural level of the interaction, examined with the use of transmission electron microscopy, it was demonstrated that the cell walls of endophyte hyphae and plant cells are mostly in close contact, and are connected by an intercellular matrix (Koga *et al.*, 1993). Similar structures have been noted in other plant-fungus associations, and it has

been speculated that the intercellular matrix, or involving layer, may facilitate the flow of nutrients. Conversely, it might protect the fungus against host defences (Bracker & Littlefield, 1971). In the mycorrhiza symbiosis, it is assumed that nutrient exchange is facilitated by passive movement of solutes into the fungus-plant interface, followed by active uptake across membranes. The latter apparently involves the activities of H^+-ATPases at the symbiotic interface, which provides the energy required for the solute uptake into fungal and plant cells (Gianinazzi-Pearson, 1996). However, whether similar processes may be involved in endophyte-grass interactions remains to be established.

1.2.2 Effects of Endophyte-Infection on Plant Growth and Photosynthesis

It is now widely recognised that endophyte-infection has effects on the growth of the host plant. But it often appears difficult to determine the reason for the improved fitness frequently noted for endophyte-infected plants. On the one hand, it is known that the production of the alkaloids in infected plants provides greater protection against herbivores (Section 1.2 and Section 1.2.3), thus enhancing plant growth performance. On the other hand, it may be due to direct effects of the endophyte on plant physiology and growth rate, and, in addition, some interactions between immediate effects on plant physiology and the alkaloid-mediated resistance to herbivores may occur.

However, as noted previously (Section 1.2), it is now known that endophyte-infection conveys a number of physiological improvements to the plant that enhance its growth and persistence, especially under adverse abiotic conditions. Likewise, in controlled environment studies, greater production of tillers and higher biomass were reported for tall fescue plants infected by *N. coenophialum* (Belesky *et al.*, 1987) and perennial ryegrass infected by *N. lolii* (Latch *et al.*, 1985) compared to their endophyte-free counterparts. However, effects of the endophyte on plant growth were not always consistent. The *N. lolii*-perennial ryegrass symbiosis in particular has often proven difficult for reproducing in field trials earlier reports of enhanced plant growth due to the endophyte (Hume, 1993). Moreover, differences in plant growth responses to endophyte-infection

were found between different levels of plant nutrients: at lower nutrient levels, endophyte-infection can cause a decrease in tiller number per plant (Cheplick *et al.*, 1989).

Changes in the physiology of endophyte-infected plants have been observed in tall fescue infected by *N. coenophialum*. In this association, the presence of the endophyte promotes levels of glutamine synthetase, a plant enzyme important for the nitrogen metabolism of its host (Lyons *et al.*, 1990), therefore, infection seems to improve efficient use of this plant nutrient (Lyons *et al.*, 1990). Unexpectedly, endophyte-infected tall fescue plants that showed generally enhanced growth (see above, this section) had, on average, lower net-photosynthetic rates than uninfected plants (Belesky *et al.*, 1987). By contrast, net photosynthesis was greater in endophyte-infected tall fescue plants under water deficit (Richardson *et al.*, 1993) and higher temperatures (Marks & Clay, 1996). The reason(s) for the greater plant growth, despite a lower rate of photosynthesis of infected plants reported by Belesky *et al.* (1987) are not known. In the view of this and other findings, it has been hypothesised that infected plant may use photosynthates more efficiently (Bacon, 1993). At present, however, aside from the noted alteration in enzyme activity levels such as glutamine synthetase in the plant (see above), little is known about the physiological basis of these and other effects of the endophyte on plant growth and physiology. The reported studies on photosynthesis were carried out only with tall fescue infected by *N. coenophialum*, therefore, prior to this study it was not known if or how photosynthesis in perennial ryegrass may be affected in response to infection by *N. lolii*.

1.2.3 The Production of Secondary Metabolites by Grass Endophytes and Their Effects on Animal Health and Plant Persistence

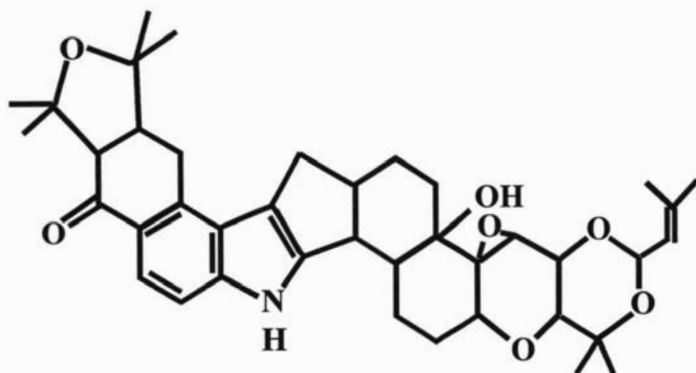
Secondary metabolites, of the alkaloid chemical class, are produced in many endophyte-grass symbioses. Of particular agricultural interest are the alkaloids produced by tall fescue-*N. coenophialum* and perennial ryegrass-*N. lolii* associations. All of these alkaloids are only produced in endophyte-infected grass plants, and have been isolated from endophytes growing in culture, which suggests that alkaloid synthesis in the different symbioses is solely or largely carried out by the fungal symbiont. However, in the case of

saturated amino pyrrolizidine (= loline) alkaloids some trace amounts have also been recently detected in uninfected grasses (Justus *et al.*, 1996).

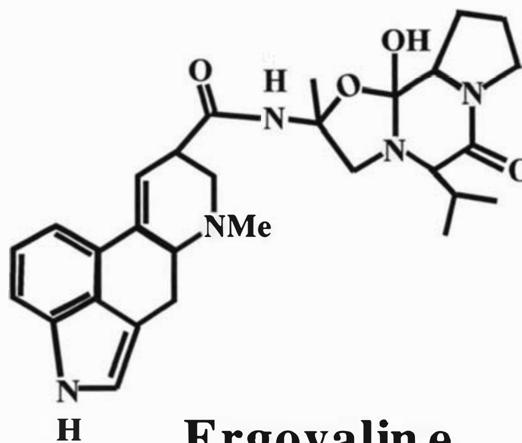
Ergopeptines, alkaloids that possess an ergoline ring system linked to a cyclol peptide chain (Didek-Brumec *et al.*, 1996), are produced in *N. coenophialum*-infected tall fescue (Lyons *et al.*, 1986). The major ergopeptide in this symbiosis is ergovaline (Lyons *et al.*, 1986; Fig. 1), which is also produced in the symbiosis of perennial ryegrass with *N. lolii* (Rowan & Shaw, 1987). Ergopeptine alkaloids are considered the causative agent for fescue toxicosis in mammals (Lyons *et al.*, 1986), a syndrome characterised by weight loss, occasional abortion and gangrene in limbs of animals grazing on tall fescue pastures infected by *N. coenophialum* (Garner *et al.*, 1993; Siegel & Bush, 1997). The pharmacological actions of these compounds are broad, and include vasoconstrictive activities and effects on the endocrinal system (Bacon *et al.*, 1986). The biological activity of the ergot alkaloids is modulation of several receptors of neurotransmitters, thus, interference with the activities of, for example, serotonine, dopamine, and noradrenaline (Wink, 1998). Controlled animal feeding studies demonstrated that an ergovaline concentration of about 0.2 $\mu g\ g^{-1}$ in tall fescue herbage was sufficient to induce measurable physiological effects in cattle (Cornell *et al.*, 1990). In endophyte-infected tall fescue plants, ergovaline is usually present in concentrations ranging from 0.5-5 $\mu g\ g^{-1}$ (Siegel & Bush, 1997), and is also produced, reaching similar concentrations, in perennial ryegrass infected by *N. lolii* (Lane *et al.*, 1997c). It has been demonstrated that ergopeptines are produced in cultures of *N. coenophialum* (Bacon, 1988), indicating that this fungus (and presumably *N. lolii* as well) possess the complete biosynthetic pathway necessary for ergopeptine production.

Peramine (Fig. 1), a pyrrolopyrazine alkaloid, is produced in endophyte-grass associations involving *Epichloë* and *Neotyphodium* species (Rowan, 1993; Siegel & Bush, 1997). This compound has also been identified in cultures of *Neotyphodium* endophytes (Rowan, 1993). Peramine is a potent feeding deterrent against Argentine stem weevil (ASW; *Listronotis bonariensis*), a major and detrimental pest of perennial ryegrass in New Zealand (Dymock *et al.*, 1989; Rowan, 1993; Rowan & Gaynor, 1986). The biological

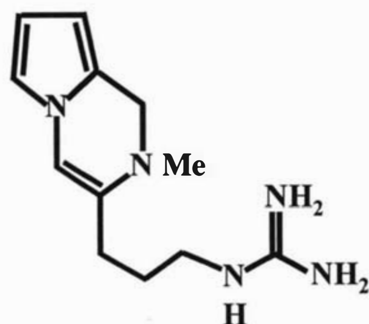
Figure 1
Structures of the three major alkaloids produced in the symbiosis of *Lolium perenne* with *Neotyphodium lolii*. Reproduced from Rowan (1993) and Garner *et al.* (1993).



Lolitrem B



Ergovaline



Peramine

activity of peramine against ASW and other insects is mainly as a feeding deterrent, and no evidence for toxicity has been found (Rowan, 1993), although some effects on the development of other insect species have been reported (Dymock *et al.*, 1989). Mammalian toxicity of this alkaloid appears to be low (Pownall *et al.*, 1995). Levels of peramine causing feeding deterrence are low: at a concentration of 0.1 $\mu g\ g^{-1}$, tested in a bioassay, ASW is significantly deterred from feeding (Rowan *et al.*, 1990). However, in no-choice situations deterrence occurs at 10 $\mu g\ g^{-1}$ (Rowan, 1993). To date, the biochemical basis for the biological activity of peramine is not known (Siegel & Bush, 1997).

Lolitrem B (Fig. 1), an indole diterpenoid, is produced in the symbiosis of *N. lolii* with perennial ryegrass and in some other *Neotyphodium*-grass associations, and is found in endophyte-infected seed and herbage (Rowan, 1993; Siegel & Bush, 1997). As with ergovaline and peramine, lolitrem B has also been identified in fungal cultures (Penn *et al.*, 1993). The lolitrems are tremorgenic neurotoxins (Gallagher *et al.*, 1981) that bring about symptoms of ryegrass staggers, a nervous disorder affecting animals grazing on endophyte-infected perennial ryegrass pastures (Fletcher & Harvey, 1981). This disorder affects cattle, sheep and horses and is characterised by tetanic muscle spasms that result in severe incoordination and hypersensitivity to external stimuli (Rowan, 1993). However, unlike the case with ergopeptine alkaloids, the effects on animals are short term and no gross pathological changes in tissues were observed (Prestidge, 1993; Rowan, 1993), but the liveweight gain rate of the animals can be affected. The biochemical mode of action of lolitrem B is to bind and thereby inhibit the GABA (γ-amino butyric acid) receptor in the brain, a primary target for the toxic action of mycotoxins (Gant, 1987). Concentrations of 2 $\mu g\ g^{-1}$ lolitrem B in endophyte infected grasses are sufficient to induce the symptoms of ryegrass staggers (Rowan, 1993).

The fourth main class of alkaloids produced in endophyte-grass associations are the saturated amino pyrrolizidine alkaloids, collectively known as lolines (predominantly N-formylloline and N-acetylloline). Lolines have been found in relative high abundance in tall fescue infected by *N. coenophialum* (Bush *et al.*, 1993; Siegel *et al.*, 1990). Exceptionally high concentrations (> 5 $mg\ g^{-1}$) of these alkaloids were found in *Festuca*

pratensis, Huds. infected by *Neotyphodium uncinatum* (Gams, Petrini & Schmidt) Glenn, Bacon & Hanlin (Bush *et al.*, 1997). The lolines have insecticidal activities and are important for the resistance of plants to insects (Bush *et al.*, 1993). Lolines also have some effects on vertebrate herbivores (Bush *et al.*, 1993). However, because of the absence of lolines in the *N. lolii*-perennial ryegrass symbiosis, which is the symbiosis pertinent to this thesis, the loline alkaloids are not discussed in further detail.

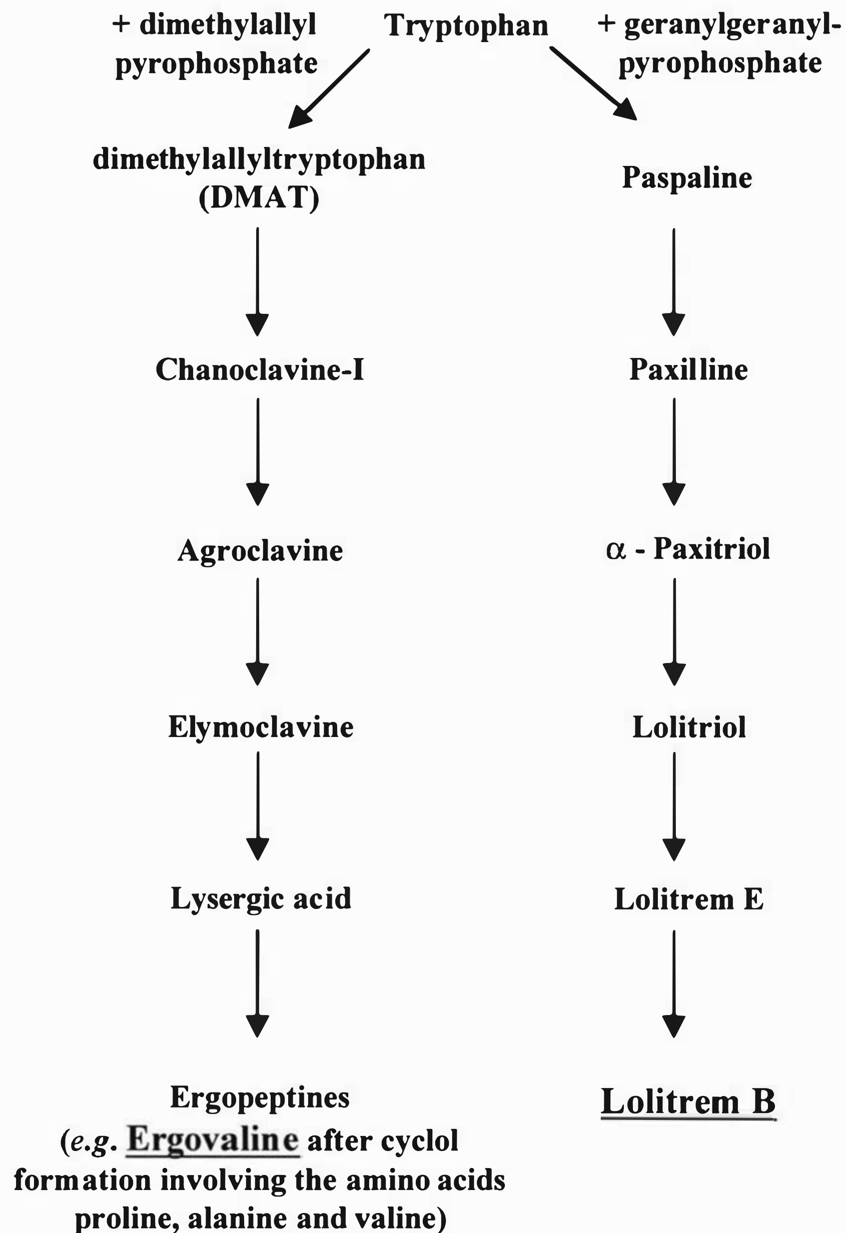
The impact of the *N. lolii*-perennial ryegrass symbiosis on animal performance, due to the production of the two anti-mammalian alkaloids lolitrem B and ergovaline, is agronomically highly undesirable. On the other hand, because infected plants have greater persistence, notably in the presence of ASW, and against abiotic stresses in general (Section 1.2), the maintenance of the symbiosis appears to be a necessity. Consequently, a major aim of recent research is the development of plant-endophyte associations that are free of anti-mammalian toxins, but at the same time retain their resistance to herbivorous insects. As toxin-producing associations of *N. lolii* with *L. perenne* are likely to be used further in pastoral farming, another area of research is aimed at understanding the physiological basis for alkaloid production as a basis for better management of endophyte-infected pastures to improve animal health.

1.3 BIOSYNTHESIS, ANALYSIS, *IN PLANTA* DISTRIBUTION, AND PHYSIOLOGY OF ALKALOID PRODUCTION

1.3.1 The Biosynthesis of Ergovaline, Lolitrem B and Peramine

To date, the complete biosynthetic pathway for any of the alkaloids in endophytic fungi has not been elucidated, but based on some recent findings putative biosynthetic pathways of ergovaline and lolitrem B have been proposed as indicated in Fig. 2. Features of the biosynthesis of ergopeptide alkaloids have been mainly studied in the ergot fungi belonging to the genus *Claviceps* (Garner *et al.*, 1993; Siegel & Bush, 1997), which are close relatives to the *Epichloë/Neotyphodium* endophytes (Schardl *et al.*, 1991).

Figure 2
Putative biosynthetic pathways of the alkaloids lolitrem B and ergovaline (Siegel and Bush, 1997).



The first committed step in the ergot alkaloid synthesis is the formation of 4-(γ,γ-dimethylallyl) tryptophan (DMAT) derived from dimethylallyl pyrophosphate (formed from mevalonic acid) and L-tryptophan (Fig. 2). Tryptophan thereby provides the later indole moiety of the ergot alkaloids (Bu'Lock & Barr, 1968). The catalytic enzyme responsible for this step is DMAT synthetase, and this synthetase has been previously purified and characterised (Cress *et al.*, 1981). The genes encoding DMAT synthetase in *Claviceps fusiformis* and *Claviceps purpurea* have recently been cloned (Tsai *et al.*, 1995; Tudzynski *et al.*, 1999). Because of its pivotal position in the ergot alkaloid pathway of *Claviceps* species, attempts are now underway to isolate the corresponding DMAT synthetase gene(s) in *N. coenophialum,* which could be a suitable target for genetic manipulation to eliminate this class of alkaloids in the *N. coenophialum*-tall fescue symbiosis (Bush *et al.*, 1997).

The biosynthetic pathway in the formation of lolitrem B also requires the amino acid tryptophan at its start (Fig.2). A tremorgenic precursor in the lolitrem biosynthetic pathway in *N. lolii* is paxilline (Weedon & Mantle, 1987), and it has been suggested that the synthesis of paspaline from geranylgeranylpyrophosphate and tryptophan is a crucial step for the synthesis of paxilline and lolitrem B (Miles *et al.*, 1995). Initial studies, aimed at understanding the biosynthesis of tremorgenic compounds at the molecular level, have recently been conducted by targeted disruption of the pathway in the paxilline-producing fungus *Penicillium paxilli* (Young *et al.*, 1998).

Little is known about the biosynthesis of peramine. This alkaloid possesses two characteristic structural features: a previously unknown pyrrolopyrazine-ring system and a rare guanidinium group, and it is thought to be biosynthetically derived from the amino acids proline and arginine (Rowan, 1993).

Most of what is known about alkaloid biosynthesis in fungi has been inferred from culture fermentation studies. Based on these studies *in vitro* it has been generally established that synthesis of secondary metabolites in most fungi takes place, or is enhanced, in the stationary growth phase (Didek-Brumec *et al.*, 1996; Finkelstein & Ball, 1992; Rehacek *et al.*, 1973; Rehacek *et al.*, 1971; Vining, 1973), that is, primary growth

and alkaloid synthesis are usually clearly dissociated (Didek-Brumec *et al.*, 1996). The most relevant example of this dissociation in the literature is that of ergot alkaloid production in *C. purpurea.* In this fungus, when growing in the plant, ergot alkaloids are not synthesised during mycelial proliferation, but at a later stage, marking differentiation with the beginning of the sexual cycle (Didek-Brumec *et al.*, 1996; Tudzynski *et al.*, 1997). Likewise, the formation of ergot alkaloids by *C. purpurea in vitro* normally occurs under conditions of decreased cell proliferation and degradation and resynthesis of nucleic acids and proteins (Rehacek, 1980).

The amino acid tryptophan, being the main precursor of ergot alkaloids, is important for the induction of the ergot alkaloid-biosynthetic pathway in *C. purpurea.* Further *in vitro* studies have demonstrated that the presence of tryptophan stimulates increased cellular levels of DMAT synthetase (Krupinski *et al.*, 1976). Moreover, the regulation of DMAT synthetase at the level of transcription in *C. purpurea* has been recently demonstrated (Arntz & Tudzynski, 1997). Other important factors for ergot alkaloid synthesis in *Claviceps* are calcium, a cofactor for the DMAT synthetase (Cress *et al.*, 1981), and inorganic phosphate, which is inhibitory to the formation of these compounds in culture (Didek-Brumec *et al.,* 1996).

1.3.2 Analytical Methods for the Quantitative Determination of the Alkaloids

A number of analytical methods have been previously developed for quantitative determination of each of the alkaloids implicated in the agricultural impact of the ryegrass endophyte *N. lolii* (reviewed by Garner *et al.*, 1993; Porter, 1994; Rowan, 1993; Siegel & Bush, 1997). These methods have generally been devised for relatively large quantities of endophyte-infected grass samples, as many previous investigations were aimed mainly at the determination of alkaloid levels representing the levels in whole pastures. The work presented in this thesis was directed at determining the concentration of these alkaloids at a more defined level within the plant, thus requiring smaller tissue quantities and improved sensitivity. Because modification of existing protocols was an important part of this thesis,

analytical methods that are commonly used for studying the endophyte-associated alkaloids will be briefly described.

Lolitrem B can be measured in plant tissue extracts by HPLC with sensitive and selective fluorescent detection (Gallagher *et al.*, 1985). As a suitable internal standard is not available, quantitation of this compound is carried out by external standardisation with a lolitrem B primary standard (Gallagher *et al.*, 1985). Due to its lipophilic properties, extraction of this compound is performed with organic solvents (Gallagher *et al.*, 1985; Garthwaite *et al.*, 1993), and concentrations of 0.2 $\mu g\ g^{-1}$ in endophyte-infected grass tissues can be reliably detected (Gallagher *et al.*, 1985). Immunological methods for quantitative analysis, involving detection by antibodies to protein conjugates with lolitrem B, have been described for the detection of lolitrem B in cultures of *N. lolii* (Garthwaite *et al.*, 1993; Penn *et al.*, 1993), but at present, have not been used for determination in plant tissues.

The determination and quantitation of peramine can also be performed by HPLC with detection by UV absorption (Rowan & Gaynor, 1986; Tapper *et al.*, 1989). Peramine is more hydrophilic than lolitrem B and ergovaline, and can be recovered and extracted from plant tissues with aqueous organic solvents mixtures (Barker *et al.*, 1993; Tapper *et al.*, 1989). The synthetic homologue, homoperamine, with similar chemical and physical properties to peramine (including UV absorbance), but is readily resolved from peramine by HPLC, has been prepared for use as an internal standard (Barker et al., 1993; Rowan, 1993).

Ergovaline can be detected and measured at low concentrations in extracts of endophyte-infected grasses by HPLC, with the advantage of sensitive and selective fluorescent detection as for lolitrem B (Garner *et al.*, 1993; Siegel & Bush, 1997). However, ergovaline is difficult to analyse because ergopeptine alkaloids are susceptible to photolytic and air oxidation, hydration and epimerisation/isomerisation (Garner *et al.*, 1993; Porter, 1994). Quantitation of this alkaloid in plant material has generally been performed with another ergopeptide (usually ergotamine or ergocrystine) as the internal standard, added to the sample prior to extraction (Barker *et al.*, 1993; Rottinghaus *et al.*,

1991; Shelby & Flieger, 1997; Yates & Powell, 1988). These ergopeptides can be resolved from the analyte by HPLC, have similar fluorescence detection properties, and, due to their similar chemical and physical properties, are expected to be recovered from extracts with a similar efficiency to ergovaline (Shelby & Flieger, 1997).

The quantitation with an internal standard will yield more accurate results than a calibration against external standards, provided that the internal standard can be sufficiently mixed with the sample, and can be recovered with an efficiency closely similar to that of the analyte (Garfield, 1991). However, for achieving accurate quantitation, it is most important that the internal standard is added at the earliest step of the extraction. In this way, inevitable losses of the compound under investigation during the extraction can be effectively compensated.

For example, recovery of ergovaline from plant samples is likely to be incomplete (Rottinghaus *et al*., 1991; Shelby & Flieger, 1997). For good solubility of this alkaloid in a polar or non-polar solvent, the pH of the solvent has to be adjusted low or high, respectively. However, especially under these extractive conditions, ergovaline is susceptible to chemical degradation (Garner *et al.*, 1993; Porter, 1994). In addition, ergovaline will be subjected to partioning between the solvent and the solvent phase matrix, which may well have some ion exchange and/or reverse phase character(s).

The use of an ergopeptide with similar chemical and physical properties to the analyte as internal standard for the extraction process can provide some compensation for the inevitable incomplete recovery of ergovaline from extracts and, hence, improve the accuracy of results. The accuracy of an analytical method based on internal standardisation depends on the relative, rather than absolute, recoveries of the analyte and internal standard from an extract. Despite the importance of relative recovery for reliable quantitation of ergovaline, this analytical parameter has not been addressed in detail in previous studies, and, therefore, as a part of this study, was examined in the experimental development of an improved method for ergovaline extraction.

1.3.3 Alkaloid Distribution within the Plant

The quantitative distributions of the major alkaloids in grass-endophyte symbioses within the grass plant have been largely established. However, many questions have remained open, for example, whether their distributions are determined by the distribution of the endophyte mycelium. Moreover, it has now become evident that the environment strongly affects alkaloid production (Section 1.3.4). Nonetheless, only few studies concerning alkaloid production and distribution in grasses have been conducted in sufficiently controlled environments. Thus, although some general trends for accumulation of each alkaloid within the plant have been established, little appears to be known about the role of, for example, plant genotype and plant tissue on alkaloid production, and, at present for all of the alkaloids, the site(s) of production within the grass plant are unknown.

In endophyte-infected perennial ryegrass and tall fescue, ergovaline is found at higher concentrations in basal tissues, that is, in the pseudostem, which is mostly comprised of leaf sheath material (Christensen *et al.*, 1998; Lane *et al.*, 1997c; Lyons *et al.*, 1986). The higher concentration of ergovaline in basal tissue relative to leaf blade in tall fescue has been interpreted as a result of the greater concentration of endophyte in these tissues (Siegel & Bush, 1997). The distribution of ergovaline in a perennial ryegrass tiller appears to be very heterogeneous; accumulation of ergovaline occurs principally in basal tissues, inflorescence and seeds (Lane *et al.*, 1997c). However, the degree to which this heterogeneity within the plant reflects that of endophyte mycelium was not known. Moreover, contrary to *C. purpurea*, for which differential synthesis of ergot alkaloids has been shown (see above, Section 1.3.1), it is not yet known whether alkaloid production occurs at similar rates in all parts of the endophyte mycelium, or whether it is spatially and/or temporally regulated within the plant.

Of the three alkaloids, peramine has the most even distribution within the plant. Levels of this alkaloid in perennial ryegrass infected by *N. lolii* are usually similar in lower and upper leaf tissues (Ball *et al.*, 1997a; Davies *et al.*, 1993; Keogh *et al.*, 1996). Peramine distribution is thought to be due to its relatively high mobility within the plant,

perhaps allowing net movement from leaf sheaths to leaf blades (Keogh *et al.*, 1996; Rowan, 1993). Experiments with endophyte-infected plant seedlings have shown that peramine is translocated within the seedlings shortly after germination, and also from seeds with non-viable endophyte (Ball *et al.*, 1993). The even distribution of this alkaloid might therefore be due, at least in part, to translocation by the plant.

Lolitrem B accumulates in tissues closer to the tiller base, but is also found in significant quantities in the leaf blade of *N. lolii*-infected ryegrass (Ball *et al.*, 1997b; Davies *et al.*, 1993; Keogh *et al.*, 1996). Higher accumulation of this compound was found to occur in older and senescent leaves (Davies *et al.*, 1993; Keogh *et al.*, 1996). As for peramine, translocation within the plant has been implicated in its *in planta* distribution (Ball *et al.*, 1993). Concentrations of *N. lolii* and lolitrem B in plant tissues follow similar seasonal trends, with significantly higher concentrations of alkaloid and fungus in plant tissues in summer, and generally lower concentrations in winter (Ball *et al.*, 1995; di Menna *et al.*, 1992). This suggests that amount and/or distribution of endophyte mycelium in the plant determine the concentration of lolitrem B. A similar influence of annual season on alkaloid concentration was reported for ergovaline in *N. coenophialum*-infected tall fescue (Rottinghaus *et al.*, 1991; Woodburn *et al.*, 1993).

1.3.4 Endophyte Concentration and Alkaloid Production as Influenced by the Environment, and Putative Roles of Physiological Parameters for Alkaloid Synthesis

Extremely high levels of all three alkaloids normally not encountered in the field, have been observed in endophyte-infected perennial ryegrass grown in a glasshouse environment (Lane *et al.*, 1997a). Basal plant tissues contained occasionally more than 60 $\mu g\ g^{-1}$ ergovaline (usually 0.5-5 $\mu g\ g^{-1}$, Section 1.2.3) and more than 30 $\mu g\ g^{-1}$ lolitrem B (usually 2-15 $\mu g\ g^{-1}$, Lane *et al.*, 1997b). These observations suggest that in the glasshouse environment, the physiological basis of alkaloid production can be strongly altered, and they indicate that the control of the plant growth environment is very important for studies on alkaloid production in the symbiosis.

Increased nitrogen levels increase the levels of ergovaline in tall fescue and perennial ryegrass plants infected by *N. coenophialum* and *N. lolii,* respectively (Lane *et al.*, 1997b; Lyons *et al.*, 1986; Rottinghaus *et al.*, 1991), and higher concentration of ergovaline were also found in plants experiencing a water deficit (Barker *et al.*, 1993; Belesky *et al.*, 1989). The dependence of ergovaline production on nitrogen available to the symbiosis has led to the speculation that ergovaline alkaloid levels may be determined by the availability of nutrients, such as nitrogen, carbohydrates and other metabolic compounds (amino acids, ions), to the fungal mycelium in the plant (Belesky & Hill, 1997; Roylance *et al.*, 1994). However, in the previous studies, only alkaloid levels were examined, and no information of the quantitative distribution of the endophyte was reported. Therefore, it was not known whether the greater availability of nutrients may lead to more extensive growth of alkaloid-producing fungal mycelium or enhance the rate of alkaloid production per mycelial biomass.

As noted (Section 1.3.3), for none of the alkaloids the site of production is known, and consequently, little is known about the physiological conditions within the plant that lead to their synthesis in endophyte-infected grass tissues, and whether production in mycelium is significantly regulated by the plant. The possible role of the environment, in which the endophyte resides, namely the different plant tissues within a single grass tiller has received little attention in studies undertaken previously. The quantitative determination of the alkaloids in these very diverse microenvironments within the plant, across a representative range of genetically different plants could significantly improve the understanding of the physiological basis of alkaloid production, and was therefore addressed as part of the work for the present study.

As discussed (Section 1.3.1), in culture, the formation of many alkaloids usually takes place towards the end of the active growth stage in fungi, with the onset of the stationary growth phase, as commonly expected for secondary metabolites. Secondary metabolism in culture is usually accompanied by measurable changes in fungal primary metabolism, that is, down-regulation of protein biosynthesis and accumulation of storage products (Didek-Brumec *et al.*, 1996; Rehacek, 1980; Rehacek *et al.*, 1971). Consequently, it has been assumed that similar physiological changes may occur with the endophyte in

the plant environment (Mantle, 1993). Moreover, in *N. coenophialum*, delayed ergot alkaloid synthesis in cultured mycelium has been shown to occur (Bacon, 1988), suggesting that ergot alkaloid production is indeed enhanced in stationary-phase mycelium.

By assessing the alkaloid production and metabolic activity of the fungus in the plant, it may be possible to characterise the *in planta* physiological state of the endophyte and its implications for the formation of the alkaloids. As a primary hypothesis, the environment within young, growing leaves may be initially favourable for the growth of the endophyte, as these receive larger quantities of photosynthates and other nutrients from older leaves for growth (Robson *et al.*, 1988). Subsequently, with the cessation of leaf extension, the fungus may then encounter physiological conditions inhibitory to its primary metabolism, being similar to a stationary phase-state in culture, and leading to secondary metabolite formation. This model may also explain why the endophyte mycelium appears relatively sparsely in leaf tissues (Section 1.2.1); at the start of this investigation it was considered possible that further growth of the endophyte may be prevented by a down regulation of its metabolism (metabolic state) in the mature plant tissues. Therefore, a main aim of the present study was to determine the physiological state of the endophyte in the different leaf tissues of the grass plant.

1.4 The GUS Reporter Gene: A Tool for Assessing the Metabolic State of Endophyte Mycelium

The availability of molecular biology-based techniques has now opened up new experimental avenues, aimed at the understanding of many physiological processes in a variety of organisms. These techniques allow for defined alteration of the regulation of metabolic processes at the genetic level, and also the consequences of a genetic change to be observed at the phenotypic level. A great advancement in the study of plant-fungus interactions was made with the possibility to transform plant-interacting fungi with gene constructs containing the ß-glucuronidase (GUS) gene of *E. coli* (Jefferson, 1987). ß-glucuronidase (ß-D-glucuronoside glucuronosohydrolase) is an acid hydrolase that catalyses the cleavage of a wide variety of ß-glucuronides. Many substrates for the GUS

enzyme are commercially available, including substrates for spectrophotometric, fluorometric, and histochemical analyses (Jefferson *et al.*, 1986). The use of the GUS system is attractive for various reasons: fluorometric detection of GUS activity offers high sensitivity, since in this method the signal-to-noise ratio is particularly high, allowing for detection with two to four orders of magnitude greater sensitivity than spectrophotometric methods; the GUS enzyme has a high stability, hence, its activity remains linear for a long time; GUS activity is absent from plant tissues, thus allowing expression to be examined *in planta* (Jefferson, 1987). The compound 4-methyl umbelliferyl glucuronide (MUG), used in the present study, is a GUS specific fluorogenic substrate. MUG is cleaved by GUS to release 4-methyl umbelliferone (MU), which fluoresces at a pH greater than 9 (Jefferson, 1987). Since linearity of the assay is maintained over a wide range of concentrations of the product MU, GUS activity can be determined from the kinetics of the reaction, rather than by a single end-point measurement, significantly enhancing the accuracy of the determination. Because of its many advantages as a reporter gene for fungal gene expression and its suitability for *in planta* studies, GUS has been used in the characterisation of various plant-fungus associations (Bunkers, 1991; Couteaudier *et al.*, 1993; Mönke & Schäfer, 1993; Oliver *et al.*, 1993; Yates *et al.*, 1999).

Murray *et al.* (1992) successfully transformed a grass endophyte with the GUS gene under control of the glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter of *Aspergillus nidulans*, and stable expression of GUS in plant tissues infected by the transformed endophyte was demonstrated (Murray *et al.*, 1992). Expression of the GUS gene under the control of the *gpdA* promoter of *A. nidulans* is essentially constitutive, as it contains sequences that facilitate, but do not regulate expression (Punt & van den Hondel, 1991).

By using a *Neotyphodium* endophyte-transformant created by Murray *et al.* (1992), Herd *et al.* (1997) developed methods for the quantitative extraction of GUS activity from plant tissues infected by the transformed endophyte. The constitutive GUS expression by the fungus was then utilised by Herd *et al.* (1997) to map the distribution of metabolically active mycelium in the plant. In this study, it was determined that the fungus was metabolically active in all parts of a tiller, and that the distribution of metabolically active

mycelium followed distinct and characteristic patterns within leaves (Herd *et al.*, 1997). However, as the GUS method alone was used in this study, it was not possible to determine as to whether the observed quantitative distribution reflected solely the distribution of fungal biomass or if it was also due to differences in metabolic activity of the fungus.

The expression rate of the constitutive promoter should largely correspond to the overall rate of protein biosynthesis in cells. The overall rate of protein synthesis may be taken as an indicator for metabolic activity, required for example, for the growth of endophyte mycelium. Hence, if constitutive GUS expression varied measurably with endophyte metabolic activity assessed by growth of the endophyte mycelium, its expression in mycelium in the plant would be a useful indicator for the metabolic activity, or metabolic state, of the endophyte. Constitutive GUS expression could then be used for the determination of the physiological state of the endophyte in different plant genotypes and plant tissues. This could then answer the question whether, as proposed (Section 1.3.4), the endophyte in mature plant tissues is in a state similar to a stationary state phase state in culture.

1.5 AIMS AND OBJECTIVES OF THIS STUDY

A number of parameters have been identified that affect alkaloid production in endophyte-infected grasses. However, very little is known about the physiological state of the endophyte in the plant environment that leads to their synthesis and accumulation, and to the observed distribution of the endophyte mycelium in the plant. As noted (Section 1.3.3), although general distributions of the agronomically important alkaloids have been investigated, previous studies were not performed in fine detail. Moreover, very little was previously known about the quantitative relation of the alkaloids to endophyte mycelium in the plant.

The intimate association between the plant and fungus presents difficulties in experimental situations, as any physical or other alteration of the symbiotic state is likely to bring about changes in the parameters that are to be assessed. A main goal of this work

was therefore to examine different symbioses under constant conditions, resembling those in its natural environment, and also allowing sequential measurements of alkaloids and endophyte metabolic activity that could be compared directly to one another. Previous work by Murray *et al.* (1992) and Herd *et al.* (1997) demonstrated the utility of the GUS reporter gene for examining endophyte-gene expression and for precise mapping of GUS-expressing mycelium *in planta*. Thus, assuming that the GUS gene does not affect the overall state of the symbiosis, it would be an effective tool for examining the physiological state (= metabolic state) of the endophyte in varying plant environments.

At the commencement of this study, an *N. lolii* strain transformed with the GUS reporter gene was available. This strain had been created by transformation of the *N. lolii* strain Lp 19, which naturally occurs in perennial ryegrass (Christensen *et al.*, 1993). This transformant contained a single copy of the GUS gene, under the control of the heterologous constitutive *gpdA* promoter (Section 1.4) and, upon incoculation into plants, produced the three agronomically important alkaloids lolitrem B, peramine and ergovaline (Saunders, 1997; Saunders & Schmid, 1995).

The aims for the present study were:

(i) To assess the physiological state of the endophyte in different plant tissues and plant genotypes,

(ii) to investigate the quantitative distribution of the three alkaloids lolitrem B, ergovaline and peramine, and of metabolically active mycelium within a single grass plant (tiller), to assess whether the distribution of the alkaloids is determined by the distribution of metabolically active endophyte mycelium,

(iii) to determine the growth rates and physiological state of endophyte-infected and endophyte-free plants under the experimental conditions.

The experimental objectives undertaken to address these aims were:

- To test in culture the usefulness of constitutive GUS expression of a transformed endophyte strain for assessment of the endophyte metabolic state in the plant.

- To establish a controlled environment for defined growth of plants during experiments.

- To develop methods for the quantitative determination of alkaloids from small tissue quantities, which would enable precise mapping of the distributions of the alkaloids within tillers.

- To determine the endophyte metabolic state (EMS) in a representative range of different plant tissues and plant genotypes.

- To determine the distribution of metabolically active endophyte mycelium, together with the distributions of the three alkaloids ergovaline, peramine and lolitrem B within tillers from a representative range of plant genotypes.

- To determine growth and photosynthesis in endophyte-infected and endophyte-free plants under the experimentally defined growth conditions.

2.0 MATERIALS AND METHODS

2.1 FUNGAL STRAINS AND PLANT LINES

Fungal strains and plant lines used in this study are listed in Table 1.

2.2 MEDIA

All media were sterilised for 20 min at 121 °C prior to use. MilliQ water was used in all preparations. Liquid media were allowed to cool to room temperature before addition of antibiotic(s) and inoculation. Solid media contained 1.5% agar (Davis) and were allowed to cool to approximately 50 °C before antibiotic(s) were added and plates were poured. Uninoculated plates were stored at 4 °C for 2-3 days prior to use.

2.2.1 Potato Dextrose Agar and Potato Dextrose Broth (PDA and PDB)

PDB contained 24.0 g dehydrated potato dextrose broth (Difco) per litre.
PDA was prepared by addition of 15 g/l of agar (Davis) to PDB.

2.2.2 Yeast Extract Glucose Medium (YEG Medium)

YEG medium contained 5.0 g yeast extract (Difco) and 2 % glucose (BDH) per litre.

2.3 BUFFERS AND SOLUTIONS

All buffers and solutions were prepared with MilliQ water.
The pH of solutions was adjusted with a pH meter (Model PHM 210, Radiometer Copenhagen).

Table 1

Fungal strains and plant genotypes used in this study.

Fungal strain or plant genotype	Relevant characteristic	Source or reference
Fungal Strains		
Neotyphodium lolii Lp19	*N. lolii* (LpTG-1) from *Lolium perenne*	Christensen *et al.*, 1993
KS1	*N. lolii* GUS-transformant (co-transformed with pAN7-1 and pFG gpd)	Saunders, 1997
FM 13	*Neotyphodium* Lp TG-2 (Lp 1, Christensen *et al.*, 1993) GUS-transformant (co-transformed with pAN7-1 and pNOM-102)	Murray *et al.*, 1992
Plant Genotypes		
Nui D	Seedling derived clonal line of cultivar "Grasslands NUI" #	Saunders, 1997
Nui G	Seedling derived clonal line of cultivar "Grasslands NUI" #	Herd *et al.*, 1997
Nui M	Seedling derived clonal line of cultivar "Grasslands NUI" #	This study
Nui UI	Seedling derived clonal line of cultivar "Grasslands NUI" #	This study
Nui UII	Seedling derived clonal line of cultivar "Grasslands NUI" #	This study
Nui UIII	Seedling derived clonal line of cultivar "Grasslands NUI" #	This study
Nui UIV	Seedling derived clonal line of cultivar "Grasslands NUI" #	This study
KLM TI	Seedling derived clonal line of cultivar "Grasslands KLM" $	This study
KLM TII	Seedling derived clonal line of cultivar "Grasslands KLM" $	This study
KLM TIII	Seedling derived clonal line of cultivar "Grasslands KLM" $	This study
KLM TIV	Seedling derived clonal line of cultivar "Grasslands KLM" $	This study
KLM TV	Seedling derived clonal line of cultivar "Grasslands KLM" $	This study
Greenstone SI	Seedling derived clonal line of cultivar "Grasslands Greenstone" $	This study
Greenstone SI	Seedling derived clonal line of cultivar "Grasslands Greenstone" $	This study
Greenstone SIII	Seedling derived clonal line of cultivar "Grasslands Greenstone" $	This study
Greenstone SIV	Seedling derived clonal line of cultivar "Grasslands Greenstone" $	This study
Greenstone SV	Seedling derived clonal line of cultivar "Grasslands Greenstone" $	This study

Cultivar of *Lolium perenne.*

$ Cultivars of *Lolium perenne* x *Lolium multiflorum* tetraploid hybrids.

2.3.1 GUS Extraction Buffer

(Jefferson, 1987) contained 50 mM sodium phosphate [adjusted to pH 7.0 by mixing of 50 mM di-sodium phosphate with 50 mM sodium di-hydrogenphosphate (BDH)], 0.1 % (v/v) Triton X-100, 0.1 % (w/v) (BDH) sodium lauryl sarcosine (Sigma), 10 mM sodium-EDTA (BDH) and 10 mM ß-mercaptoethanol (Sigma, added prior to use).

2.3.2 GUS Assay Buffer

(Jefferson, 1987) was prepared by addition of 4-methylumbelliferyl-ß-D-glucuronide (Sigma) to GUS extraction buffer (Section 2.3.1) to a final concentration of 1.2 mM.

2.3.3 GUS Stop Buffer

(Jefferson, 1987) contained 0.2 M Na_2CO_3 (BDH) in water.

2.3.4 GUS Calibration Solution

contained 2 μM 4-methylumbelliferone (MU, Sigma) in GUS extraction buffer (Section 2.3.1).

2.3.5 Saline Solution

contained 8 g NaCl (BDH) per litre.

2.3.6 Lacto-Glycerol Solution

was an aqueous solution containing 20% lactic acid (BDH), and 50% glycerol (BDH).

2.3.7 Decolorisation Solution

contained 2 parts 95 % ethanol and 1 part Lacto-Glycerol Solution (Section 2.3.6).

2.3.8 Bleaching Solution

contained 2.5 g chloral hydrate (BDH) per ml water.

2.3.9 Mounting Solution

was an aqueous solution containing 25 % lactic acid (BDH) and 25 % glycerol (BDH).

2.3.10 Aniline Blue Solution

was prepared with 0.1 % (w/v) aniline blue (Michrome) in bleaching solution (Section 2.3.8).

2.3.11 10x Fungal Nuclear Staining Solution

contained 100 μg/ml Hoechst dye (H 33258, Sigma) in water.

2.3.12 10x Fungal Septa Staining Solution

contained 10 μg/ml fluorescent brightener Calcofluor white M2R (Sigma) in water.

2.3.13 Chlorine Bleach

was a 1:10 dilution of commercially available sodium hypochlorite solution (Regular Bleach, Sophora Products Ltd.) in water.

2.4 GROWTH AND MAINTENANCE OF FUNGAL CULTURES AND PLANTS

2.4.1 Fungal Isolation from Plant Tissues

Isolation of endophyte from leaf tissues was performed as described by Christensen *et al.* (Christensen *et al.*, 1998). All procedures were performed using aseptic technique. An endophyte-infected leaf was excised from a tiller and surface sterilised for five minutes in chlorine bleach (Section 2.3.13), followed by one minute in 80% (v/v) ethanol, rinsed with sterile MilliQ water and dried on sterile filter paper. Small tissue pieces were cut with a heat sterilised scalpel and transferred onto PDA (containing 2.5 μg/ml tetracycline, Sigma) and incubated at 22 °C in the dark. Fungal growth from plant tissues appeared usually after 4-6 days incubation, depending on the fungal isolate and tissue type.

2.4.2 Preparation of Inoculum and Growth in Liquid Cultures

All manipulations were performed using aseptic technique and sterilised media (Section 2.2), pipette tips, scalpels, micropestles and tubes for transfer of mycelium. For small-scale cultures, colonies obtained by isolation from plant tissues were sub-cultured in liquid medium as follows: a small square (about 2-3 mm in length and width) from the margin of a fungal colony (with a diameter > 20 mm) was removed with a scalpel and transferred into liquid growth medium for incubation.

Neotyphodium cultures were grown at 22 °C on potato dextrose agar (PDA, Section 2.2.1) or at 25 °C in nutrient broth (PDB, or YEG, Section 2.2.1). Liquid cultures were grown in 50 ml medium in 250 ml Erlenmeyer flasks fitted with cotton stoppers on a rotatory shaker (150 rpm, Orbit Shaker No. 3591; Labline Instr. Inc.). Growth in liquid cultures was terminated after 25-30 days of incubation.

On occasions, these liquid cultures were used as an inoculum for a subsequent liquid culture. In this case, aliquots of mycelium previously grown in liquid cultures were dispensed into 1.5 ml microcentrifuge tubes, harvested by centrifugation (13 000 x g, 5 min; Biofuge 13; Heraeus Sepatech GmbH), washed twice in saline, and ground and suspended with a micropestle (0030120973, Eppendorff) in growth medium. The suspensions (15-20 mg fresh weight per ml) were used for inoculation of fresh medium (one ml of inoculum per 50 ml of medium).

In experiments with GUS-transformed fungal strains, the presence of the genetic marker was verified prior to all inoculations by examination of GUS expression in mycelium. Therefore, separate pieces of mycelium from a colony were qualitatively analysed for GUS on microtitre plates as described (Section 2.6.3). After the presence of the GUS marker was confirmed, mycelium from the same colony was used as inoculum as described above.

2.4.3 Determination of Fungal Biomass as Dry Weight in Liquid Cultures

After thorough agitation in the flask, 1 - 3 ml of culture containing fungal mycelium were removed aseptically with automatic pipettes for transfer into pre-weighed microcentrifuge tubes with perforated lids. Mycelium was pelleted by centrifugation in a Biofuge 13 for 5 min at 15, 000 x g and washed two times with MilliQ water (centrifugation for 5 min at 15, 000 x g). Samples were frozen in methanol (-30 °C) and dried in the tubes for 16 hours in a freeze-drier with a Model JDX 220 double stage high vacuum pump (Levingston Bros. Ltd.). Immediately after removal from the freeze-dryer, tubes containing mycelium were sealed into an airtight box with desiccant (silica blue gel, BDH) for transfer to a balance. The dry weight of mycelium was then determined on an AE 100 automatic balance (Mettler) by subtracting the previously determined weight of the tube from the total weight of the tube with mycelium. Weight reduction of the tube in the freeze drier was accounted for by adding 0.2 mg to the weight determined (0.2 mg had been determined to be the average loss of a tube after freeze drying).

2.4.4 Plant Culture and Maintenance of Symbioses

Plants were grown in 0.6 l pots containing potting mix (AgResearch Grasslands) to which Osmocote slow-release fertiliser had been added. To initiate a new plant, a single tiller from an existing plant was transferred into a new pot. To ensure the presence of GUS-expressing endophyte in tillers used for further vegetative propagation, five tillers per plant and of uniform size and age were selected and marked. Three tissue pieces of the youngest mature leaf (Section 2.5.1.2) of each tiller were then examined for GUS expression in a qualitative microtitre assay as described in section 2.6.3. Tillers with uniform and strong expression of GUS were separated from the grass tuft and planted to initiate the growth of new plants.

To grow up a new plant, leaves and roots of a tiller were trimmed to 2-4 cm length, and the tiller base was inserted into the soil to a depth of 1-2 cm. Immediately after potting, tillers were watered to saturation. Plants were grown either in a glasshouse or in a controlled environment cabinet (see below, this section). They were watered 2-3 times per week (at each watering to full saturation: water was applied to the drainage pans under each pot, and plants were soaked for 15 minutes, then excess water was removed), and after one month fertilised. To each plant were applied weekly 80 ml of nutrient solution [prepared with a commercially available fertiliser ("Thrive") according to the recipe supplied by the manufacturer (Yates): 2 g nitrogen (3 % nitrate, 2.6 % ammonium, 21.4 % urea), 413 mg phosphate, 663 mg potassium per litre and trace elements (Co, Mn, Mb, Zn, Cu)]. Plants were maintained for 3-4 months. A new plant was then initiated as described above.

Plants were regularly examined for disease symptoms. Diseased plants were removed from experiments, and sprayed with Vydate (DuPont) or Omite (Uniroyal Chemical Company) for control of aphids and mites, respectively. Systhane (active compound mycobutanil, Rohm and Haas) or Karathane (Rohm and Haas) were sprayed to combat fungal diseases. All applications were carried out according to the supplier's instructions (Rohm and Haas). Diseased plants were sprayed on one day and the application repeated after five days. For experiments conducted to determine the effect of

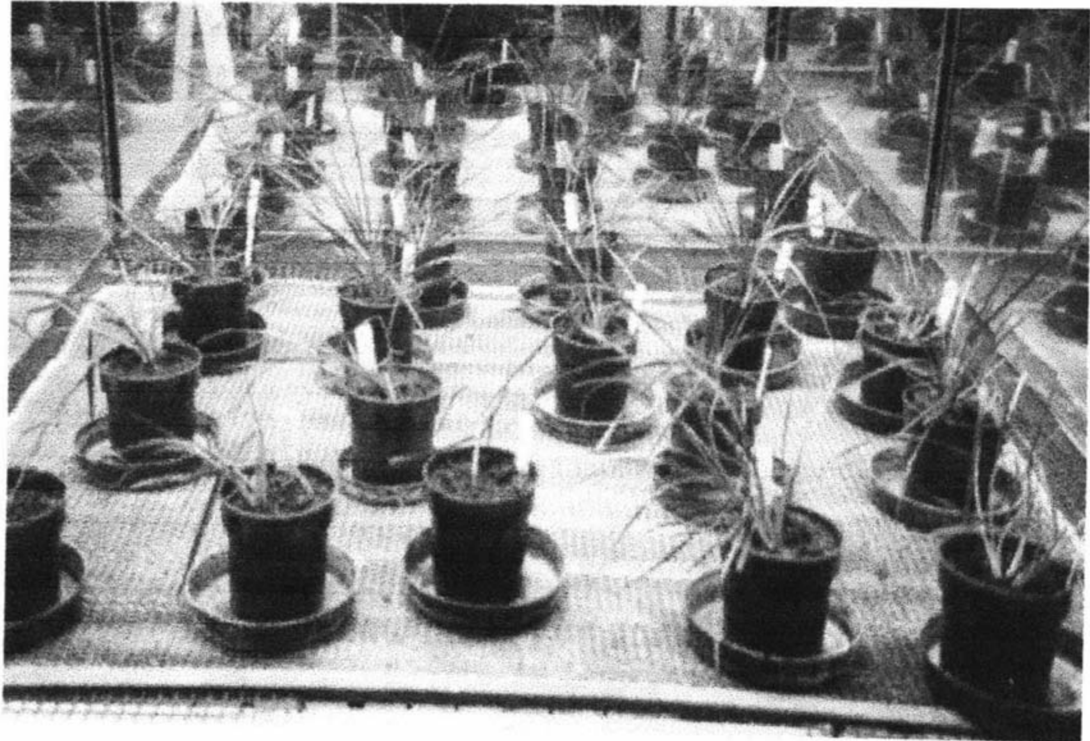
pesticides on the endophyte, the dosages for the two fungicides were modified as described in section 3.1.3.2.

In experiments aimed at determining the effect of a combined pesticide treatment on the endophyte, the insecticide Vydate was mixed with fungicides by adding 2 ml of a 0.5 % solution to 1 litre. Omite was added at a concentration of 4 g per 1 litre. One gram Karathane was dissolved into one litre of the pesticide mix prior to application. A volume of 0.5-ml Systhane stock solution (125 g per l) was added to one litre pesticide mixture (without Karathane added).

Spraying of the plants was performed with a hand held sprayer fitted with a vaporising outlet. All pesticides were applied for 10-20 seconds on plants until thorough coverage of leaves was achieved. Per plant 25-30 ml of the mixtures or water (controls) were applied.

Plants used for controlled environment studies were grown in a controlled environment growth cabinet (Temperzone limited; Fig. 3). An area of 110 x 120 cm was illuminated with two 1000 W halogen lamps (Philips) and six 400 W HPI-T lamps (Philips). The vertical walls enclosing the inner chamber were lined with mirrors for light dispersion. Light intensity at canopy height was 450 ± 30 µmol photons per square metre per second; measurements were taken at the four corners of the chamber and at four points along the median [measured with a quantum sensor (LI-190S, LiCor, Lincoln, Neb., USA) and recorded on a solar monitor (LI-1776, Li-Cor, Lincoln, Neb., USA)] at the beginning and completion of the experiments in this study. Lamps were situated 140 cm above the growing plants and a water screen was interposed to mitigate introduction of heat into the cabinet. The temperature in the cabinet was 15 ± 2 °C and plants were illuminated for 12 hours followed by 12 hours complete darkness. Plants were equally spaced on wire mesh and were weekly rearranged at random within the cabinet, to avoid possible effects of environmental gradients within the cabinet.

Figure 3
Grass plants grown and maintained in an environmentally controlled plant growth chamber. All experiments in this study were carried out on plants grown in this environment, if not indicated otherwise. Plants were grown at 15 °C and a 12 h light : 12 h darkness cycle.
See Section 2.4.4 for more details.



2.4.5 Inoculation of Plants with Endophyte Mycelium

Artificial plant infections of plants with endophyte mycelium were carried out as described by Latch & Christensen (Latch & Christensen, 1985). Grass seedlings were grown on water agar for six days to a length of about 2-6 cm. With a scalpel, each seedling was cut aseptically and longitudinally under a dissecting microscope at the region between mesocotyl and coleoptile. Into the resulting slit of 2-3 mm length a small piece of *N. lolii* mycelium was inserted. The mycelium used for the inoculations had been isolated from plant tissues (Section 2.4.1), and had been grown for 3-4 weeks (on PDA, Section 2.4.2). The presence of the GUS gene in the fungal isolates was verified for tillers from which the endophyte was isolated, and also checked on 4-5 small mycelial pieces taken from the margins of the colonies as described (Section 2.6.3). Seedlings were incubated for 7-10 days in the dark (25 °C) on water agar, followed by ten days in the light. Thereafter, they were planted in root trainers, and after one month examined under the microscope for the presence of hyphae in epidermal leaf sheath strips stained with aniline blue (Section 2.7).

2.4.6 Generating of Endophyte-Free Clonal Plants by Fungicide Treatment

Endophyte-infected tillers of plants were separated from tufts and thoroughly rinsed with tap water to remove all soil attached to the roots. Leaves and roots were trimmed to 2-4 cm length and tillers completely submersed in tap water containing 2 g l^{-1} Benlate [active compound benomyl (methyl 1,2-benimidazole carbamate), DuPont] and incubated for six hours at room temperature. After the initial incubation, tillers were planted into 300 g sand (with Osmocote slow releasing fertiliser added), containing 40 ml Benlate solution (final concentration of benomyl: 200 $\mu g\ g^{-1}$) in 150 ml plastic containers without holes for drainage, and then grown in the glasshouse. Every 2-3 days, pots were watered for maintenance of initial pot weight.

Newly emerging tillers were examined for the presence of fungal mycelium by microscopic examination of aniline blue-stained leaf sheath strips (Section 2.7) 4 - 5 weeks

after beginning of the treatment. After it was confirmed that they were free of endophyte, these tillers were transferred into new pots for further growth. In tillers emerging from the tillers previously re-potted, absence of the endophyte was again determined by microscopic examination and qualitative GUS assay (Section 2.6.3). These tillers were again separated from their mother tillers and grown up to plants that were then further propagated as described (Section 2.4.4). All other fungicide treated plants were discarded

2.5 HARVESTING OF PLANTS, ISOLATION OF ENDOPHYTE AND PLANT TISSUE EXTRACTIONS

2.5.1 Plant and Tiller Dissection for Analyses of GUS Activity and Alkaloids

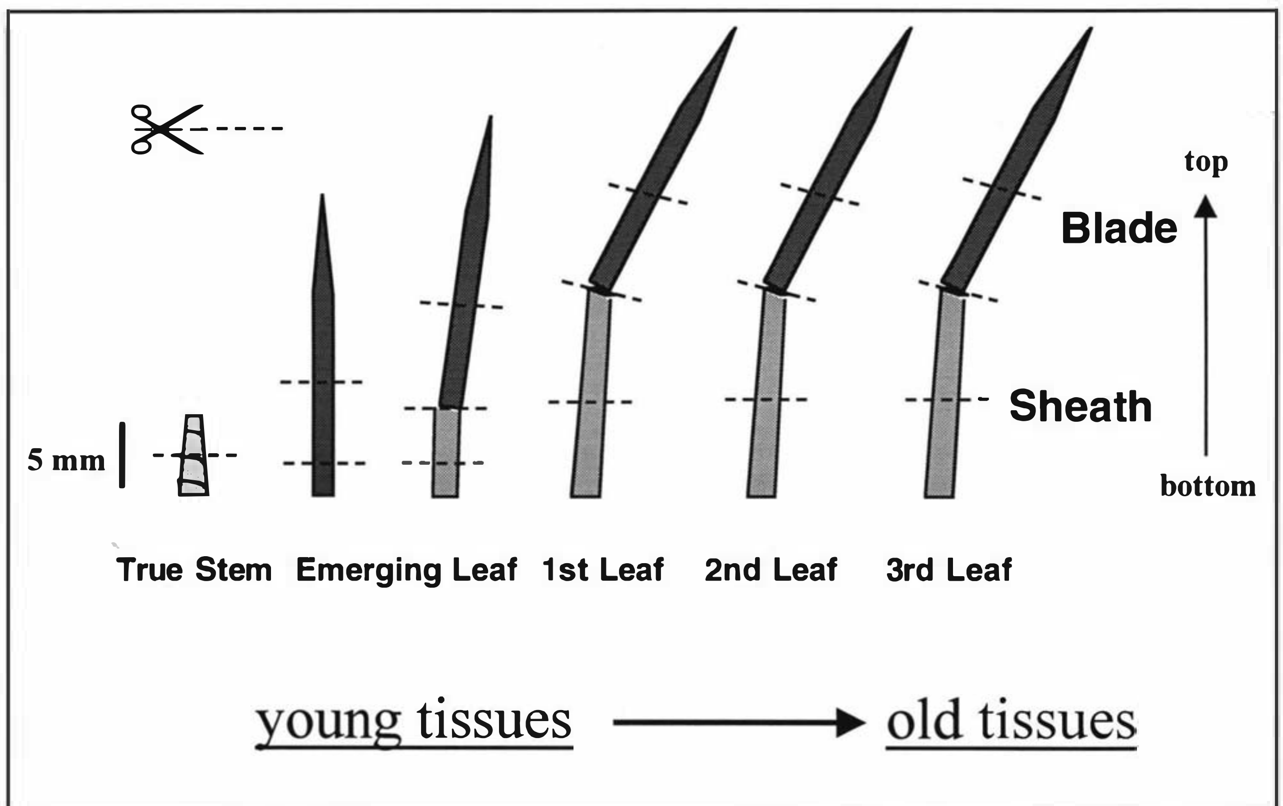
2.5.1.1 Plant Dissection

A whole plant was comprised of a grass tuft of about 30 – 120 tillers and was derived from a single tiller planted (Section 2.4.4). From these plant tufts, tillers were removed by a careful cut at the tiller base including 1-2 cm of roots. Plants from which tillers had been removed were not sampled for additional tillers for at least one week.

2.5.1.2 Tiller Dissection

A diagrammatic view of a dissected tiller is shown in Fig. 4. All dissections were performed under a dissecting stereomicroscope (217146 Olympus). A single tiller typically consisted of 1-3 mature leaves and 1-2 emerging leaves. Mature leaves were identified by the presence of a fully developed ligule, a thin protuberance on the upper side of the leaf, dividing the leaf into sheath and blade. Each mature leaf was removed from a whole tiller by first slitting the leaf sheath with a fine tip of a pair of tweezers. The leaf was carefully peeled away and gently torn off from the base of the tiller. Mature leaves were numbered according to leaf age and further fractionated as indicated in Fig.4.

Figure 4
Dissection scheme of a grass tiller. Dashed lines indicate where leaf sections were cut for sampling. Emerging leaves (longer than 20-30 mm; younger leaves were discarded) were differentiated into two types, according to their developmental stage (sheath formation), and fractionated as indicated. The lowest emerging leaf part (10-15 mm) constituted (sheath or blade) meristem, further sections were classified as mid-leaf and top (blade). Sheaths (light grey) and blades (dark grey) were separated into bottom/lower and top/upper sections, respectively. Scale bar indicates for the dimension of the true stem only. The true stem was fractionated into lower stem and stem apex.



Emerging leaves were identified by the absence of a fully developed ligule and were dissected for fractionation as described in Fig. 4. The true stem (the region at the base of the tiller, Fig.4) was divided into stem apex and stem (the region between the attachment point of the first mature leaf and roots).

2.5.2 Simultaneous Assessment of GUS Activity and Alkaloids in Plant Tissues

Tissues were harvested and fractionated (Section 2.5.1.2) and ground in liquid nitrogen as described (Section 2.5.3). Endophyte-linked GUS activity concentration was determined on weighed plant tissue samples of between 5–12 mg dry weight as described (Section 2.6.1). Alkaloid analyses were carried out three to eight weeks after harvests on subsamples from the same plant material assessed for GUS activity. Ergovaline and peramine were simultaneously extracted from subsamples and quantitatively analysed as described (Sections 2.8.1, 2.8.3.3, 2.8.3.4). Lolitrem B was extracted and analysed on further subsamples as described (Section 2.8.2.2, 2.8.2.3).

2.5.3 Extraction of Endophyte-Linked GUS Activity from Fungal Mycelium and Plant Tissue

Freeze-dried mycelium (4 - 8 mg dry weight) was mixed with ice-cold GUS extraction buffer (600 µl, Section 2.3.1), and the mixture was homogenised with a micropestle (0030120973, Eppendorff) for 5 minutes in 1.5 ml microcentrifuge tubes. Tubes containing buffer with the homogenised mycelium were then placed into an ethanol ice-bath for cooling, and the mycelium was further disrupted using a ultrasonic disintegrator (Soniprep-150, MSE Scientific Instr.) fitted with an exponential titanium microprobe (tip diameter 3 mm, transformation ratio 7:1). For sonication, the tip was immersed into the suspension to a depth of about 0.5 cm. A sonicator setting of 15 µm was used. Taking into account the transformation ratio of the microprobe, the actual movement of the tip in the solution was 105 µm. Bursts for 15 seconds were followed by 15 seconds cooling of samples. Ten bursts were applied to the mycelium. Mycelial debris in the

extracts was pelleted by centrifugation (15 000 g, 5 min, Biofuge 13, Heraeus) and the supernatant collected and kept on ice at 4 °C for GUS determination with the fluorometric quantitative assay (Section 2.6.1). For experiments aimed at determining the extraction efficiency from mycelium, the mycelium was subjected to 4, 8, 10, 12, 15, 18, 20, and 22 bursts. Mycelial breakage was then also examined under the microscope (470600-9901, Zeiss Ikon; 400fold magnification).

Extractions of plant tissues were carried out as described by Herd *et al.* (1997). Plant tissue was harvested and placed into microcentrifuge tubes with perforated lids. These were transferred into a styrofoam insulated metal block pre-cooled to – 70 °C. To each sample, liquid nitrogen was applied, and the tissue was quickly ground with a micropestle (0030120973, Eppendorff). During the grinding, care was taken that the tube was always filled with nitrogen. The resulting fine powder was freeze-dried for 16-20 hours as described previously (Section 2.4.3). Immediately after drying, the dry weight was determined with a Mettler AE 100 automatic balance. If GUS activity was not determined immediately, samples were treated as described in the following: ground and freeze-dried plant tissue samples were purged with nitrogen for 10-20 seconds, and sealed in the tubes for storage at –20 °C. GUS activity in these samples was measured within one week (it was assessed that storage under nitrogen for up to ten days did not measurably affect GUS activities, data not shown; longer storage periods were not assessed).

To 10-15 mg of freeze dried material were added 600 μl GUS extraction buffer, and GUS activity was extracted as previously described for fungal mycelium (this section), except that only 4 sonication bursts were used for the extraction.

2.6 ß-GLUCURONIDASE (GUS) ASSAYS

2.6.1 Quantitative Extractive Assay

GUS enzyme activities in plant tissue and mycelial extracts were quantitatively analysed with a modification of the method described by Herd *et al.* (1997). Assays were

carried out in a final volume of 200 µl GUS assay buffer (Section 2.3.2). Extracts from endophyte mycelium, prepared as described in Section 2.5.3, were 100-fold diluted in GUS extraction buffer (Section 2.3.1), and 10 µl of the diluted extract were used for the assay.

Prior to mixing, extracts and assay buffer were equilibrated to 37 °C in 1.5 ml microcentrifuge tubes placed in a water bath (JB1, Grant Instruments Ltd.). Enzyme reactions were started by addition of the extracts to the assay buffer containing the GUS substrate MUG (final concentration 1.2 mM, Section, 2.3.2). At regular time points (usually every 10-15 min), 40 µl assay volume were removed and reactions stopped by addition to 1.95 ml GUS stop buffer (Section 2.3.3). Per assay 3-4 time points were taken.

Using a Hoefer TK 100 fluorometer (excitation 355 nm, emission 460 nm; Hoefer Scientific Instr.), fluorescence in stop buffer was read in a fluorometry glass cuvette (TKO 105, Hoefer Scientific Instr.) as arbitrary units. For conversion of fluorescent units into amounts of the fluorescent GUS product in stop buffer, the fluorometer was calibrated according to the manufacturer, prior to measurements with GUS calibration solution (Section 2.3.4). The fluorometer was set to zero with stop buffer only, and then 50 µl calibration solution were added to 1.95 ml stop buffer. The cuvette was inserted and the fluorometer was quickly adjusted, by setting of the appropriate scale knob, to a reading of 500. After calibration, a fluorometer reading of 1 equalled 0.2 pmoles of the fluorescent product MU in 2 ml stop buffer.

Readings of fluorescence at regular time points were plotted and linear regression on increase of fluorescence over time performed. The slope of the regression (= increase of fluorescence per min) was multiplied by the factor (= 0.2, see above, this section), converting fluorescence to amounts of GUS product to calculate increase in product (methyl-umbelliferone; MU) as pmol MU min^{-1}.

Enzyme activity in the whole extracts was calculated by multiplying the increase with a factor taking account of the dilution steps:

$$\text{Factor} = \frac{\text{assay volume } (\mu l)}{\text{volume removed per time point } (\mu l)} \times \frac{\text{extract volume}}{\text{volume of extract used in assay } (\mu l)}$$

The enzyme activity in a whole extract was then used to calculate GUS activity per dry weight of the tissue used in the extraction.

In some plant tissues interference with the quantitative GUS determination from extracts was detected, leading to an underestimation of GUS activity in these tissues (Section 3.1.9). GUS activity values obtained from extracts of these tissues were therefore corrected, using the factor determined in Section 3.1.9 (Fig. 14), taking into account the weight of the sample extracted and the volume of the extract used in the assay.

2.6.2 Semi-quantitative Microtitre Plate GUS Assay

Multiple transverse cuts from a grass leaf (1 mm length, 2-3 mm width) containing GUS-expressing endophyte were transferred into wells of a 96-well microtitre plate (Nunclon). The outermost columns and rows of the plate were thereby excluded. To each tissue piece were added 100 µl GUS assay buffer (Section 2.3.2). After incubation for 1-2 hours at 37 °C in the dark, microtitre dishes were placed onto a UV TMW-20 Transilluminator (Alpha Innotech Corp.), preheated for at least 5 min.

A digital gel documentation system (Alpha Innotech Corp.) was used for quantitative determination of fluorescence in each microtitre well. A microtitre dish was placed on the transilluminator, and an image capturing 8 wells was acquired with a digital camera (Model 4912 2010/0000, heliopan; Germany) at a fixed height (55 cm), aperture (5.6), zoom (33-35), focus (1.6), and exposure (1 s). The ELISA function was called up

from the window of the image analysis system program (IS 1000, Alpha Innotech Corp.), and ELISA area rings where adjusted (size 20) for measurements of light intensities in individual wells. Light density in each well was determined as arbitrary units. A calibration curve was created with sequential dilutions of known concentrations of the fluorescent product MU (ranging from 0.2 – 3 nmoles per 100 µl GUS extraction buffer), using the same settings. Light density values were then plotted against amounts of MU per well.

Fluorescence in wells with plant tissue pieces was assessed at 3-4 regular time points (every 60 - 90 min). Using the fluorescence measured, amounts of MU were determined, using the regression equation of the calibration curve. With linear regression of amounts of MU per well over time, GUS activity was determined as pmol MU per min per tissue piece.

2.6.3 Qualitative Microtitre Plate GUS Assay

Samples from endophyte-infected leaves were prepared and distributed on microtitre plates as described (previous section). If fungal mycelium was used, small pellets, or mycelial fragments of liquid culture or from plates were placed into wells of the microtitre plate. After incubation for 2 - 3 hours in GUS buffer with substrate as described (previous section), the plate containing plant tissue or fungal mycelium was examined under UV on the transilluminator as used for semi-quantitative determination of GUS (previous section). The presence of GUS activity was analysed qualitatively by comparison of the fluorescence observed with a negative control (endophyte-free plant tissue pieces, endophyte without the GUS gene).

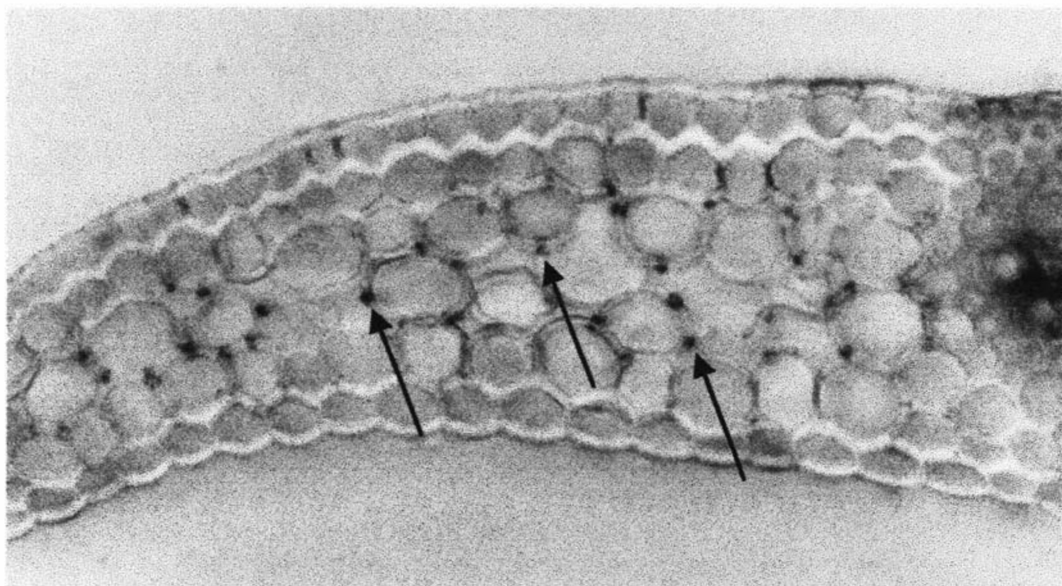
2.7 DETERMINATION OF HYPHAL BIOMASS CONCENTRATION IN PLANT TISSUES

Leaves were sampled and dissected as described (Sections 2.5.1.1, 2.5.1.2). Immediately after sampling, each leaf section was weighed on a Mettler AE 100 automatic

balance, and the fresh weight recorded. Sections were then measured with a ruler under a dissection microscope (217146 Olympus) and the length of each recorded. Transverse sections (3-5 mm) were then cut from either side of a leaf section. These were used to determine hyphal numbers in a cross-section. Therefore, they were cleared and stained as follows (Y.Y. Tan, personal communication, Massey University): each section was placed into a 1.5 ml microcentrifuge tube and 0.5 ml decolorisation solution (Section 2.3.7) were added. Leaf sheath and emerging leaf sections were incubated at room temperature for 2-4 hours, and leaf blade sections for 20-24 hours, or until leaf pigments were completely removed. After clearing of the sections, they were transferred into 50% (v/v) ethanol for 15 minutes, washed in MilliQ water and mature leaf tissues placed into 0.5 ml bleaching solution (Section 2.3.8) for 16-20 hours. For emerging-leaf sections (except for the emerged leaf blade part) the bleaching solution step was omitted.

Cutting of a cleared transverse section for microscopic examination was performed in mounting solution (Section 2.3.9), with a scalpel under a stereomicroscope (217146; Olympus), on a microscope slide to obtain fine (< 0.05 mm in width) transverse sections. A cut section was placed, with the cross-section exposed for examination, into a drop of aniline blue solution (Section 2.3.13) and sealed with a cover slip. To hasten the staining, and for removal of air-bubbles the slide was gently passed through a flame. After cooling, the cross-section was examined under the microscope (470600-9901; Zeiss Ikon) at 400x magnification. Aniline blue-stained hyphae appeared as blue dots between cells of the leaf mesophyll (Fig. 5). The number of hyphae in a cross-section was determined by scanning the entire width of the cross-section and counting of all hyphae in the section with a manual cell counter. Counts were made on two specimens per cleared leaf section. For determining the average hyphal number in a cross-section of a leaf section, the hyphal numbers per cross-section, determined on the cleared and stained sections previously cut from either side of the leaf section, were pooled.

Figure 5
Light microscopy of a cross section (400x magnification) through a leaf section (young leaf sheath) of an endophyte-infected plant. The leaf tissue was cleared from background and incubated in the selective stain aniline blue as described (Section 2.7). Endophyte hyphae stained by aniline blue are visible as blue dots (some indicated with arrows) between the mesophyll cells of the leaf.



The hyphal biomass in the whole leaf section was calculated as a volumetric unit (mm^3) with the equation:

$$V = \mathbf{n} \times \mathbf{r}^2 \times \mathbf{h} \times \pi$$

With **n** being the average number of hyphae in the section, **r** the hyphal radius, **h** the length of the section, and $\pi = 3.14$.

The hyphal radius was determined with a calibrated eyepiece micrometer at 1000x magnification (with immersion oil) in transverse leaf cross-sections, after clearing and staining with aniline blue (carried out as described above). The width of a cross-section was scanned for hyphae, and the diameter (= 2 x radius) of each hypha appearing in the field of vision assessed, until more than 50 hyphae per sampling had been measured.

Fungal biomass concentration was assessed per leaf dry weight (mm^3 endophyte biovolume per mg dry weight). The dry weight of a leaf section was estimated from the previously determined fresh weight of the section (see above, this section). Therefore, the fresh weight was converted to dry weight with the use of the appropriate fresh weight-dry weight coefficient for the tissue type examined, given by the equations determined as described in Fig. 11.
The equations used were:

Leaf sheath:	$DW = -0.141 + 0.388 \times FW$
Leaf blade:	$DW = 0.092 + 0.332 \times FW$
Emerging leaf:	$DW = -0.025 + 0.291 \times FW$

To check the presence of endophyte hyphae in plant tissues, the method by Latch & Christensen (Latch & Christensen, 1985) was used on the leaf sheath of the youngest mature leaf of a tiller. The leaf was removed from the tiller and placed under a stereo microscope (217146 Olympus). The epidermis of the adaxial side of the leaf sheath was cut with a scalpel, and a fine pair of tweezers used to strip away 1-5 mm of the epidermis. The specimen was transferred into a drop of aniline blue solution (Section 2.3.13) on a

microscopy glass slide and the slide carefully heated in a flame to remove air-bubbles, and for rapid staining of hyphae. After cooling, the leaf sheath strip was examined at 400 x magnification in mounting solution (Section 2.3.9). Hyphae appeared as blue, sparsely branched strands between cells extending in direction of the leaf axis.

2.8 ANALYSES OF ALKALOIDS IN PLANT TISSUES

Samples used in alkaloid analyses were either ground in liquid nitrogen as described for plant samples in section 2.5.3 and freeze-dried for 24 h, or freeze-dried and milled in a coffee grinder. If necessary, material was stored in airtight plastic bags at –20 °C for 1-2 months. This period does not affect stability or extractability of the alkaloids examined (G.A. Lane, B.A. Tapper, personal communication, AgResearch Grasslands). Plant tissue samples were weighed on an AE 240 automatic balance (Mettler). Immediately before aliquots were removed for extraction, the material was thoroughly mixed, as with storage and handling of samples separation into different-sized particles occurred. Centrifugation during preparations was carried out with a Megafuge 1.0 (Heraeus Sepatech GmbH). HPLC data was recorded and chromatograms analysed with a Class GC data system and software (Shimadzu).

2.8.1 Extraction and Quantitation of Peramine

2.8.1.1 Extraction with Isopropanol-Lactic Acid

This procedure was a modification of the method of Barker *et al.* (1993) to allow simultaneous extraction of peramine and ergovaline. Extractions were carried out in 2.0 ml plastic vials (Sarstedt) with aqueous isopropanol - 1% (w/v) lactic acid (1:1 v/v). The efficiency of the modified method was tested by serial extractions on endophyte-infected plant material, and gave results equivalent to those obtained with the method of Barker *et al.* (1993) (B.A. Tapper, personal communication, AgResearch Grasslands). The extraction was carried out as described for ergovaline extraction (Section 2.8.3.3). The solvent for the extraction was prepared with a standard amount of the nitrate hydrogen salt

of homoperamine [3(-4'-guanidinylbutyl)-2- methylpyrrolo[1,2-a]pyrazin-1(2H)-one] (obtained from AgResearch Grasslands, about 1.5 µg of the free base in 0.5 ml MeOH) as internal standard for the quantitation of peramine.

2.8.1.2 Separation and Quantitation of Peramine by HPLC

Chromatography of peramine and homoperamine was carried out by a modification of procedures described by Barker *et al.* (1993) and Cox and Stout (1987), utilising a column-switching procedure for initial ion-exchange separation for the removal of compounds likely to interfere with the quantitative detection. This procedure was facilitated by a six port electrically-switched controlled valve system (Valco Instruments). An aliquot of extract (15-30 µl) was injected (SIL-9A autoinjector, Shimadzu) into a stream of 0.4 ml/min (M-6000A pump, Waters Associates) of methanol:water:ammonium hydroxide (33 %); 670:330:6 (v/v), onto a silica-based weak anion exchange cartridge (Macrosphere 300 WCX All-Guard, 7.5 x 4.6 mm, 7 µm, Alltech Associates) for two minutes. Peramine and the internal standard homoperamine were selectively retained in the cartridge, while other UV absorbing compounds were washed through to waste. The cartridge was back flushed at 1 ml/min with a solvent of 50 mM ammonium acetate, 5 mM guanidinium carbonate, 0.2% (v/v) acetic acid in water:methanol (4:1 v/v) with an Perkin Elmer LC Pump 250 on to a silica HPLC column, 250 x 4.6 mm (Sphereclone 5 µm, Phenomenex) for separation of peramine and homoperamine. Peramine and homoperamine peaks were detected by UV absorption at 280 nm (UV-970 detector, Jasco Corp.). Peramine concentrations in extracted samples were determined from chromatograms with the following equation:

$$\text{Concentration of analyte} = \frac{\text{MW of analyte x mass of standard x peak ratio analyte:standard}}{\text{MW of standard x mass of sample}} \ \mu g\ g\text{-}1$$

Calculations were performed using digital data collection with Class CG 10 software (Shimadzu).

2.8.2 Extraction and Quantitation of Lolitrem B

2.8.2.1 Extraction with Chloroform-Methanol

The procedure was carried out as described by Barker *et al.* (1993), with 50 mg dried plant tissue per sample. To each sample was added a chloroform-methanol mixture (1 ml, 2:1 v/v) and extraction allowed to proceed for 1 h. The extraction solvent was separated by aspiration through an immersion filter, and residual material was rinsed with two further 0.5 ml portions of solvent. The solvent was evaporated under reduced pressure and residues were taken up in 1 ml of 1,1-dichloroethane-acetonitrile (4:1 v/v). The extract was transferred through a syringe filter into HPLC sampling vials.

2.8.2.2 Extraction with 1,2-Dichloroethane, 10% Methanol

To allow disruption and extraction of samples directly in plastic vials (2.0 ml, Sarstedt), a modified method developed by B.A. Tapper (AgResearch Grasslands), utilising 1,2-dichloroethane-methanol (9:1 v/v) was used. This solvent was chosen because of its lower volatility compared with chloroform, important in avoiding evaporative losses in the absence of an internal standard, its compatibility with polypropylene extraction vials, and as to minimise chromatographic interference with lolitrem B. The efficiency of recovery of lolitrem B from reference samples and from standard additions was found to be equivalent to that of the chloroform-methanol method (B.A. Tapper, personal communication, AgResearch Grasslands). The preparation and analysis of samples was carried out as follows, using methods developed at AgResearch Grasslands (B. A. Tapper, personal communication).

After addition of four 0.5 mm glass beads, weighed samples (5-50 mg dry weight) were suspended in 0.5 or 1 ml solvent, tightly capped and agitated for 1 min in a cell disrupter (FP 120 Savant FastPrep; BIO 101 Inc.) followed by extraction (room temperature, darkness) for 60 min on an orbital shaker. Extracts were separated by filtration through polyethylene filters fitted between two vials under centrifugation (6000

g, 5 min). The filtered extracts were transferred into HPLC vials and analysed for lolitrem B immediately thereafter, or stored for 1 – 2 days at –20 °C in sealed HPLC vials.

2.8.2.3 Separation and Quantitation of Lolitrem B

For quantitative analysis of lolitrem B, aliquots of 15 – 30 µl of extract were injected (SIL-10A autoinjector; Shimadzu) into a silica column (Sphereclone, 5 µm, 250 x 4.6 mm, Phenomenex) at 29 °C with acetonitrile - dichloromethane - water (BDH, 14:86:0.1 v/v), and eluted at a flow rate of 1 ml/min (LC 10 AD pump, Shimadzu). Lolitrem B was detected by fluorescence (RF 535 detector; Shimadzu, λ_{ex} 268 nm and λ_{em} 440 nm). Concentrations of lolitrem B in samples were estimated by comparison of the integrated peak areas with those of pure lolitrem B (Barker *et al.*, 1993), kindly provided by Dr C. O. Miles of AgResearch Ruakura, Hamilton, New Zealand, in the same batch of samples, taking into account the sample weight.

2.8.3 Extraction and Quantitation of Ergovaline

2.8.3.1 Chloroform-Methanol-Ammonia Extraction

The extraction procedure was carried out as described by Barker *et al.* (1993). Freeze-dried plant material (2-3 g) was finely milled in a coffee grinder and a sub-sample (50 mg) weighed into a small glass vial. To each sample was added a known amount of di-ergotamine-monotartrate (< 1 µg, Sigma) in 50 µl methanol-25 mM aqueous tartaric acid (BDH, 1:1 v/v) as an internal standard, and chloroform-methanol-ammonia (75:25:2 v/v, 1 ml) as extraction solvent. Samples were allowed to stand overnight at room temperature in the dark. After extraction, the solute was separated by aspiration through a filter and the residual plant material rinsed with two 0.5 ml portions of extraction solvent. The solvent was evaporated (1-2 hours) under reduced atmospheric pressure in 10 ml vials. The dried residue was suspended in and partitioned between carbon tetrachloride (0.5 ml, BDH) and a methanol - 25 mM aqueous tartaric acid solution (0.5 ml, 1:1 v/v, BDH). Upon standing

two phases separated, and the upper (aqueous) fraction was separated and filtered (0.2 μm filter), and transferred into a glass vial for HPLC analysis (Section 2.8.3.4).

2.8.3.2 Acetic Acid Extraction

Extraction was carried out essentially as described by Shelby & Flieger (1997). Milled samples (20 mg) were extracted with aqueous acetic acid (20% v/v, BDH) in 2.0 ml plastic vials (Sarstedt), for 1 h in darkness, at room temperature, without agitation. Samples were re-extracted overnight with the same solvent (darkness, without agitation). Extracts were filtered as described (following section) and analysed by HPLC (Section 2.8.3.4).

2.8.3.3 Isopropanol-Lactic Acid Extraction

A known quantity of between 1-50 mg of milled or ground dried plant tissue was weighed into 2.0 ml plastic vials (Sarstedt). A defined amount of between 0.3 - 1 ml 50% (v/v) aqueous isopropanol (BDH) + 1% (w/v) lactic acid (BDH) containing < 1 μg di-ergotamine-monotartrate (Sigma) was added to each vial, and vials were vortexed for 60 seconds in a cell disrupter (FP 120 Savant FastPrep; BIO 101 Inc.). Samples were extracted for 2 hours in darkness, at room temperature, without agitation. Particulate material in the extract was removed by filtration (6000 g, 5 min, Macrofuge 10, Heraeus) through a polyethylene filter, fitted between two extraction vials. Extracts were analysed immediately after preparation or stored for 1–2 days at –20 °C in sealed HPLC vials.

2.8.3.4 Separation and Quantitation of Ergovaline

Separation and quantitation of ergovaline by HPLC was carried out essentially as described by Barker *et al.* (1993). An aliquot of extract (10-30 μl) was injected with an 851 AS autosampler (Jasco Corp.) onto the HPLC column. Ergovaline and ergotamine and their respective isomers, ergovalinine and ergotaminine, were separated by reverse phase HPLC (Prodigy C18 column, 5 μm, 100 x 4.6 mm, Phenomenex) with a RP-18 Newguard pre-column) at 28 °C, with a gradient solvent system of 0.1 M ammonium acetate in water

and aqueous acetonitrile. Linear binary gradients were run as follows: 0 min, 95 % (v/v) solvent A, 5 % (v/v) solvent B; 20 min, 80% solvent A, 20% solvent B; 35 min, 50% solvent A, 50 % solvent B; 40 min, 30% solvent A, 70% solvent B; 47 min, 70% solvent A, 30% solvent B; 52 min, 95% solvent A, 5% solvent B. Solvent A was acetonitrile – aqueous ammonium acetate (0.1 M) (1:3 v/v); solvent B was acetonitrile – aqueous ammonium acetate (0.1 M) (3:1 v/v). The flow rate during runs was 1 ml/min, controlled by a PU-980 pump (Jasco Corp.). Each compound was identified and measured by fluorescent detection (λ_{ex} 310 nm, λ_{em} 410 nm, RF 551 detector, Shimadzu).

The concentration of ergovaline in the sample was estimated as described for peramine (Section 2.8.1.2), except that the analyte : standard chromatographic peak area ratio was based on the combined peak areas of the isomeric pairs ergovaline and ergovalinine, and ergotamine and ergotaminine. By pooling of the isomers, total amounts of standard and analyte remained largely constant, as over time isomerisation, that is, formation of the –inine isomers, increased (Section 3.1.4.4).

2.9 FLUORESCENT MICROSCOPY OF NUCLEI AND SEPTA IN FUNGAL MYCELIUM

Mycelium was harvested from liquid culture, washed with saline as described (Section 2.4.2), and suspended in saline using a micropestle (0030120973, Eppendorff). An aliquot (50 – 100 µl) of this suspension was transferred into 10x fungal nuclear staining solution (Section 2.3.11) to give a final concentration of 10 µg/ml of Hoechst dye, and vortexed. After incubation for 1 hour at room temperature in the dark, 10x fungal septa staining solution (Section 2.3.12) was added (to a final concentration of 1 µg/ml calcofluor white), and the suspension quickly vortexed. A drop of the suspension was placed on a microscope slide and sealed with a cover slip. A Diaphot-TMD (Nikon) inverted microscope with "TMD-EF" (Nikon) epi-fluorescent equipment and a DM 400 (Nikon) UV filter box fitted with a 450 nm barrier filter was used to examine the stained mycelium under UV light. The stained septa and nuclei in hyphae were visualised by fluorescence at 365 nm excitation. An F-601 camera (Nikon) with a Fuji Neopan 400 film was used for photographic documentation.

2.10 OBSERVATIONS DURING PLANT GROWTH

2.10.1 Tiller Number and Tillering Rate

Tiller numbers per plants were obtained by manually counting all tillers present. New tillers were included as soon as tips of their leaves appeared between leaves of the respective mother tiller.

2.10.2 Leaf Extension Rates

Leaf extension was determined by placing a small drop of ink at the area of the emerging leaf encircled by mature leaves; the distance of the ink dot from this point after growth for 24 hours was then measured with a ruler.

2.11 ASSESSMENT OF LEAF NET-PHOTOSYNTHESIS

Measurements of leaf net photosynthesis were conducted with a single-chamber open infrared gas analysis system.

2.11.1 Principle of the Open Infrared Gas Analysis System

In an open or differential system, air is drawn from the environment into the system and passed through a leaf chamber. With an infrared gas analyser (IRGA), the difference in CO_2 concentration between the chamber entrance (measured in a reference chamber) and outlet is measured. The incoming air is passed through an air conditioning system for control of humidity, and airflow to reference and leaf chamber is measured and controlled by flow meters and flow controllers. To determine CO_2 assimilation of leaves, it

is necessary to use an IRGA in calibrated differential mode, and that flow rate of air and actual humidity, as well as leaf surface area are known (Long & Hällgren, 1993).

2.11.2 Configuration of the Analysis System for Measurements of Leaf Net Photosynthesis

For all experiments, a brass metal leaf chamber (50 mm wide, 70 mm deep, 4 mm high, Griffiths Engineering) based on the design by Field *et al.* (1982) was used. Into the lid of the chamber, a 44 x 60 mm glass top was fitted for illumination of the inner chamber, and the rims of the lid were lined with styrofoam for sealing of the leaves into the chamber. Temperature of the incoming air was regulated by a computer-controlled peltier element (12 V, 14 mm^2, Tropicool), integrated into the chamber at the air inlet, and a fan (41 mm^2, Advanced Technology Company), facilitated airflow in the chamber. The CO_2 concentration was measured using an infrared gas analyser (Binos, Leybold Heraeus), and water vapour pressure was determined from dew point measurements (Dew Point Hygrometer, 1100DP; General Eastern Instruments Corporation). Additional control of the internal chamber air temperature was facilitated by a water jacket, built into the interior of the chamber and connected to a refrigerated water bath (RB5; Techne Corporation). Control of water vapour in the leaf chamber was achieved by circulating air in water through a fine bubbler, maintained in a refrigerated water bath (LTD6; Grant Instruments). The airflow rate was controlled by a mass-flow controller (AFC 2600 Pro; Aalborg Instruments) and measured by mass-flow meters (GFM 1700 E; Aalborg Instr.). A temperature electrode at the abaxial side of a leaf and inserted into the chamber was used to determine the actual leaf temperature during experiments. During measurements, CO_2 concentration in air entering the chamber was assessed after passing for 30 seconds through the IRGA; airflow through the IRGA was then switched as to allow 30 seconds of equilibration with air leaving the chamber. In this way, the differential between the two CO_2 concentrations, equalling the carbon exchange rate across the leaf was logged and recorded every 60 seconds, simultaneously with chamber and air temperature, and humidity. The instruments and data were controlled and monitored using a Macintosh LC computer with A/D converter (Lab LC, National Instruments Corporation), and a purpose-

written programme using data-acquisition software (Lab View 2, National Instruments Corporation).

2.11.3 Measurements of Leaf Net-Photosynthesis

For measurements of CO_2 exchange rates, grass leaves were inserted and sealed into the chamber, while being attached to the plant. For illumination, an incandescent light source (Philips) situated above the leaf chamber was used, and incident light was measured with a light meter prior and after each measurement. For heat dissipation, a fan was installed between chamber and light source.

All measurements were performed at ambient CO_2 (360 µmol mol^{-1}), on the youngest mature leaf of a tiller. Each measurement was carried out on two leaves from two different tillers. After sealing the leaves in the leaf chamber, photosynthesis was allowed to stabilise and monitored for about 20-30 min, until a constant rate was reached. Leaf areas were determined after completion of each measurement, for calculation of CO_2 exchange per unit leaf area. The computer-logged photosynthesis data were entered into formatted data spreadsheets and photosynthesis per leaf area determined.

2.11.3.1 Determination of Light Response

Prior to all measurements, for complete light-induction of photosynthesis, plants were exposed for at least two hours to the light in the controlled environment in which plants were grown (450 µmol m^{-2} s^{-1}). In the experiments, leaves were inserted into the leaf chamber, and leaf photosynthesis was allowed to equilibrate to a constant level (usually reached after 20 min) at 20 °C leaf temperature, and a photon flux density of 1900 µmol m^{-2} s^{-1}. By interposing neutral density filters (Coherent Scientific Pty, Ltd.) of varying transmittance, incident light was gradually reduced to photon flux densities of 1550, 1270, 1000, 800, 500, 460, 370, 240, 175 and 0 µmol photons m^{-2} s^{-1}. At each light intensity step, leaves were illuminated for 8-10 minutes.

As light intensity could not be determined simultaneously with measurements of photosynthesis, before and after experiments a light calibration was carried out with the filters used (variation was less than 10% at each light intensity step). Temperature changes of leaf temperature with varying light intensities were corrected by adjustment of the air temperature as described (Section 2.11.2).

2.11.3.2 Determination of Temperature Response

As described previously (previous section) plants were pre-exposed to light for induction of photosynthesis. During all experiments, the light intensity was adjusted to a photon density of 2000 µmol m^{-2} s^{-1}. After equilibration and recording of photosynthesis at 22 °C (see above, previous section), the leaf temperature was raised by increasing the air temperature of the chamber (Section 2.11.2) to a leaf temperature of 40 +/- 1 °C. Leaves were exposed to this temperature for 10–12 min, and then the air temperature was decreased over a time of 10–15 min to a leaf temperature of 22 °C. Leaf photosynthesis was measured and recorded as previously described (Section 2.11.3).

3.0 RESULTS

3.1 METHOD DEVELOPMENT

To address the biological questions listed under Aims and Objectives (Section 1.5) a number of new methodologies had to be developed.

One objective was to use the GUS system to determine the physiological state of the endophyte in the plant. However, prior to *in planta* studies, it was required to establish whether and how GUS activity expression changes in mycelium of the GUS-transformed endophyte grown *in vitro*. Therefore, it was necessary to complement the established method for GUS extraction from plant material with a method for extraction from cultured mycelium.

In addition, a selection system for the GUS gene had to be implemented, to ensure that mycelium of the transformed endophyte was homogenous for the genetic marker.

A controlled environment for growth of grass-endophyte symbioses during experiments had to be established, since variation in the environmental conditions impacts on *in planta* levels of the alkaloids.

For precise mapping of the *in planta* distributions of the alkaloids produced in the symbiosis, methods had to be devised that would allow for their determination from small sample quantities.

The development of a more rapid method for quantitative GUS determination in endophyte-infected plant tissues was necessary to accommodate larger sample sizes.

At a later stage during the experimental work an interference with the quantitation of GUS activity in some plant extracts was detected. The influence of this interference on GUS measurements was therefore quantitatively determined.

3.1.1 Development of a Method for Quantitative Extraction of GUS Activity from Fungal Mycelium

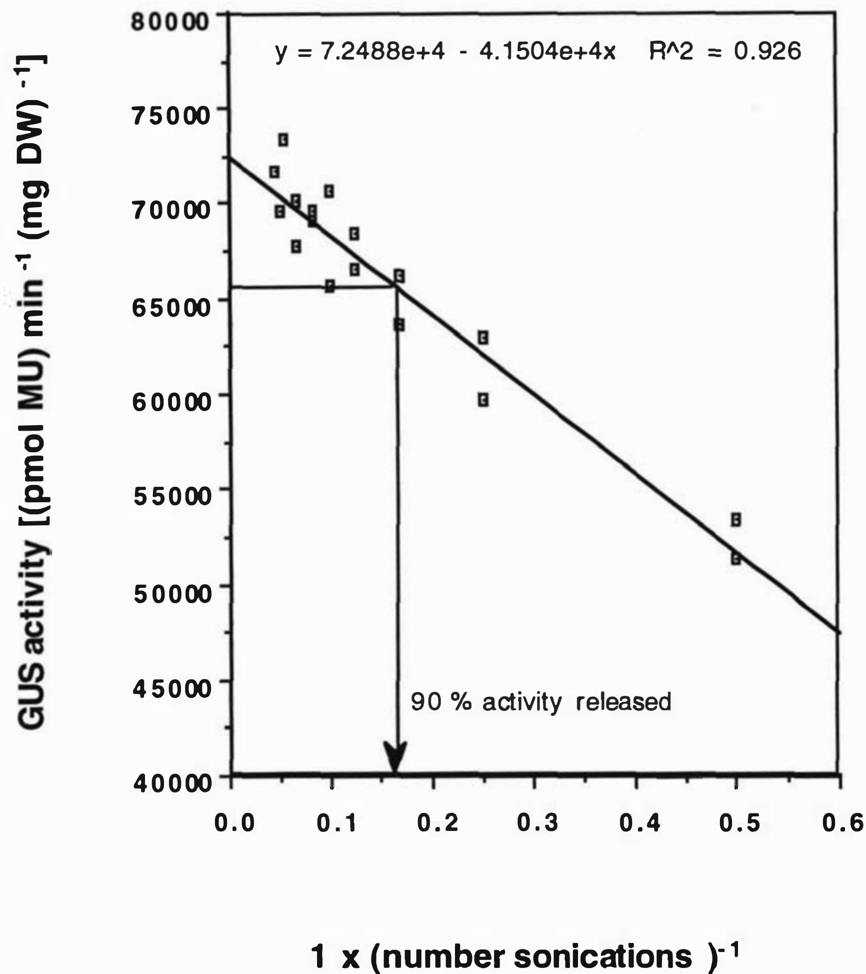
Quantitative measurements of GUS activity in cultured endophyte mycelium required reliable extraction. The method of Herd *et al.* (1997) for extraction of endophyte-linked GUS activity was designed to release more than 90 per cent of GUS activity in plant tissues by sonication. To determine the number of sonication cycles for release of 90 per cent GUS activity from endophyte mycelium grown in liquid culture (Section 2.4.2), a time course experiment was performed. Homogenisation and sonication of mycelium was carried out as described (Section 2.5.3). After each pre-determined number of cycles, 30 μl extract were removed and replaced with 30 μl fresh buffer. GUS activities in the extracts removed during the time course were determined as described (Section 2.6.1).

To determine the number of cycles that reliably extracts more than 90 per cent of GUS activity the GUS enzyme activities were plotted against the inverse number of sonication cycles applied. To estimate the maximum GUS activity extractable, the intercept of a linear regression (= activity released after an infinite number of cycles) was used, and it was determined that after six sonication cycles 90 per cent GUS activity had been released (Fig. 6). Examination of sonicated mycelium under the microscope confirmed that hyphae were thoroughly disrupted after this time. For all further extractions of GUS activity from cultured endophyte mycelium, ten sonication cycles were then used. This number of cycles was chosen to ensure that more than 90 per cent of activity were released every time extractions were performed (Fig. 6).

Because these extractions were carried out with mycelium of exponentially growing cultures, further experiments were conducted to test if more sonication cycles were needed for stationary phase mycelium. Compared to the activity released by ten cycles, no significant additional GUS activity was released from stationary phase mycelium subjected to more than ten sonication cycles (data not shown), indicating that no significant growth phase-specific differences in the extraction of GUS activity occurred.

Figure 6

GUS activity released from endophyte mycelium *versus* inverse number of sonication cycles applied. The arrow indicates the number of cycles required to release 90% of the maximum extractable GUS activity (intercept of the linear regression) that would be extracted if an infinite number of sonication cycles were applied.



3.1.2 Development of a Selection System for Obtaining Genetically Homogeneous Endophyte Isolates from Non-Sporulating Mycelium

The GUS gene construct was artificially introduced into the genome of the endophyte. Because of its metabolic cost, that is, constitutive expression of a foreign protein, not matched by an apparent value of the gene product for the fungus, the presence of the GUS gene was probably a disadvantage for the transformed endophyte strain. This gene had therefore a higher probability than the natural genes of the endophyte to be eliminated or inactivated by a mutation (Miller & Sands, 1992). Thus, a reliable selection system for the genetic marker was required. Genetically homogenous isolates from fungal mycelium are commonly obtained by sporulation and plating of spores for phenotypic selection of the developing colonies (Fincham, 1989). However, spore production by the *N. lolii* strain Lp 19 is seldom and erratic (personal observations, M.J. Christensen, personal communication, AgResearch Grasslands). Therefore, an alternative procedure had to be devised that ensured that mycelium used for plant inoculations and culture experiments was genetically homogeneous for the GUS gene.

For some fungi, an alternative method for obtaining genetic homogenous mycelium may be feasible, because of their mode of nuclear distribution in actively dividing cells. The division of the nuclei in growing hyphae takes place in the apical compartment of a hypha (Trinci, 1979). In this way, every newly formed hyphal compartment below the hyphal apex is supplied with one or several nuclei. Based on the number of nuclei in the apical compartment, two types of nuclear division (the mononucleate *Schizophyllum* or multinucleate *Aspergillus* type) can be distinguished in septate fungi. As shown in Figure 7, genetically pure isolates can be obtained by subculture from colonies with greater confidence when hyphae contain only one nucleus per apical compartment. Thus, if the nuclear division in the *N. lolii* strain Lp 19 followed the *Schizophyllum* type this should allow obtaining genetically homogenous mycelium of the GUS-transformed strain. Therefore, the nuclear distribution and segregation in hyphae of the *N. lolii* strain Lp 19 was examined.

Figure 7

Scenarios of nuclear segregation after a mutation occurred (indicated by grey nuclei), for two known types of nuclear and cell division. In type **A**, a number of nuclei are present in the apical compartment of a hypha (*e.g.* in *Aspergillus,* Trinci, 1979), which may lead (if some mixing "**M**"of nuclei in the compartment occurs) to a colony **(A')** with many sectors. In type **B,** the apical compartment contains only one nucleus (*e.g.* in *Schizophyllum,* Trinci, 1979), leading to a clear-cut segregation into two sectors within a colony **(B')**. As more sectors are present in colony **A'**, subculture from this colony is more likely to result in genetically heterogeneous mycelium than subculture from colony **B'**.

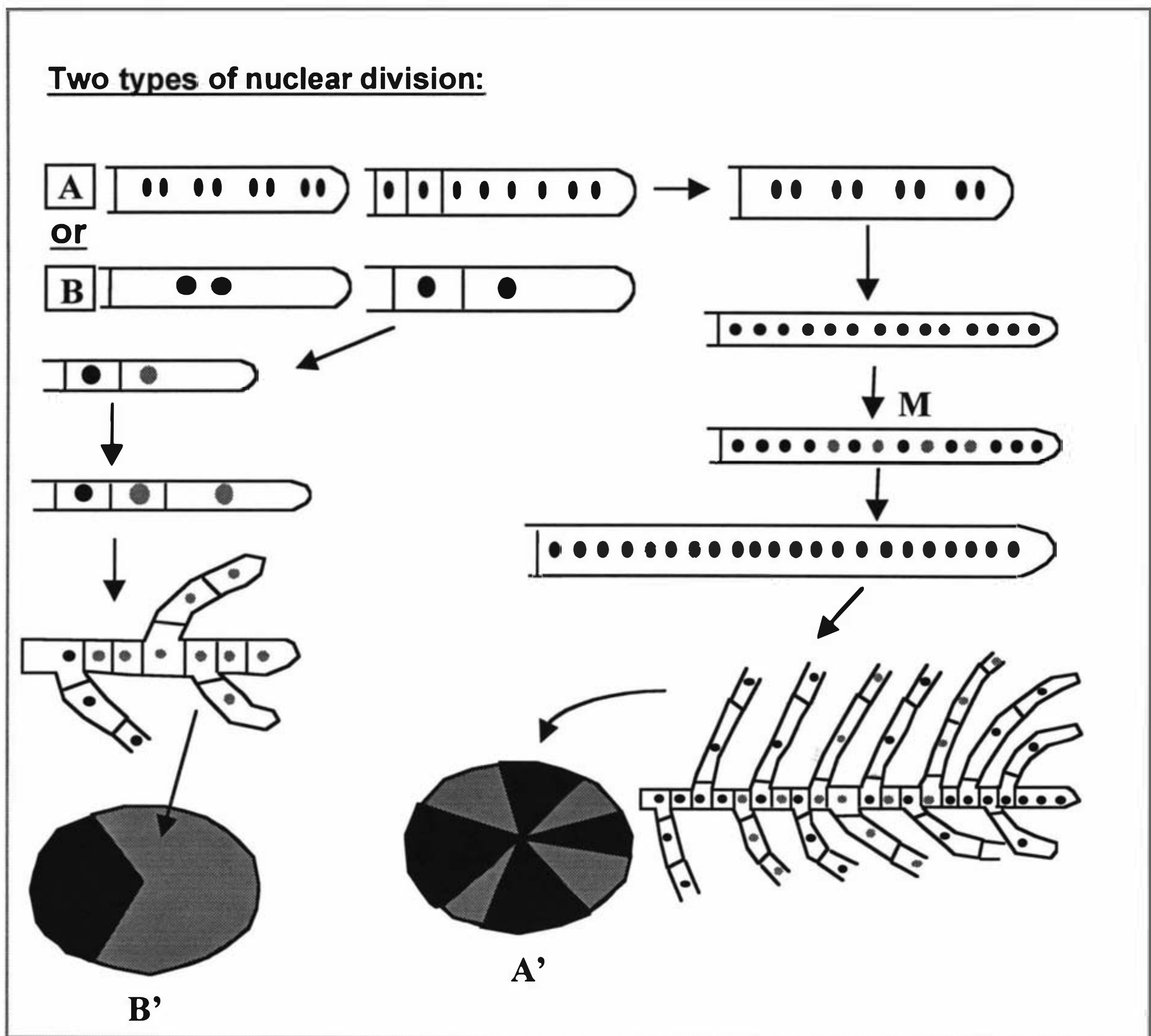
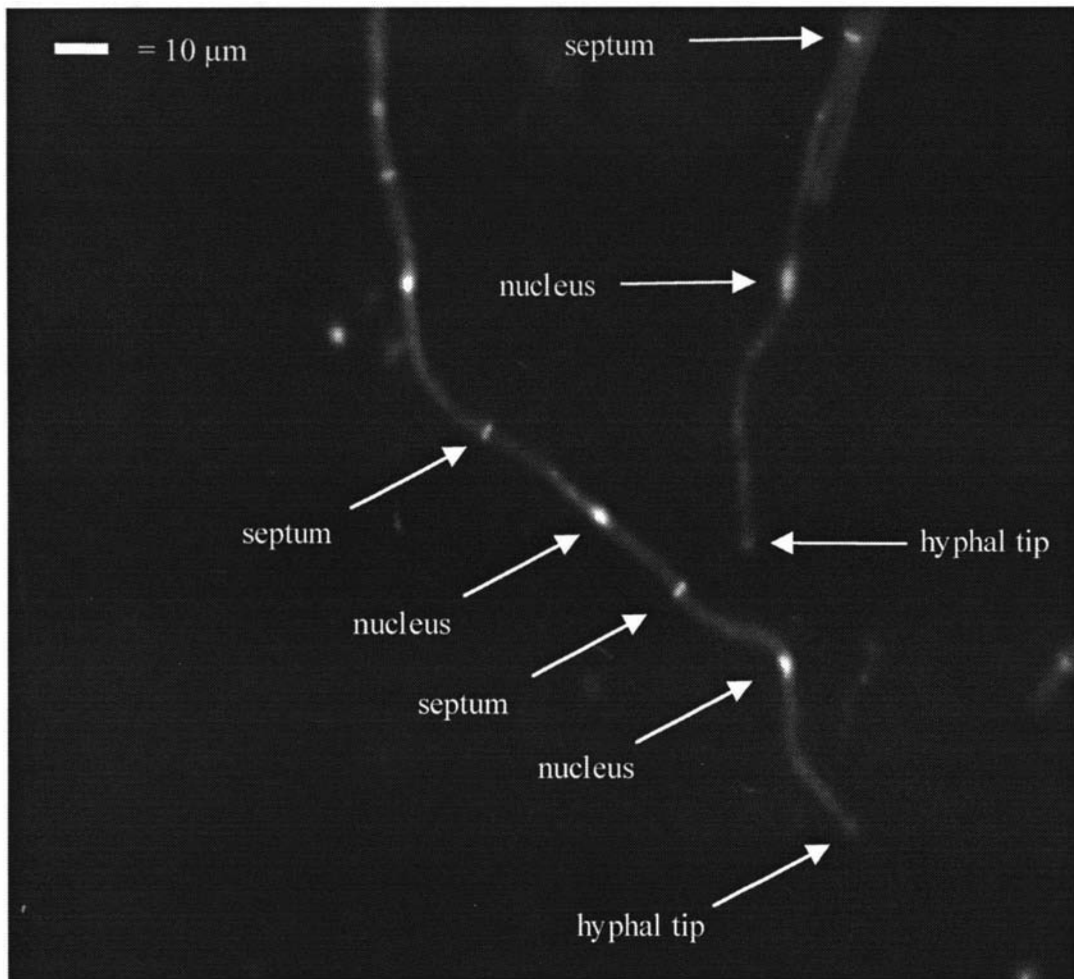


Figure 8

Flurorescent microscopy of mycelium of the endophyte strain Lp 19 (GUS-transformant KS1) grown in liquid culture. The mycelium was simultaneously stained with the fluorescent stains Calcofluor and Hoechst dye (Section 2.9) for visualisation of septa and nuclei in hyphae. More than 100 hyphal tips were examined for the number nuclei in the apical compartment. As shown for two hyphae and indicated by arrows, only a single nucleus is present in the apical compartment of a growing hypha of Lp19.



The number of nuclei in the apical compartments of hyphae of Lp 19 was determined. These experiments were carried out with fluorescence microscopy on hyphae from exponentially growing mycelium in culture (7 days of growth, 4-5 mg DW mycelium per ml culture PDB, Section 2.4.2). In all hyphae examined, one nucleus was present per hyphal apical compartment (Fig. 8). This demonstrated that the nuclear distribution in Lp 19 followed the *Schizophyllum* type (Fig. 7). As in this type defined segregation into sectors occurs, sub-culturing from colony margins (as described in Section 2.4.2) was used to obtain genetically pure isolates for experiments.

Endophyte mycelium in cultures was regularly quantitatively and qualitatively assessed (as described in Sections 2.6.1 and 2.6.3), to verify the presence of the GUS gene. In addition, expression of GUS in single hyphae under fluorescent microscopy (400 x magnification) was examined. Therefore, more than 100 pellets of a 14 days old liquid culture (approximately 5-6 mg DW mycelium per ml PDB medium) were stained (in GUS extraction buffer, Section 2.3.1) with the GUS selective stain Imagene Green (Molecular Probes). Neither in these pellets nor in cultured mycelium qualitatively checked for GUS-expression loss of GUS activity was detected (data not shown).

3.1.3 Establishment of a Controlled Environment for Studies of Endophyte-Plant Interactions

A goal of this study was to determine the quantitative relationship between endophyte metabolic activity and the production of the alkaloids ergovaline, peramine and lolitrem B (Section 1.5). As it is not possible to assess all of these parameters simultaneously, measurements had to be performed by sequential sampling. However, environmental conditions in the field show variation over time that affect the concentration of the endophyte and the alkaloids (Section 1.3.3). Light and temperature in the glasshouse are often variable and conditions in the glasshouse can bring about extreme levels of alkaloid concentrations (Section 1.3.4). Growth and maintenance of plants in a more controlled environment was therefore required. Therefore, plants were introduced into a controlled environment chamber that allowed for the regulation of temperature and light period (Fig. 3). Regulation of the light period was important for restricting the grass plants to vegetative growth. The control over the plant growth status was crucial, because concentrations of alkaloids change with the developmental stage (vegetative or reproductive) of the grass plant (Ball *et al.*, 1997b; Lane *et al.*, 1997c; Rottinghaus *et al.*, 1991). Vegetative growth of plants was chosen, because the generation of continuously flowering plants is not feasible.

3.1.3.1 Plant Growth in the Growth Cabinet

Plants in the growth cabinet were grown at 15 °C, and initially at a 16:8 h light to darkness cycle. However, this light period led to the development of reproductive structures. To have the plants growing in a vegetative state, the light period was adjusted to a 12L:12D h cycle. This prevented flowering from occurring. The plants were supplied with water as needed and fertilised regularly as described (Section 2.4.4). Under these conditions, growth of the plants was rapid (about 20-30 tillers per plant after 1-2 months of growth) and uniform for each genotype (Section 3.3.1), and occurred at a constant rate for up to 3-4 months, after which plants were re-planted (Section 2.4.4).

3.1.3.2 Assessment of the Impact of Fungicide and Insecticide Application on *In Planta* GUS Activity

Incidence of pathogenic fungal infections and other pests impacting on the health of plants was low in the growth cabinet. However, occasional application of pesticides was necessary to combat pathogens. Spraying against pathogenic fungi was conducted with either Karathane or Systhane (Section 2.4.4). Mites were controlled with Omite (Section 2.4.4), and infestation with aphids was treated with Vydate (Section 2.4.4). The fungicides especially could have an effect on the metabolic activity and viability of the endophyte. Therefore, the impact of the pesticides on the *in planta* viability and GUS activity of the endophyte was determined on endophyte-infected plants. Plants of the perennial ryegrass cultivar 'Grasslands Nui', genotypes M and G infected by the GUS-transformed endophyte strain FM13 (Table 1) were used. Because of space limitations in the growth cabinet, plants used in this experiment were grown in the glasshouse at AgResearch Grasslands in late winter early spring (August-September), with temperatures ranging between 15 and 22 °C.

Plants of the genotype G were assigned to treatment with the fungicide Karathane in combination with Vydate and Omite. Plants of the genotype M were treated with Systhane also in combination with Vydate and Omite. Controls (one plant per treatment) received spraying with water only. Spraying of plants was performed as described (Section 2.4.4).

Two tillers per treatment and of each control were harvested seven days after each application for determination of GUS activity concentration (Section 2.6.1) in the pseudostem (the basal region of a tiller, comprised of leaf sheaths of all leaves on the tiller and emerging leaf). Seven days after the final application, tillers of plants sprayed with Systhane and the respective control were quantitatively analysed for GUS activity concentrations in 14 sections of each tiller.

Table 2

GUS activity concentrations in fungicide-treated plants and untreated controls. Fungicides used were Karathane or Systhane, both in combination with two insecticides, section 2.4.4.

Table **A** lists means (n = 18-20) of GUS activity concentration in pseudostems (per cent of controls) of fungicide-sprayed plants and controls. Table **B** lists GUS activity concentrations in tissue sections of plant genotype M assessed after last application of Systhane. Means of sprayed plants and controls in Table **A** were not significantly different ($P > 0.1$, t-test on data paired according to date of sampling). A significant difference was found between sections of sprayed plants and controls, shown in Table **B** ($P = 0.04$, t-test, data paired according to tissue section). However, this difference was not due to a negative fungicide effect on GUS, as activities in the sections of sprayed plants were on average greater than in control sections. Controls had 86 % (63 % lower limit, 109 % upper limit of 95 % confidence intervals) of the activities of fungicide treated plants.

A

Treatment	GUS activity concentration [%]	Standard deviation
Genotype G control	100	27
Genotype G Karathane	99	28
Genotype M control	100	53
Genotype M Systhane	97	41

B

Tiller Section	Treatment	
	control	Systhane
	GUS Activity Concentration [(pmol MU) min^{-1} $(mg\ DW)^{-1}$]	
upper emerging leaf	2	5
lower emerging leaf	2	8
1 st leaf upper blade	4	4
1 st leaf lower blade	4	3
1 st leaf upper sheath	16	27
1 st leaf lower sheath	38	39
2nd leaf upper blade	3	3
2nd leaf lower blade	2	4
2nd leaf upper sheath	24	26
2nd leaf lower sheath	44	63
3rd leaf upper blade	3	2
3rd leaf lower blade	3	4
3rd leaf upper sheath	38	59
3rd leaf lower sheath	140	186
Average	23.1	30.9
	control	Systhane
Average (per cent of control)	100 %	134 %

No negative effects of the fungicide treatments on GUS activity concentration were observed in composite samples (pseudostem, Table 2A) and individual tiller sections (Table 2B). In addition, the endophyte was isolated from leaf tissues of pesticide treated plants and controls, to determine whether fungal growth from the plant tissues was affected by the spraying. No differences in outgrowth of endophyte from plant tissue between treatments were detected (data not shown).

3.1.4 Development and Optimisation of a Method for Extraction of Ergovaline from Small Quantities of Endophyte-Infected Plant Material

Available extraction methods for quantitative analyses of the alkaloids ergovaline, peramine, and lolitrem B required, for each alkaloid, plant tissue quantities of 50-100 mg dry weight (Barker *et al.*, 1993). Improved methods for the quantitative extraction of lolitrem B and peramine that required significantly less sample material were developed by modification of existing protocols (Barker *et al.*, 1993) by others (B. A. Tapper, personal communication, AgResearch Grasslands) contemporaneously with this work. For this study, there was particular interest in the distribution of the alkaloid ergovaline in relation to that of endophyte metabolic activity. The development of a procedure for quantitative extraction of ergovaline was therefore part of the work presented. Ergovaline is susceptible to chemical degradation under extractive conditions (Section 1.3.2). Therefore, modifications of extraction procedures had to be carefully monitored, especially since small sample quantities would raise the limit of detection of ergovaline in the HPLC. These modifications were aimed at utilising an aqueous organic solvent, suitable for extraction in plastic that allowed a rapid clean up of extracts by filtration.

In this study, the practice of other researchers in the field of analytical alkaloid chemistry was followed of adding the ergopeptide ergotamine as an internal standard prior to extraction (Barker *et al.*, 1993; Hill *et al.*, 1993; Rottinghaus *et al.*, 1991; Shelby & Flieger, 1997). In this method, simultaneously with the extracting solvent, a known

amount of the standard is added to a sample that is to be analysed (Section 1.3.2). Ergotamine is generally considered a suitable internal standard for analysis of ergovaline in endophyte-infected perennial ryegrass, as it occurs naturally only in negligible concentrations (< 0.05 ppm) in endophyte-infected grasses (Rottinghaus *et al.*, 1991; Shelby & Flieger, 1997). As noted (Section 1.3.2), the ergopeptine alkaloid ergotamine is structurally similar to ergovaline, with similar physical and chemical properties. Ergotamine, added as an internal standard prior to extraction, should thus be recovered from samples with comparable efficiency to ergovaline.

Hence, in the method development, particular attention focussed on selecting extraction conditions that yielded efficient and equivalent recovery of both internal standard and analyte for obtaining accurate ergovaline estimates. Therefore, the raw peak area data for the analyte (the sum of ergovaline plus ergovalinine) and the internal standard (the sum of ergotamine plus ergotaminine) have been monitored as measures of recovery, and are presented in addition to the ergovaline estimates.

Ergopeptides are subjected to isomerisation under mild conditions, including protracted storage of plant material, as well as usual extraction procedures. Isomerisation of the ergopeptides leads to mixtures of C8-epimers such as, for example, ergovaline/ergovalinine, and ergotamine/ergotaminine. By measuring the ergopeptides as the sum of both epimers, estimates remain largely constant with varying isomerisation during sample storage and analysis (Barker *et al.*, 1993; Shelby & Flieger, 1997). Therefore, this procedure has been adopted in the present study (Section 2.8.3.4). During the method development, isomerisation of the standard was also monitored as an indicator of possible degradation of standard as well as analyte.

3.1.4.1 Sample Material for Method Development

Variation in ergovaline concentration in samples due to heterogeneity of ergovaline within plant material has been observed previously (G. A. Lane and B. A. Tapper, personal communication, AgResearch Grasslands). This heterogeneity is highly undesirable in the development of an analytical method, and poses a particular problem if the analysis is to

be performed with small sample quantities. Therefore, plant material suitably homogenous for ergovaline was used for the method development. A pooled high-ergovaline sample, previously utilised as a control and known to give consistent ergovaline estimates (G. A. Lane and B. A. Tapper personal communication) was used. In addition, a sample with an expected lower ergovaline concentration was analysed for homogeneity of ergovaline content, as described in the following, for utilisation in the method development.

For the analysis of homogeneity, the pseudostem fractions of four (clonal) endophyte-infected plants were collected and divided into small (<5cm length) and large (>5cm length) pseudostems. These samples were freeze-dried and milled (Section 2.8), and an aliquot removed from each and weighed (50 mg dry weight). Using a previous standard procedure (Barker *et al.*, 1993), the samples were then extracted in chloroform-methanol-ammonia (Section 2.8.3.1), and the ergovaline concentration was determined as described (Section 2.8.3.4). Estimates for ergovaline concentration in the different fractions are listed in Table 3. The low concentration of ergovaline in this material was suitable for the planned development of a method for quantitative extraction from small samples potentially low in ergovaline concentration, and the low variation in ergovaline content between the fractions allowed pooling of the material. The combined samples thus provided sufficient material with a known and homogenous concentration of ergovaline for experiments on the extraction efficiency of different solvent systems.

Table 3

Ergovaline concentrations in pseudostem fractions of four clonal endophyte-infected perennial ryegrass plants extracted with chloroform-methanol-ammonia.

Sample	Ergovaline concentration [ppm]
Plant A large tillers	0.64
Plant A small tillers	0.55
Plant B large tillers	0.41
Plant B small tillers	0.57
Plant C large tillers	0.55
Plant C small tillers	0.56
Plant D large tillers	0.46
Plant D small tillers	0.43
Average ± std dev.	0.52 ± 0.08

3.1.4.2 Extraction with Solvents Containing Isopropanol and Lactic Acid

To minimise loss of ergovaline during extractions by reducing the steps required for sample preparation and clean up, extractions were performed in plastic extraction vials. Extraction of ergovaline was therefore carried out with an organic solvent consisting in aqueous isopropanol (= propan-2-ol) with lactic acid. Isopropanol is suitable for extraction of another important alkaloid – peramine -, is compatible with polyethylene vials and filters that are necessary for rapid small-scale filtration, and is directly compatible with HPLC, thus requiring no further evaporation and partitioning step. Lactic acid was included to provide an acidic environment, which is required for extraction of the ergopeptides (Section 1.3.2). In addition, this organic acid had been previously found to have good qualities for extraction and stabilisation of ergovaline (Moubarak *et al*., 1993; Testereci *et al*., 1990).

In an initial experiment, sample material previously assessed for ergovaline by the chloroform-methanol-ammonia method (see above, previous section) was used in extractions with two different concentrations of aqueous isopropanol (IP), 20% and 50% (v/v) each with 1% (w/v) lactic acid (LA). For these extractions, 20 mg dried herbage was suspended in 0.5 ml solvent, vortexed, and extracted at room temperature for one hour. An additional set of samples (same solvent treatments) was extracted overnight in darkness on a rotary shaker (horizontal agitation, 30 rpm).

The filtration of extracts (Section 2.8.3.3) removed particulate material rapidly. The filtered extracts were suitable for analysis by HPLC without further purification, which significantly reduced requirements for time and work, especially compared with the chloroform-methanol-ammonia procedure (Section 2.8.3.1). Separation and quantitation of ergovaline was carried out as described in Section 2.8.3.4. Concentrations of ergovaline and the peak areas of ergovaline and internal standard from which the concentrations were estimated (see equation in section 2.8.1.2) are listed in Table 4. For all IP-LA solvents, concentrations were very similar to the concentrations obtained with the chloroform-methanol-ammonia method (see above, previous section). This demonstrated that ergovaline could be extracted for quantitative analyses with isopropanol-lactic acid-based

Table 4

Estimated ergovaline concentrations and peak areas of internal standard (ergotamine), ergovaline and after extraction with isopropanol (IP) and lactic acid (LA). Samples were extracted for 60 min and overnight with two concentrations (20% and 50%, v/v) of aqueous IP, 1% (w/v) LA.

All samples analysed were from pooled material previously analysed by the chloroform-methanol-ammonia method (Table 3). Each value is the mean of three replicate extractions (± 1 standard deviation).

Concentration isopropanol & extraction time	Estimated ergovaline concentration [ppm]	Peak area ergovaline x 10^3 [lysergyl units]	Peak area internal standard x 10^6 [lysergyl units]
50%, 60 min	0.45 ± 0.04	3.00 ± 0.33	1.64 ± 0.07
20%, 60 min	0.51 ± 0.03	1.81 ± 0.08	0.88 ± 0.01
50%, overnight	0.51 ± 0.05	2.17 ± 0.21	1.06 ± 0.02
20%, overnight	0.58 ± 0.03	1.92 ± 0.06	0.82 ± 0.03

Recovery of internal standard in overnight re-extractions with 50% isopropanol was 7%, and with 20% isopropanol 36% of the first extractions.

solvent systems. A lower recovery of analyte and standard was observed in samples extracted with 50% IP agitated overnight (Table 4). Therefore, agitation of samples during extraction was omitted in subsequent experiments. In addition, lower recoveries of ergovaline and internal standard were observed with 20% IP than with 50% IP (Table 4). This indicated a sensitivity of extraction to solvent proportions, which was therefore tested in a second experiment.

In this experiment (Table 5), batches of different concentrations of IP in 1% (w/v) LA and of 50% (v/v) IP with varying concentrations lactic acid were prepared. A volume of 0.5 ml of each solvent was used for extraction of 20 mg samples. Plant material chosen for this experiment was from the high-ergovaline sample (see above, previous section). This sample was used, as its higher ergovaline content allowed a comparison between solvents well above the detection limit in the HPLC. The samples were vortexed and extracted for one hour. After separation of the extracts by filtration, samples with 30% and 70% IP in 1% LA and 50% IP in 2%, 5% and 10% LA were re-extracted overnight, with the same solvents as used for the first extractions. As noted (see above Section 3.1.4), in choosing an appropriate solvent, it was important that both ergovaline and internal standard were extracted with equal efficiency. Performing the re-extractions, to determine whether analyte and internal standard had been equally recovered in the initial extraction tested this.

Estimates for ergovaline concentration were similar at all isopropanol concentrations. The highest estimate was obtained with 50% IP-1% LA, coinciding with the highest recovery of both analyte and internal standard (Table 5A). Varying concentrations of lactic acid (from 1–10%, w/v) in 50% isopropanol gave only small effects on extraction (data not shown). The amount of isomerisation of the internal standard increased with increased isopropanol content (Table 5A), suggesting that higher organic solvent content compromised the stability of the ergopeptides. Isomerisation was similar over the range 1%-10% lactic acid in 50% IP (data not shown), indicating that it is relatively insensitive to variation in lactic acid concentration.

Table 5

Estimated ergovaline concentrations and peak areas of ergovaline and internal standard ergotamine after extraction with five concentrations of aqueous isopropanol, 1% (w/v) lactic acid (**A**). Recoveries of ergovaline and internal standard after re-extraction overnight at two concentrations isopropanol (**B**).

Each value is the mean of three replicate extractions (± 1 standard deviation). Plant material used was an ergovaline reference standard.

A

Concentration isopropanol [%, v/v]	Estimated ergovaline concentration [ppm]	Peak area ergovaline x 10^4 [lysergyl units]	Peak area internal standard x 10^5 [lysergyl units]	Isomerisation of internal standard [% ergotaminine]
30	2.33 ± 0.02	5.75 ± 0.18	3.93 ± 0.15	1.71 ± 0.05
40	2.27 ± 0.12	6.87 ± 0.71	4.83 ± 0.29	1.88 ± 0.10
50	2.63 ± 0.01	8.31 ± 1.31	5.06 ± 0.79	2.90 ± 0.71
60	2.19 ± 0.04	6.70 ± 0.37	4.91 ± 0.23	3.62 ± 0.27
70	2.28 ± 0.09	6.73 ± 0.12	4.69 ± 0.11	4.77 ± 0.47

B

Concentration isopropanol [%, v/v]	Peak area ergovaline [% of first extraction]	Peak area internal standard [% of first extraction]
30	18.3	15.9
70	6.6	7.7

After re-extraction, the relative recovery of ergovaline was similar to that of ergotamine in both 70% IP-1% LA and 30% IP-1% LA (Table 5B), and also in 50% IP with 2% or 5% lactic acid (16% recovery for both compounds, data not shown). In 50% IP with 10% lactic acid, recovery of the internal standard was reduced (data not shown). The relative recoveries after re-extraction depend on both the efficiency of the initial extraction, and on the stability of both internal standard and ergovaline in the extraction solvent. The recovery of both compounds after re-extraction was lower with 70% IP – 1% LA than with 30% IP – 1% LA (Table 5B), perhaps due to losses associated with the higher isomerisation rate, and/or due to the higher initial recovery with 70% IP – 1% LA (Table 5A).

In conclusion, with the isopropanol-lactic acid solvent system a generally excellent consistency in relative recoveries of standard and analyte could be demonstrated. Therefore, the aqueous isopropanol - 1% (w/v) lactic acid 1:1 (v/v) solvent was used in all further extractions, since this solvent gave highest recovery, with little isomerisation of the standard, suggesting that it has only small effects on stability of standard and analyte.

3.1.4.3 Extraction and Recovery of Ergovaline and Internal Standard with an Acetic Acid Solvent

The high efficiency and reliability of the isopropanol-lactic acid solvent could be demonstrated in a further experiment. During the development of the new extraction method, Shelby and Flieger (1997) reported an analytical method utilising 20% aqueous acetic acid as extraction solvent for quantitative extraction of ergovaline. The ergovaline estimates obtained with this method were considerably higher than estimates obtained by a method of Hill *et al.*, (1993) utilising chloroform-methanol-ammonia (Shelby & Flieger, 1997). Aqueous acetic acid was therefore compared with the newly developed isopropanol-lactic acid method. Extractions with the two solvents were carried out as described (Section 2.8.3.2 and 2.8.3.3). Both solvents yielded similar amounts of extracted ergovaline both in first extractions and re-extractions, but recovery of the internal standard was substantially lower in extractions with 20% acetic acid (Table 6).

Table 6
Listed are estimated concentrations of ergovaline and chromatographic peak areas of ergovaline and internal standard ergotamine from plant tissue extractions with aqueous acetic acid (20% v/v); 50% (v/v) isopropanol, 1% (w/v) lactic acid, and peak areas in re-extractions with the same solvents. Indicated are means ± 1 standard deviation (n=3). nd = not determined.

Solvent	Estimated ergovaline concentration [ppm]	Peak area ergovaline x 10^4 [lysergyl units]	Peak area internal standard x 10^5 [lysergyl units]	Isomerisation of internal standard [% ergotaminine]
20% acetic acid	3.94 ± 0.16	9.19 ± 0.19	3.69 ± 0.10	8.26
50% isopropanol, 1% lactic acid	2.27 ± 0.05	9.67 ± 0.37	6.72 ± 0.13	2.17
re-extractions				
20% acetic acid	nd	1.99 ± 0.05	1.33 ± 0.14	nd
50% isopropanol, 1% lactic acid	nd	1.64 ± 0.05	1.14 ± 0.07	nd

Re-extractions of both acetic acid and isopropanol-lactic acid had similar amounts of internal standard (Table 6). This further indicated poor recovery of the internal standard in the first extraction with 20% acetic acid, resulting in a greater estimated ergovaline concentration in extractions with this solvent (Table 6). Another indicator that the ergovaline estimate from the acetic acid solvent did not accurately assess the true ergovaline concentration is the differential in recovery of the two ergopetides on re-extraction. The relative recoveries of ergovaline and ergotamine on re-extraction with isopropanol-lactic acid were nearly identical (17% of the initial extract), but for acetic acid there was relatively more ergotamine than ergovaline recovered from the residue (ergotamine 36%, compared to ergovaline 22% of the initial extract). Isomerisation of the internal standard was higher with 20% acetic acid than in extractions with isopropanol-lactic acid (Table 6), indicating that the stability of the ergopeptides was also more affected by the acetic acid solvent.

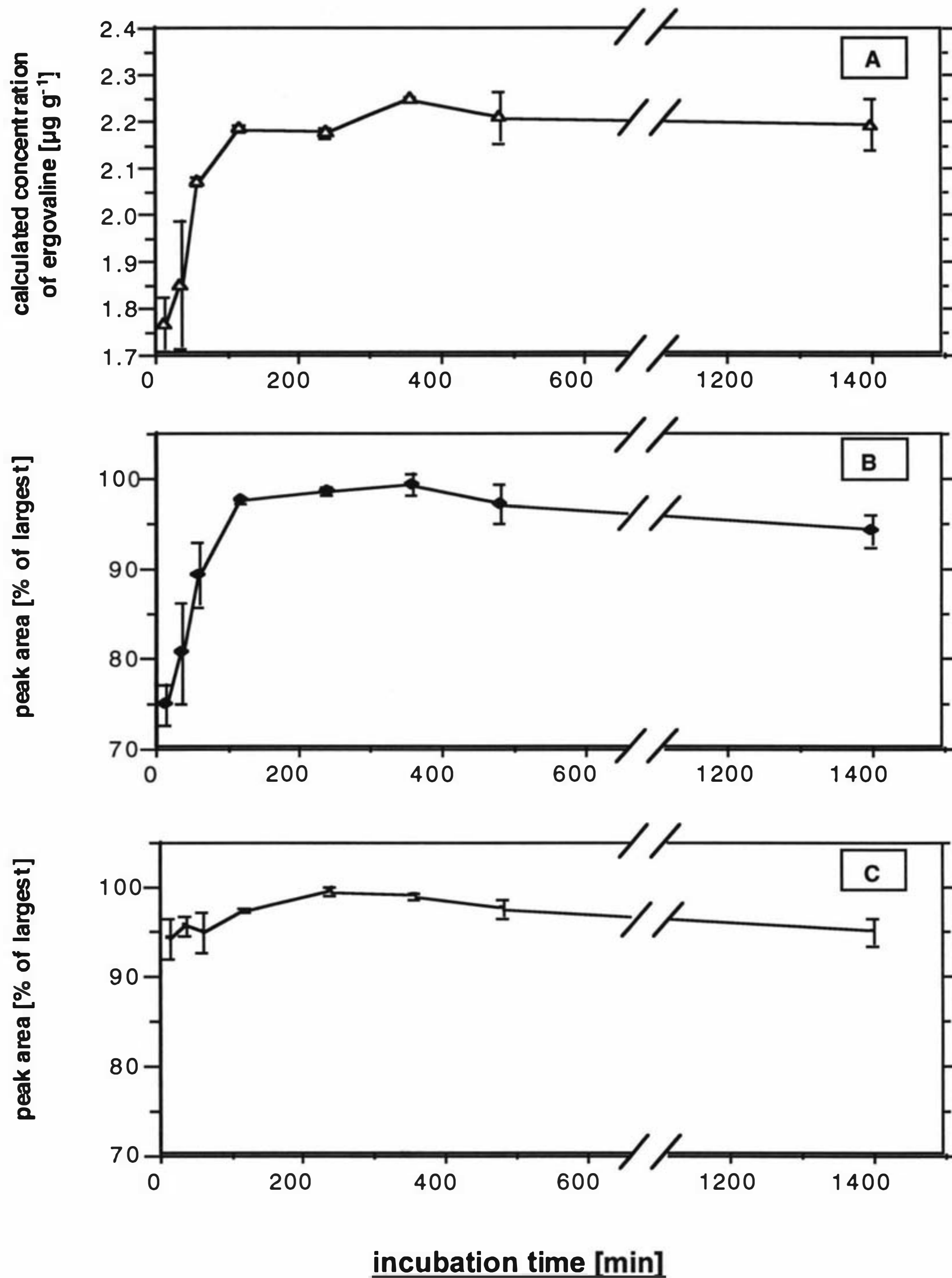
This comparison indicated that the isopropanol-lactic acid solvent, under conditions previously carefully selected for improved extraction, gave more reliable estimates of ergovaline concentrations than the aqueous acetic acid solvent method by Shelby and Flieger (1997). This was mainly due to the poor recovery of internal standard with the latter solvent. The acetic acid system was therefore not further used for extraction of ergovaline.

3.1.4.4 Time Course of Ergovaline Extraction and of Recovery of Internal Standard

The extraction time for optimum release of ergovaline and recovery of internal standard remained to be established. A time course experiment was performed for extraction with isopropanol-lactic acid on 20 mg samples of high-ergovaline plant material. Each sample was extracted in 0.5 ml solvent. At regular time points, extracts of designated samples were harvested for analysis as described (Section 2.8.3.3). The estimated ergovaline concentration increased rapidly during early stages of the extraction (15 – 120 min, Fig. 9A). After two hours extraction, the ergovaline estimates stabilised at about 2.2 ppm, with subsequent changes less than 0.1 ppm. As indicated by the peak area

Figure 9

Time course of extraction of ergovaline and recovery of internal standard ergotamine from grass tissue extracts. Graph **A** indicates concentrations of ergovaline estimated from the absolute values for the peak areas in graph **B** (ergovaline, per cent of greatest area measured) and **C** (internal standard, per cent of greatest area).
Error bars = ± 1 standard deviation (n=3).



increase over time (Fig. 9B) extracted ergovaline rapidly increased during the initial incubation (15 – 120 min), and then increased slightly to a maximum at 360 min. With protracted extraction, the concentration in the solvent slightly declined. Initially, extracted internal standard also increased, but significantly less than did ergovaline (Fig. 9C). This suggests initial binding, with subsequent release, of the internal standard to plant material. Highest recovery of the internal standard was achieved after extraction for two to four hours, near to the peak of extraction of ergovaline (Fig. 9C).

In this experiment, the decline in recovery of ergovaline and internal standard after overnight extraction (Fig. 9B, C) was much less than in the initial experiments with 50% IP – 1% LA (Section 3.1.4.2). However, in the initial experiments, samples were shaken during the overnight extraction, causing heating and higher aeration, which might have increased degradation of ergovaline and internal standard. The stability of the internal standard was initially not affected; isomerisation of the internal standard was low (1-2 %) after incubation for 15 – 120 min, but increased thereafter to 5 - 6 % after 24 hours (data not shown).

Extraction of samples for two hours was used in all subsequent extractions, since after this time most ergovaline and internal standard had extracted into the solvent and only little isomerisation occurred.

3.1.4.5 The Influence of Sample Dry Weight in Extraction of Ergovaline and Recovery of Internal Standard

The differences in recovery with different solvents observed earlier (Section 3.1.4.2, 3.1.4.3) indicated that partitioning of analyte and internal standard between the extract and plant tissue material varied. Differences in the amounts of sample in extractions might therefore affect the quantitation of ergovaline. In this work, the aim of the method development was to extract ergovaline from small sample quantities (< 10 mg dry weight). Thus, it was essential to investigate the influence of sample amount on extraction by comparing extraction efficiencies with varying sample quantities. Plant material samples

Table 7

Estimation of ergovaline concentrations from samples varying in dry weight. Extractions were performed with two types (high- and low-ergovaline) of plant material using the isopropanol-lactic acid solvent.

Values are means of three replicate extractions (± standard deviation), each in 50% (v/v) isopropanol, 1% (w/v) lactic acid.

Dry weight of sample [mg]	Estimated concentration in high – ergovaline [ppm]	Estimated concentration in low – ergovaline [ppm]
50	2.41 ± 0.07	0.52 ± 0.01
20	2.33 ± 0.07	0.54 ± 0.07
10	2.27 ± 0.03	0.59 ± 0.02
5	2.21 ± 0.05	0.55 ± 0.03
2	2.19 ± 0.09	0.36 ± 0.08

high (> 2 ppm) and low (< 1ppm) in ergovaline (Section 3.1.4.1) were used, and from each of these samples aliquots of 2, 5, 10, 20, and 50 mg dry weight were extracted. All extractions were performed as described (Section 2.8.3.3). Ergovaline concentrations determined from these extractions are listed in Table 7. For samples containing more than 5 mg plant material and samples with high ergovaline content down to 2 mg ergovaline estimates did not significantly ($P > 0.05$, ANOVA) vary with sample size. For the low ergovaline material (mean 0.51 ppm), estimates from 2 mg samples were significantly lower (mean 0.36 ppm, $P < 0.05$, Scheffé's test). This was due to very small peak sizes in chromatograms. In this sample, the detection of ergovaline was below the chromatographic limit of quantitation.

The results of this experiment demonstrated successful extraction and quantitation of ergovaline with the newly developed extraction method from samples of 2 mg (ergovaline content of plant material > 2.0 $\mu g\ g^{-1}$) or 5 mg (content of plant material > 0.5 $\mu g\ g^{-1}$). With the isopropanol-lactic acid solvent, the sample amount in extracts did not significantly affect extraction of ergovaline and recovery of internal standard. The result for the smallest plant tissue sample (2 mg) indicated that with this tissue quantity, the quantitation limit in the HPLC was approached, if plant material was low in ergovaline content.

3.1.4.6 Summary and Conclusions

With a solvent consisting of aqueous isopropanol with 1% (w/v) lactic acid 1:1 (v/v) quantitative analysis of ergovaline from samples of 2 mg (if ergovaline concentration was > 2 ppm) or 5 mg (if ergovaline concentration was < 0.5 ppm) could be accomplished. This was a significant reduction in sample quantity compared to previous methods for analysis of ergovaline, matching amounts used for determination of GUS activity. Therefore, the new quantitative method for ergovaline allowed for resolution of the ergovaline distribution within the plant, equivalent to the resolution achieved with the GUS method. By careful selection of the proportion of the components in this solvent system, reliable and consistent extraction of ergovaline and recovery of internal standard were achieved. Variation in the proportion of isopropanol in the solvent showed that

relative recovery of ergovaline and internal standard depends on the relative amount of the organic solvent component. Employing isopropanol as the extracting solvent facilitated extraction in plastic vials, allowing for rapid clean up for HPLC analysis, significantly reducing time requirements with this procedure.

3.1.5 Comparison of the Quantitative Extractive GUS Assay with a Semi-Quantitative Microtitre Plate Assay

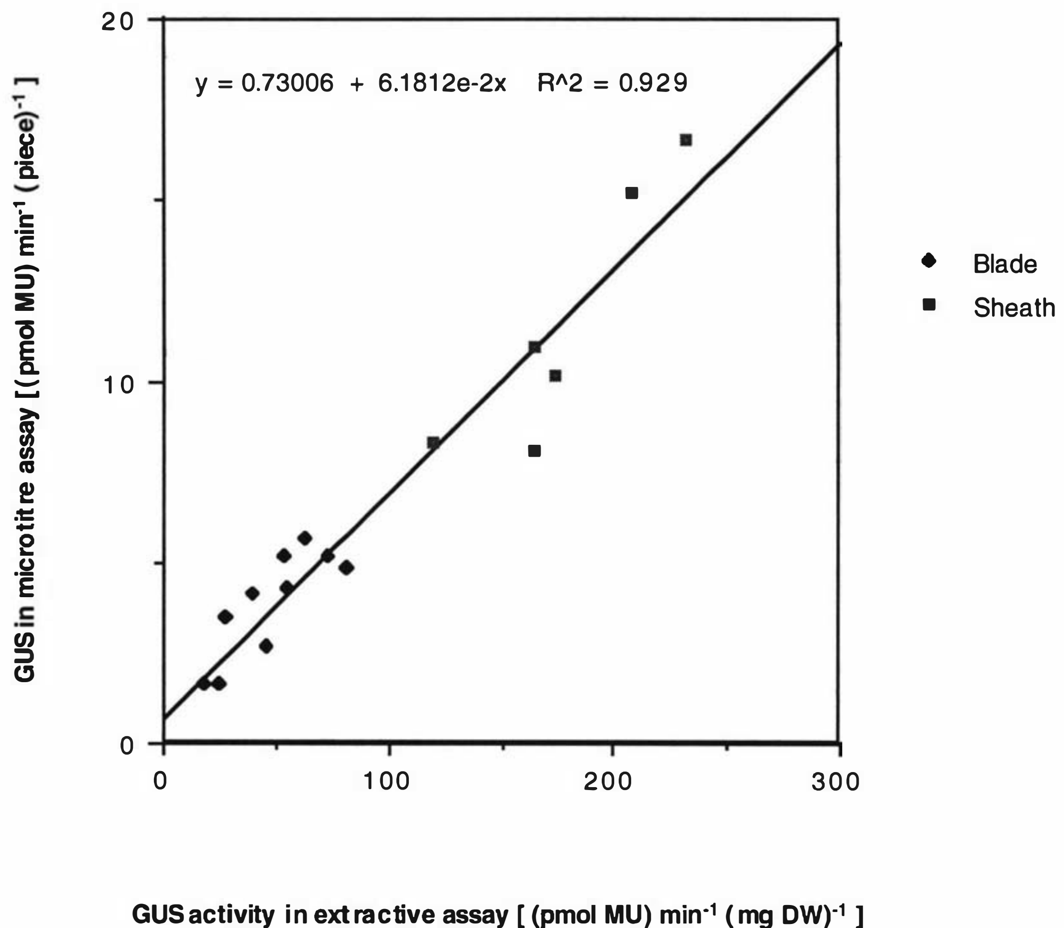
Although it is highly reliable and sensitive, the extractive method for quantitative determination of GUS activity from plant tissues by Herd *et al.* (1997) is also relatively time consuming because samples are ground and sonicated for extraction (Section 2.5.3). For quantitative screening of more than 20 samples, it was desirable to employ a more rapid method for determination of GUS activity. Jefferson (1987) recommended the use of microtitre plates for semi-quantitative determination of GUS activity concentration in plant tissues. Therefore its usefulness for this purpose was evaluated. Leaf tissues were prepared and analysed for GUS activity by the extractive and microtitre method (Sections 2.6.1 and 2.6.2). In the microtitre method, light densities in wells containing tissue pieces were assessed at regular time points. They were converted to amounts of the fluorescent product MU with a calibration curve created with the product (Section 2.6.2), and GUS activities in the assay determined as pmol of MU min^{-1}. Measurements were performed with the tissue pieces in the microtitre wells. This was afforded by a calibration at the start of the assay. For the calibration, the light densities in the microtitre wells containing the tissue pieces were recorded (Section 2.6.2), and then used as blank values during subsequent measurements.

For the two different tissue types (sheath and blade) used, a linear and similar relationship existed between the two assays (Fig. 10). A higher background was observed in the microtitre assay, limiting the use of this assay to tissues where GUS activity concentration exceeded this value. As indicated in Fig. 10, this was fulfilled in leaf sheaths, permitting reliable assessment of GUS activity in this plant tissue section with the

Figure 10

Comparison of the extractive method with the microtitre assay method for determination of GUS activity. Each data point represents a simultaneous measurement with the extractive method and with the microtitre method. Data points for leaf sheath (squares) and leaf blade (tilted squares) are indicated. Linear regression was performed on all data.

Small tissue pieces (length 1 mm, width about 3mm, weight approx. 0.2-0.5 mg DW) were cut out of the middle of a leaf segment (sheath or blade) and analysed for GUS activity in the microtitre assay (Section 2.6.2). The remaining leaf part was quantitatively analysed with the extractive GUS assay (Section 2.6.1).



microtitre method. With this method, more than 50 tissue samples could be assessed for GUS activity within one day. Moreover, less handling of samples was required making this procedure less laborious than the extractive GUS method.

3.1.6 Determination of *In Planta*-Endophyte Mycelial Concentration by Microscopy

For determining the endophyte metabolic state in mycelium *in planta* (Section 1.5), the GUS method had to be complemented with a method for assessing endophyte biomass in the grass plant. Attempts by others to quantify endophyte mycelium in grass tissues with a method for detection of chitin (Ride & Drysdale, 1972) were not successful (Y.Y. Tan, personal communication, Massey University). Previously ergosterol has been used for quantitative detection of endophyte mycelium (Richardson & Logendra, 1997). However, since larger quantities (1 g) of seeds were used in this study, the development of a method for extraction from small leaf tissue samples would have been required. Enzyme-linked-immunosorbent assay (ELISA), based on antibodies raised against endophyte antigens produced by cultured mycelium, is frequently used for assessing the endophyte concentration in plant tissues (Ball *et al.*, 1995; Hiatt III *et al.*, 1997; Musgrave, 1984). In this method, larger quantities of plant tissue are also required, because of a greater number of replications per sample (O.J.-P. Ball, personal communications, AgResearch Grasslands). The sensitivity of this method for quantitation of mycelium is also lower since antibodies are raised against an unknown composition of proteins produced by the endophyte in culture.

Because no other methods for determination of mycelial concentration in plant tissues were available that met the requirements of high sensitivity and compatibility with the GUS method, light microscopy was used for quantitation of endophyte biomass. Endophyte hyphae are relatively evenly distributed within tissues and hyphal branching in tissues is low (Y.Y. Tan, personal communication). Therefore, a method had been devised (Y.Y. Tan, personal communication), involving clearing of tissue sections from pigments and subsequent staining of endophyte hyphae with the selective fungal stain aniline blue.

With this method, quantitative estimates for hyphal concentrations can be obtained by determining the number of hyphae in a leaf cross-section. Plant tissue pieces were cleared from background and hyphae selectively stained as described in Section 2.7. An example for a cleared and aniline blue-stained leaf cross-section used for hyphal enumeration is shown in Fig. 5.

The diameter of endophyte hyphae, the dimension of which is required for determination of endophyte biomass in tissues (Section 2.7), was previously estimated to be on average 4 μm in plant tissues, as determined by measuring aniline blue-stained hyphae on enlarged photographs taken from light microscopy leaf-cross sections (Y.Y. Tan, personal communication). However, this measured diameter was evidently greater than previously reported for *N. lolii* (Koga *et al.*, 1993; Weedon, 1987). Therefore, hyphal diameters were measured directly under the light microscope as described in Section 2.7.

Estimates obtained in this way (Table 8) were comparable to diameters measured from published electron micrographs of *N. lolii* in leaf tissues (Koga *et al.*, 1993). In cultured mycelium of the same fungal species, hyphal diameters varied between 1.8-2.2 μm (Weedon, 1987), thus also very close to the estimates obtained here. However, hyphal diameters apparently varied between leaf blade and leaf sheath. In the leaf sheath, the diameter was on average 17 per cent smaller, and the difference between sheath and blade was statistical significant (Table 8). The reason(s) for the difference in hyphal diameter between blade and sheath was not determined, due to the relatively limited resolution in the light microscope.

Table 8

Hyphal diameters of the endophyte strain Lp 19 (GUS-transformant KS1) in leaf sheaths and leaf blades of two plant genotypes. Measurements of diameters were carried out by light microscopy as described in section 2.7.

Means were determined on 50 - 100 hyphae per tissue. The difference in hyphal diameter between leaf sheath and leaf blade was statistical significant ($P < 0.01$, t-tests within genotypes).

Genotype/ tissue	Mean diameter [μm] ± std. dev.
Nui UIII sheath	2.05 ± 0.51
Nui UIII blade	2.41 ± 0.51
Nui UIV sheath	2.00 ± 0.53
Nui UIV blade	2.33 ± 0.41
all sheaths	2.03 ± 0.52
all blades	2.37 ± 0.46

Since the difference in hyphal diameter was measured across plant genotypes and because of the magnitude of this difference, the respective estimates for the different leaf tissues were used for determining the endophyte biomass concentration in these. Hyphal diameters of 2.0 and 2.4 µm were used for assessing the *in planta* fungal biomass (Section 2.7) in leaf sheaths and blades, respectively.

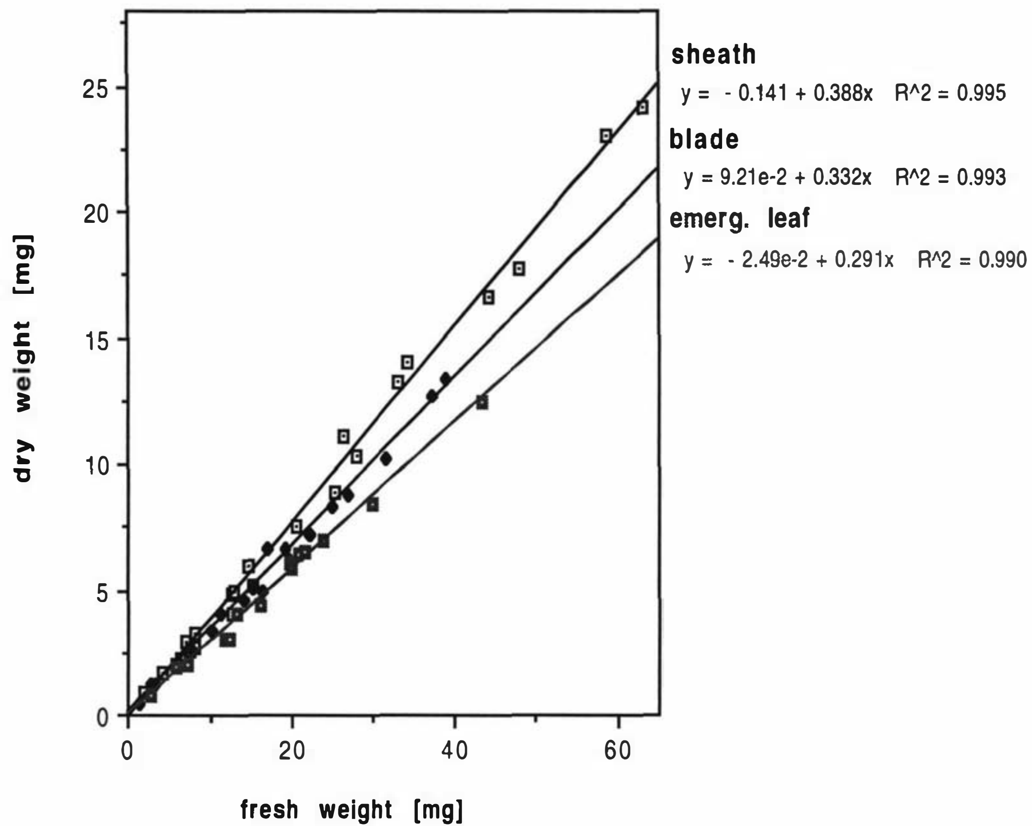
3.1.7 Assessment of Fresh Weight-Dry Weight Coefficients for Plant Tissues

For measuring the endophyte metabolic state in the plant (Section 1.5), it was required to determine GUS activity and mycelial concentrations in a grass leaf section at the same time. To relate the two parameters to each other, the dry weight of the section had to be determined. Since losses of plant material during the grinding in the extractive GUS method (Section 2.5.3) inevitably occurred, it was necessary to assess the weight of the leaf section before the grinding step. As the fresh weight of a leaf tissue was readily measurable, and assuming that fresh and dry weight showed a significant and sufficiently close correlation, the relationship between the two was determined for different leaf tissues. Leaf tissues were obtained by tiller dissection (Section 2.5.1.2), and fresh and dry weight for each assessed as described (Fig. 11). Measurements were performed on fractionated leaf material (sheath, blade, emerging leaf), as it was expected that these tissues varied significantly in water content. In experiments, to determine the dry weight of a leaf section from its measured fresh weight, in accordance to the tissue type of this leaf section, one of the three quantitative relationships shown in Fig. 11 was used.

Figure 11

The relationship of fresh weight to dry weight in three different leaf tissues. Linear regression equations are indicate at each line for each tissue type.

Tissues were dissected, sealed into pre-weighed microcentrifuge tubes and weighed within twenty minutes. Immediately after weighing, samples were freeze-dried in the tubes for 20 h, after drying transferred into an airtight box with desiccant (silica blue gel) and weighed in the tubes within 15 min. The dry weight of each piece was assessed by subtracting the weight of the tube.



3.1.8 Positive Selection for GUS Reporter-Gene Activity in Endophyte Mycelium - Segregation of GUS-Expressing and Non-Expressing Mycelium into Grass Tillers

GUS expression was stable in cultured mycelium (Section 3.1.2). However, loss of GUS activity from plants infected by the GUS-transformed endophyte occurred. This loss was characterised by lack of GUS activity in all parts of a grass tiller. To verify infection, five GUS-negative tillers were randomly selected and endophyte-infection was confirmed in all of them, as determined by light microscopy (Section 2.7, not shown).

Prior to the establishment of an *in planta*-selection for GUS expression, up to 20% of all tillers within a plant showed lack of GUS expression (personal observation, Schmid *et al.*, 2000). The lack of GUS expression characteristically appeared at the single tiller level, as exemplified by one plant where loss was detected: one endophyte-infected tiller showed complete absence of GUS activity expression, whereas a second tiller from this plant exhibited normal expression (Fig. 12).

Further investigations revealed that other tillers in this plant also showed complete lack of GUS expression (data not shown). The plant lacking GUS expression in some tillers was immediately derived from a seedling inoculated with the GUS-transformed strain (Section 2.4.5). To determine whether the lack of GUS expression was possibly due to physiological suppression in the plant, endophyte mycelium was isolated from the two tillers varying in expression as described (Section 2.4.1). These endophyte isolates were both identified as Lp 19 (M.J. Christensen, personal communication, AgResearch Grasslands), thus confirming that they were derived from mycelium of the GUS-transformed Lp 19 used previously to infect the plant. *In vitro*, the two isolates showed the respective GUS phenotypes as previously observed *in planta*, and were each continuously cultured in PDB liquid medium (Section 2.4.2), and regularly examined for GUS expression. The mycelium isolated from the GUS-negative tiller maintained lack of GUS expression in culture (repeated subculture over 3-4 months), and the mycelium from

Figure 12
GUS expression in two endophyte-infected tillers, grown from one plant.
GUS was assessed by the qualitative microtitre assay (Section 2.6.3) in leaf tissue pieces of two tillers from a single plant (genotype KLMTV), infected by the GUS-transformant strain KS1. Presence of GUS is verified by fluorescence of the GUS-specific product MU under UV. In tiller 1 (row **A**) no GUS expression was detectable, in tiller 2 (row **B**) GUS expression is clearly present, indicated by strong fluorescence in each microtitre well.
Lanes 1-4 contain leaf sheath tissue pieces, lanes 5-8 contain leaf blade tissue pieces.
The lower activity in the leaf blade sections (lanes 5-8 of row **B**) is due to a lower endophyte concentration in these tissues.
Leaf tissue pieces were cut as described in Section 2.6.2 and incubated in the microtitre dish for four hours at 37 °C in GUS extraction buffer containing the GUS substrate MUG.

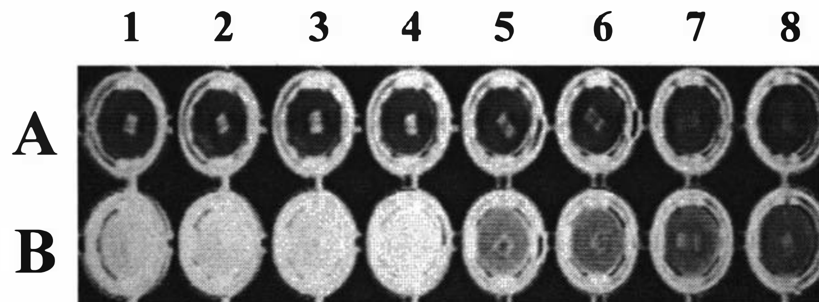
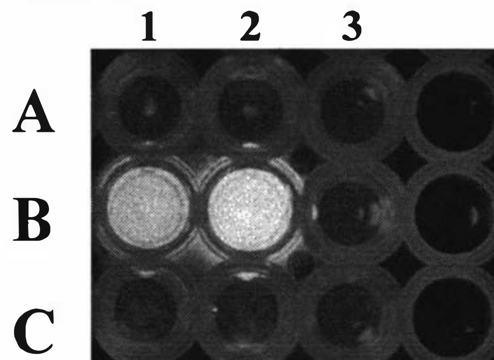


Figure 13
Maintenance of the GUS phenotype in endophyte mycelium.
Endophyte mycelium was isolated from plant tissues of the tillers for which differences in GUS expression are indicated in Fig. 12 (above).
Row **A** contains mycelium isolated from tiller 1 (row **A**, Fig. 12), row **B** contains mycelium from tiller 2 (row **B**, Fig. 12). Row **C** contains un-transformed Lp 19 as a negative control.
Lane 3 of each row contains buffer with substrate only.
Fungal mycelium was harvested from exponentially growing liquid cultures and incubated in the presence of the GUS substrate MUG as described in Fig. 12.
Activities measured in the extractive GUS assay (Section 2.6.1) were not detected in the isolate KLMTV tiller 1 and Lp 19. The isolate from KLMTV tiller 2 had a GUS activity concentration of 7.7×10^4 (pmol MU) min^{-1}(mg DW)$^{-1}$.



the GUS-positive tiller maintained expression over the same period of subculture (Fig. 13). This indicated that the loss of GUS expression in mycelium was stable and heritable, thus that it probably was due to a mutation affecting the expression of the GUS gene.

The finding of complete loss of GUS activity in a single tiller suggests that GUS expressing and non-expressing mycelium segregates in a defined way into different tillers, and thus seems to indicate that the hyphae in a tiller are clonal. This was further supported by observations on a number of GUS expressing and non-expressing tillers; differential staining of single hyphae growing from tissues of these tillers with a GUS-specific stain revealed that in none of these tillers both GUS phenotypes were present at the same time (Y. Y. Tan, personal communication, Massey University). This indicates that the occurrence of tillers infected by mycelium constituting in genetically different hyphae is very low.

Accumulation of endophyte-infected tillers lacking GUS expression within plants was observed previously, as plants had been replanted without selection for the GUS marker (see above, this section). Therefore, for protection against uncontrolled loss of the marker during plant propagation, a procedure was employed for positive selection at re-planting and carried out as described (Section 2.4.4). Only GUS positive tillers were used for plant propagation.

After implementation of this protocol numbers of GUS-negative tillers within plants were low: six screenings for GUS, involving three plant genotypes, were performed and per screening 15 tillers (five per plant genotype, randomly selected from three plants, respectively) were assessed. During four screenings no negative tillers were detected; one negative tiller was found during one screening, and two negative tillers (originating from one plant) during another screening. No increase of negative tillers in plants over time was observed. In additional extractive GUS analyses on individual leaves from 14 tillers no lack of GUS activity was detected.

3.1.9 Estimation of Inhibition of GUS Activity in Plant Tissue Extracts

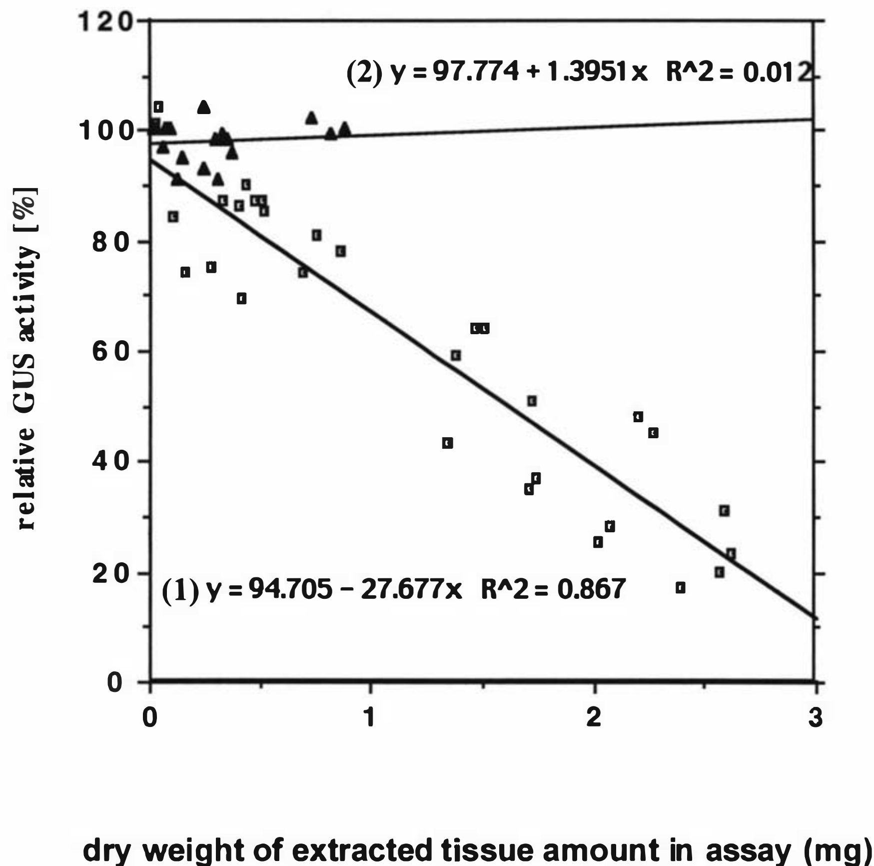
In some plant tissues, inhibition of GUS activity was detected in the quantitative GUS assay (Section 2.6.1). The inhibition in the assay was therefore quantitatively examined. Plant tissues of different types (emerging leaf upper/lower section; mature leaf sheath/blade) from three different plant genotypes infected by the GUS-transformed endophyte were assessed for GUS activity inhibition as described in Fig.14. Tissues varied in GUS activity concentration due to variation in the concentration of metabolically active mycelium. Therefore for comparing inhibition in the different tissues relative activities were calculated for all tissues: for each series varying in extracted amount of sample, linear regression was performed on the absolute values for GUS activity; the intercept of the regression (= GUS activity if an infinitely small amount of extract would have been used) was set to 100 per cent activity and relative activities in this series calculated.

A significant dependence of measured GUS activity concentration on the amount of extracted sample existed for leaf blades and outer (third) leaf sheath; a higher amount of extracted sample in the assay led to a lower GUS activity measured. No measurable effects on GUS activity were found in extracts from tissues enclosed in other leaf tissues. Therefore, the relative values for tissues with and without GUS inhibition were combined, respectively (Fig. 14). The equation of the regression the leaf tissues with inhibition was then used (assuming that an infinitely small volume of extract had been applied) to correct GUS activities in extracts from third leaf sheath, and leaf blades of all leaves.

Figure 14

GUS activity in assays with different dry weight amounts of extracted plant tissue infected by the GUS-transformant KS1. Tissues extracted were lower tissues (first leaf sheath and lower emerging leaf) indicated by triangles, and tissues from leaf blades and outer sheaths of three plant genotypes (squares). Relative activities were plotted to account for differences in absolute levels of activities present in different extracts (Section 3.1.9). Linear regressions were performed separately on tissues with inhibition (1) and without inhibition (2).

Per GUS extraction (Section 2.5.3), a weighed amount of 6-10 mg (DW) tissue in 700 µl GUS extraction buffer was used. From each extract, aliquots of 5, 20, and 50 µl (leaf sheath) and 30, 100, and 150 µl (leaf blade) were used for quantitative GUS determination in 200 µl final assay volume (Section 2.6.1). The amount of tissue used in each assay was calculated from the amount of extract in the assay and the amount of tissue (in dry weight) extracted in 700 µl.



3.2 ENDOPHYTE METABOLIC ACTIVITY AND ALKALOID SYNTHESIS IN *N. LOLII* ENDOPHYTE-INFECTED PERENNIAL RYEGRASS

3.2.1 Assessment of the Correlation of GUS Activity with Endophyte Metabolic Activity in Fungal Transformants

In this study, the *N. lolii* strain Lp 19 (Christensen *et al.*, 1993) transformed with the GUS gene of *E. coli* under the control of a constitutive promoter was used. Constitutive GUS expression may be an indicator for overall protein biosynthesis and, therefore, metabolic activity in endophyte mycelium (Section 1.4). However, the proposed relationship between GUS activity and endophyte metabolic activity had not been tested previously. Moreover, since turnover rates of the GUS enzyme in mycelium were not known, it was necessary to determine how closely measured GUS activity in endophyte mycelium is correlated with the metabolic activity of the endophyte.

Measured levels of enzyme activity may largely reflect accumulated GUS activity rather than GUS synthesis. If the rate of degradation of this enzyme was slow in fungal mycelium, it was thus possible that GUS would hardly vary with metabolic activity in the mycelium. Therefore, the usefulness of the GUS system as a quantitative marker for endophyte metabolic activity needed to be tested, by assessing the effect of changes in the metabolic state of the fungus on levels of GUS activity. This was carried out in laboratory culture where changes in the metabolic state of the mycelium can be readily induced and observed.

3.2.1.1 GUS Activity in the Endophyte Correlates with Active Growth of Mycelium

Metabolic activity as, for example, rates of protein biosynthesis, should be highest during exponential growth in fungal mycelium and reduced when growth slows or ceases in stationary phase (Greasham, 1992; Prosser, 1993; Righelato, 1979). If GUS was a valid indicator of endophyte metabolic activity, measured levels of GUS activity in mycelium

should therefore increase during active growth and decline in stationary phase. GUS activity concentration in endophyte mycelium was therefore examined during different growth phases in YEG liquid medium (Section 2.2.2). In order to observe the hypothesised increase in GUS activity during active growth, starved stationary phase mycelium was used as an inoculum (as described in Fig. 15) and grown as described (Section 2.4.2). Growth of the endophyte was assessed as biomass increase (measurement of fungal dry weight per culture volume at regular time points, Section 2.4.3). GUS activity in mycelium was extracted as described (Section 2.5.3), for quantitative determination as detailed in section 2.6.1.

Exponential growth set in after three days of incubation and GUS activity also increased at this time (Fig. 15). Growth continued until day 14 (rate of dry weight increase between day 4 and day 14 was 0.8 mg $ml^{-1}day^{-1}$, approximated by linear regression, r^2 = 0.990). GUS activity per dry weight increased during exponential growth at a rate of 4900 (pmol MU) min^{-1} $(mg\ DW)^{-1}$ day^{-1} (r^2= 0.937), and reached its maximum a day after the culture entered stationary phase. Subsequently it declined, with a half-life of 8-10 days in early stationary phase and 10-12 days in late stationary phase. The maximum difference in GUS activity per dry weight mycelium between actively growing and stationary phase mycelium was 26-fold in this culture [7.9×10^4 (pmol MU) min^{-1} $(mg\ DW)^{-1}$ at day 15 compared to 3.0×10^4 (pmol MU) min^{-1} $(mg\ DW)^{-1}$ at day 62 in late stationary phase].

Figure 15

GUS activity concentrations in fungal mycelium and mycelial concentration per culture volume of the GUS-transformant KS1 during growth in liquid YEG medium.

Preparation of inoculum was carried out as follows: mycelium of a stationary culture (>3 weeks old, 7-8 mg DW mycelium per ml) was harvested and washed, ground in saline as described in section (2.4.2) and suspended in 20 mM potassium phosphate buffer (pH 5.0). The ground mycelium was incubated in this buffer for four days at room temperature without shaking. The mycelium was harvested and washed (saline) and between 10-20 mg fresh weight were used to inoculate an Erlenmeyer flask with 50 ml YEG medium (Section 2.2.2).

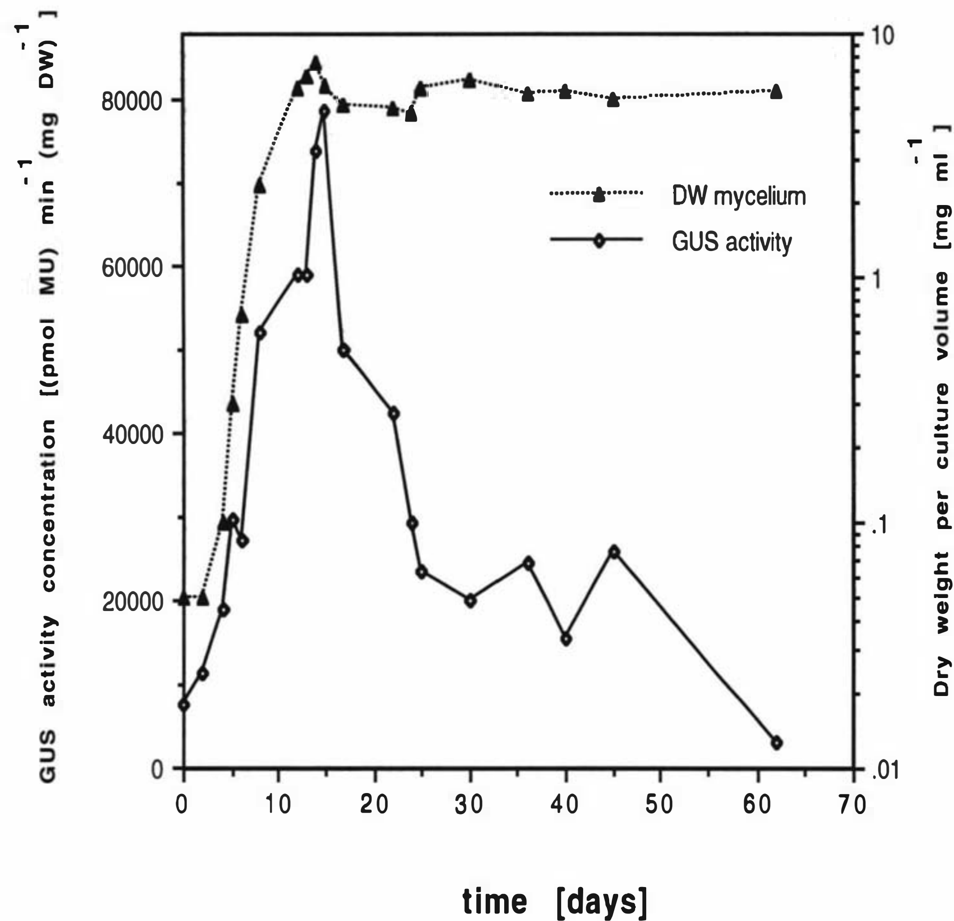
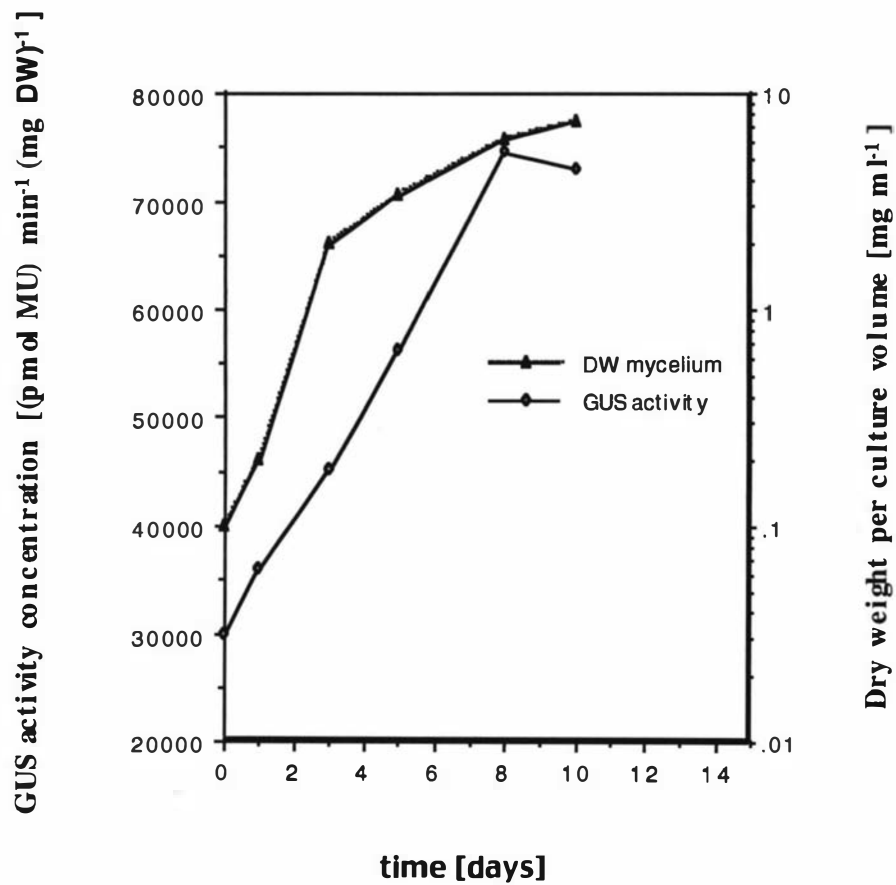


Figure 16

GUS activity concentration in fungal mycelium and mycelial concentration per culture volume of the GUS-transformant KS1 in PDB medium.

Preparation of inoculum and inoculation were carried out essentially as described before (legend Fig. 15), the incubation step in potassium phosphate prior to inoculation used in the previous growth experiment was omitted in this experiment. The culture volume was 50 ml.



In the previous growth experiment, YEG medium had been used. To determine whether different culture media lead to differences in GUS activity expression, the growth experiment was repeated as before, except with PDB medium (Section 2.2.1), until the onset of stationary growth phase (Fig. 16). GUS activity concentration was higher in the inoculum, as the incubation step in phosphate buffer had been omitted. In PDB, the growth rate was 0.8 mg dry weight mycelium per ml culture per day (between days 1 and 10, approximated by linear regression, r^2= 0.993) and the increase in GUS activity was 4700 (pmol MU) min^{-1} $(mgDW)^{-1}$ day^{-1} (r^2= 0.963), both rates being very similar to YEG (see above, this section). Therefore, growth and GUS activity in PDB was comparable to growth and GUS activity in YEG medium.

These experiments demonstrated that metabolically active mycelium had high levels of GUS activity, whereas stationary growth phase mycelium had substantially and significantly lower GUS activity concentration. However, these experiments also indicated that the onset of growth does not immediately lead to high levels of GUS activity in cells. For stationary phase, a half-life of GUS could be determined, and it can be expected that the GUS levels in stationary culture would eventually decline to below measurable concentrations. For exponential growth, the rate of increase could likewise be determined, but not the maximum possible GUS activity per dry weight in exponentially growing cultures, since GUS activity continued to increase until stationary phase was reached. It seemed probable that its maximum during exponential growth would be higher than the value observed here, provided that exponential growth would have been allowed to proceed longer.

3.2.1.2 Determination of the Maximum GUS Activity Level in Metabolically Active Mycelium

In order to determine the highest attainable level of mycelial GUS activity concentration in culture, mycelium was grown (Section 2.4.2) in PDB under semi-continuous conditions, and GUS activity assessed repeatedly (Table 9). GUS activity concentration varied slightly between samplings from this culture (Table 9), but no measurable increase over time was detected, as determined by linear regression GUS over

Table 9

GUS activity concentration in exponentially growing endophyte mycelium (GUS transformant strain KS1) grown in serial liquid cultures (PDB growth medium).

Mycelial GUS activity concentration in culture no. 1 was determined on late-exponential culture (14 days of growth, 6 mg dry weight per ml). About 20 - 30 % of the mycelium was then transferred into a flask with fresh medium, and after 2 - 4 days GUS was again determined (culture no. 2), and sub-culturing was repeated continuously as previously and GUS determined for additional three times (cultures nos. 3 - 5).

Culture no.	GUS activity concentration x 10^{-4} [(pmol MU) min^{-1} (mg DW)$^{-1}$]
1	7.1
2	12.8
3	8.1
4	12.5
5	9.2
average ± std dev	9.9 ± 2.3

the number of re-cultures (R squared = 0.057, $P > 0.6$). The average GUS activity per mycelial dry weight was 9.9×10^4 (pmol MU) min^{-1} mg^{-1} (Table 9). The rate in GUS increase observed in the previous experiments was 4700 - 4900 (pmol MU) min^{-1} (mg $DW)^{-1}$ day^{-1} (previous section). Therefore, given this rate of increase, maximum GUS activity per fungal mycelium should be reached after about 20-21 days of exponential growth, provided the inoculum was relatively low in GUS activity [$\leq 1.0 \times 10^4$ (pmol MU) min^{-1} (mg $DW)^{-1}$].

3.2.1.3 GUS Activity Declines in the Presence of an Inhibitor of Endophyte Metabolism

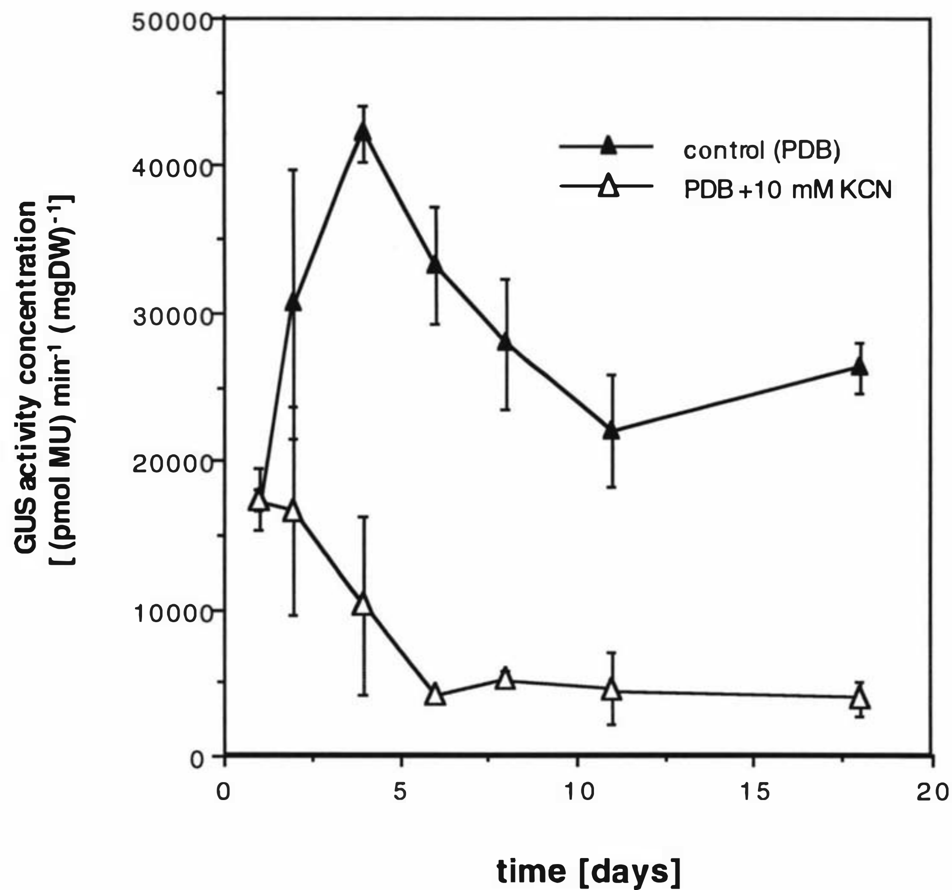
Mycelium growing in liquid culture showed the expected decline in GUS activity concentration in stationary phase (previous sections). However, stationary growth phase does usually not involve a complete cessation of protein synthesis (Prosser, 1995). It is conceivable that in the plant conditions may arise where the metabolism of the endophyte is strongly impaired, for example at extreme ambient temperatures, or if the plant becomes antagonistic towards the endophyte, as reported previously (Koga *et al.*, 1993, Christensen *et al.*, 1995). To determine the effect of conditions affecting metabolic activity of the endophyte on GUS activity, its concentration in endophyte mycelium after addition of a metabolic inhibitor (KCN) was examined.

Each culture flask containing PDB (Section 2.2.1) was inoculated with 10 mg DW mycelium and grown as described (Section 2.4.2). To determine the effect of culture conditions (with or without KCN) on endophyte metabolic activity, flasks were harvested at completion of the experiment, mycelia were washed, freeze-dried and weighed (Section 2.4.2), for determination of mycelial dry weight per flask. The cumulative weights from the samplings for GUS determination and the weights of the complete harvests were combined, to determine total mycelial dry weight in each flask as an indicator for growth (= metabolic activity).

Figure 17

GUS activity concentration in endophyte mycelium in PDB and PDB + 10 mM KCN. KCN was added for inhibiting metabolic activity in the endophyte.

Mycelium from a stationary liquid pre-culture was used to inoculate 25 ml of PDB per flask. After 24 hours of growth, GUS activity concentration in mycelium was assessed, and a stock solution of 1 M KCN added to designated flasks to a final concentration of 10 mM. All flasks including the controls were then further incubated. GUS activity was assessed at regular time points. Each data point represents the mean of two measurements. Error bars = ± 1 standard deviation of the mean.



The addition of KCN led to a three-fold reduction in GUS activity within 48 hours relative to the GUS activity concentration after 24 hours of growth (Fig. 17). The estimated half-life of the GUS enzyme activity in KCN cultures was about three days during the first 1- 5 days after incubation with KCN, and about six days after seven days incubation. Addition of KCN stopped metabolic activity, indicated by the mycelial dry weights in the different flasks. Unlike the control culture, which increased its dry weight five-fold (to 50.6 ± 4.31 mg DW) during the experiment, the dry weight of the cultures with KCN (10.0 ± 1.27 mg DW) did not increase measurably (indicated are mean ± standard deviation, n=2). GUS measurements in the control cultures were used to demonstrate that the mycelium expressed the GUS gene normally in the absence of KCN. The rate of GUS activity increase in the controls was 5000 pmol MU min^{-1} $(mg\ DW)^{-1}$ day^{-1}), thus similar to rates measured previously in growing cultures (Section 3.2.1.1), until the control cultures reached maximum dry weight concentration upon depletion of nutrients (at six days of growth).

Potential interference of KCN with activity of the GUS enzyme in the assay was tested with extracts from the cultured controls. Two extracts were quantitatively assessed for GUS activity as before and then spiked with 1 mM KCN (final concentration; this concentration exceeded the expected concentration in KCN-cultured mycelium more than 10-fold). After incubation for one hour (at room temperature), GUS activity in the KCN-spiked extracts was measured. Measured activities after KCN addition were identical to activities measured prior to addition (data not shown).

3.2.1.4 Summary and Conclusions

The results of these experiments indicated that levels of GUS activity concentration in mycelium were high in metabolically active endophyte mycelium, and significantly lower in stationary phase or metabolically inhibited mycelium. This demonstrated the utility of constitutive GUS expression for determining the metabolic state of endophyte mycelium. The culture experiments indicated some delay before an alteration of the metabolic state (stationary phase *versus* actively growing) brings about the maximum GUS activity levels, but they also showed that significant changes occur within days. As most

plant tissues within a grass tiller are probably older than 15-20 days (Skinner & Nelson, 1995, Durand *et al.*, 1999), GUS would be a valid and useful indicator for the endophyte metabolic state in the grass plant.

3.2.2 Selection of Different Plant Genotypes Infected by a GUS-Transformed Endophyte Strain

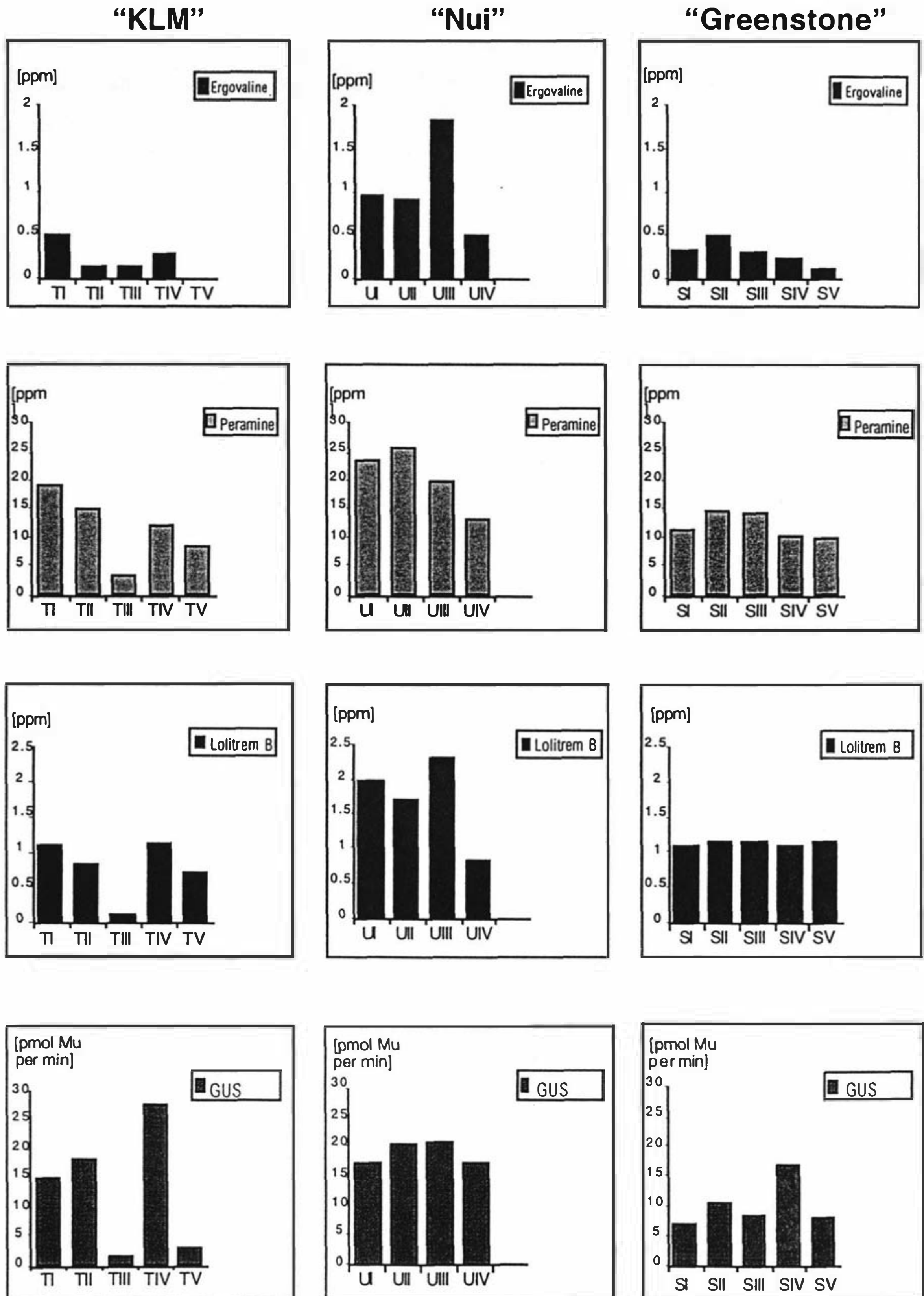
An aim of the present study was to determine the quantitative relationship between endophyte metabolic activity, and the alkaloids ergovaline, peramine and lolitrem B in the perennial ryegrass-*N. lolii* symbiosis (Section 1.5). Previous studies have reported that levels of the alkaloids can vary significantly between plant genotypes (Agee & Hill, 1994; Latch, 1994), indicating that the plant genotype is an important determinant for alkaloid production. Thus, to allow some general conclusions about alkaloid synthesis and endophyte metabolic activity in this symbiosis, it was necessary to include a representative range of plant genotypes.

As in this study only a limited number of plants could be investigated, a preliminary investigation was carried out, with the objective to select for suitable plant genotypes. The criteria for selection were that genotypes differed in endophyte metabolic activity concentration, and in some or all of the alkaloids. Seedlings of the three cultivars "Grasslands Nui", "KLM" and "Greenstone" were inoculated with the *N. lolii* strain Lp 19 (GUS-transformant KS1, Section 2.1) as described (Section 2.4.5). One month following the inoculation, plants derived from the seedlings were examined for the presence of *N. lolii* hyphae in leaf tissue as described (Section 2.7). Five plants (= plant genotypes) per cultivar for which endophyte-infection was verified were randomly selected and, because of space limitations in the growth cabinet, vegetatively propagated in the glasshouse. This investigation was carried out in late winter early spring (August - October), providing relative stability in environmental conditions in the glasshouse, with no temperature extremes impacting on alkaloid levels especially (Section 1.3.4).

Three months after inoculation, the selected plants were analysed for GUS activity on one day. Harvest of plant material for the analysis of alkaloids was carried out on one day, two days after the determination of GUS. Each plant genotype infected by the strain KS1 was assessed for endophyte metabolic activity and the alkaloids ergovaline, peramine, and lolitrem B as follows:
Two tillers per plant genotype were removed from plants as described (Section 2.5.1.1), and assayed for GUS activity in the leaf sheath with the previously developed (Section 3.1.5) semi-quantitative GUS microtitre assay (Section 2.6.2). Four tissue pieces of the youngest mature sheath of each tiller were assessed for GUS, and activities in these were pooled for each tiller and average GUS activity determined from the two tillers for each genotype. The leaf sheath was selected for the GUS analysis, as the concentration of GUS activity is usually high in this leaf fraction (Herd *et al.*, 1997). The remaining tillers were harvested, and combined for each genotype for quantitative determination of the alkaloids ergovaline (Section 2.8.3), peramine (Section 2.8.1), and lolitrem B (Section 2.8.2). Alkaloid concentration was assessed on the pseudostem of tillers (cut approximately 0.5 cm above the base, and comprised of leaf sheaths of 2-4 leaves and immature leaves). The pseudostem was chosen for the determination as it consists of basal tissues of all leaves of a tiller, thus for being representative for the whole plant.

The *N. lolii* strain Lp19/KS1 showed significant variation in endophyte metabolic activity (= GUS activity) and alkaloid concentration in different plant genotypes (Fig. 18). Overall differences between cultivars seemed to exist: in "Greenstone", levels of endophyte metabolic activity and alkaloid concentration tended to be lower than in the other two cultivars. In "Nui", levels of all three alkaloids tended to be higher than in "Greenstone" and "KLM". In most plants, no consistent trends in endophyte metabolic activity were apparent that were followed by all or even some of the alkaloids. However, in plant genotype KLM TIII concentrations of all alkaloids and endophyte metabolic activity were both comparatively low. The lower metabolic activity was probably due to a lower fungal concentration in this genotype, as assessed by examination of fungal growth from leaf tissues on PDA (Section 2.4.1, data not shown). In the cultivar "KLM"

Figure 18
GUS activity concentration and concentrations of the three alkaloids ergovaline, peramine and lolitrem B in 5 (4) genotypes of the three cultivars KLM, NUI and Greenstone. All plants were infected by the GUS transformant strain KS1.



(genotype TV), an unexplained loss of GUS activity from fungal mycelium occurred. At this stage of the study, the reason(s) for the GUS loss was unknown, and therefore plants of this cultivar were excluded from further investigations.

Two associations of the cultivar "Nui", Nui UIII and Nui UIV were selected for further studies. In this cultivar, levels of ergovaline were comparatively high, which facilitated better resolution of differences in ergovaline concentration between plant tissues and genotypes in later experiments. In addition, the two plant genotypes showed nearly threefold differences in the concentration of ergovaline and lolitrem B, and about 15 per cent variation in endophyte metabolic activity (Fig. 18). A third association, Nui D with KS1, had already been established in a controlled environment (Saunders & Schmid, 1995) and was therefore also included. The three associations were introduced and further grown in a controlled environment cabinet (Section 2.4.4).

3.2.3 The Endophyte Metabolic State is Uniformly High in Different Plant Tissues and Plant Genotypes

For this study, a major aim was to determine the metabolic state of the endophyte in the plant (Sections 1.4 and 1.5). As shown previously (Section 3.2.1), levels of GUS activity in mycelium were substantially different in culture, depending on the growth phase and, therefore, metabolic state of the endophyte mycelium. Hence, by combining extraction and quantitation of GUS (Sections 2.5.3 and 2.6.1) with the method for determining endophyte biomass concentration *in planta* (Section 2.7), the endophyte metabolic state (EMS) in the plant could be assessed. The EMS was calculated by the equation:

$$\text{EMS} = \frac{\text{endophyte metabolic activity (GUS activity) in plant tissue}}{\text{endophyte biomass (biovolume) in plant tissue}} \quad \text{(pmol MU) min}^{-1}\,(\text{mm}^3)^{-1}$$

Differences in the concentration of endophyte metabolic activity between plant genotypes were observed previously (Section 3.2.2). At the start of this investigation, a

question was whether these differences were due to variation in fungal biomass and/or in the EMS, and whether significant differences in the EMS between different plant tissues existed.

Plants used in the experiments were grown environmentally controlled (Section 2.4.4). At sampling, the plants were between 5-6 weeks old (from last planting), and tillers that possessed three mature leaves were sampled. The method for the determination of *in planta* endophyte biomass is relatively time consuming because leaf sections have to be weighed and a number of hyphal counts performed (Section 2.7). Therefore, to have a sample size manageable for handling in the laboratory, the EMS was determined in pre-selected tissues. Selection of these tissues was aimed at determining the EMS in the different plant genotypes, and in leaf tissues representing different leaf-developmental stages and leaf sections. Leaves varying in age were chosen, as a change in EMS with increase in leaf age had been hypothesised. Therefore, sections of emerging, first, and third leaves were assessed. For comparing plant genotypes, the EMS was determined in the first leaf sheath. The EMS in leaf tissues varying in age was determined in one genotype, and in sheath *versus* blade in two genotypes. Endophyte biomass and GUS activity concentrations were determined on the same samples of selected leaf sections as described (Sections 2.7 and 2.6.1).

The level of the EMS was very similar in the genotypes Nui D, Nui UIII and Nui UIV. Significant differences in endophyte metabolic activity/GUS activity and endophyte biovolume concentrations existed between these genotypes (Table 10), indicating that variation in endophyte metabolic activity concentration is largely due to a corresponding variation in endophyte biomass concentration. The difference in endophyte metabolic activity concentration between Nui UIII and Nui UIV was largely similar to the difference observed in a previous experiment (Section 3.2.2), indicating for a consistent effect of plant genotype on endophyte concentration. Two plant genotypes (NUI UIII and NUI UIV) differed nearly four-fold in endophyte and endophyte metabolic activity concentration in the leaf blade, but again the EMS was highly similar for the two (Table 11). However, in both genotypes, the EMS in the leaf blade was significantly lower than in the leaf sheath

Tables 10, 11, and 12

Averages (± 1 standard deviation) for endophyte biovolume concentration, GUS concentration, and endophyte metabolic state (EMS) in the plant genotypes Nui D, Nui UIII, and Nui UIV (Table **10**), in leaf blades of Nui UIII and Nui UIV (Table **11**), and in different-aged leaves of Nui UIII (Table **12**). The EMS was calculated for each tissue section and values for replicates were then pooled.

10 Values are listed for first leaf sheaths.

Differences in biovolume and GUS concentrations between plant genotypes were statistical significant ($P < 0.01$), but the differences in the EMS were not significant ($P > 0.05$, one-way ANOVA).

Variable	Nui D (n=4)	Nui UIII (n=4)	Nui UIV (n=4)
Biovolume concentration x 10^{-3} [$mm^3 (mgDW)^{-1}$]	2.18 ± 0.95	2.18 ± 0.22	0.99 ± 0.30
GUS concentration [(pmol MU) min^{-1} $(mgDW)^{-1}$]	265 ± 81	358 ± 84	134 ± 35
EMS x 10^5 [(pmol MU) min^{-1} $(mm^3)^{-1}$]	1.30 ± 0.36	1.64 ± 0.21	1.54 ± 0.26

11 Values are listed for first leaf blades.

The lower EMS in leaf blades than in leaf sheaths (Table **10**) of both Nui UIII and NUI UIV was statistical significant ($P < 0.05$, paired t-test on leaves).

Variable	Nui UIII (n=4)	Nui UIV (n=4)
Biovolume concentration x 10^{-3} [$mm^3 (mgDW)^{-1}$]	1.24 ± 0.17	0.38 ± 0.06
GUS concentration [(pmol MU) min^{-1} $(mgDW)^{-1}$]	130 ± 28	29 ± 12
EMS x 10^5 [(pmol MU) min^{-1} $(mm^3)^{-1}$]	1.05 ± 0.08	0.92 ± 0.04

12 Values listed are from sheaths of genotype Nui UIII.

The difference in EMS between the emerging leaf and each of the mature leaves was significant at $P < 0.05$ (ANOVA, Scheffé's test).

Variable	Emerging leaf (n=3)	First leaf (n=4)	Third leaf (n=2)
Biovolume concentration x 10^{-3} [$mm^3 (mgDW)^{-1}$]	6.70 ± 2.67	2.18 ± 0.22	1.43 ± 0.30
GUS concentration [pmolMU min^{-1} $(mgDW)^{-1}$]	443 ± 81	358 ± 84	253 ± 14
EMS x 10^5 [(pmol MU) min^{-1} $(mm^3)^{-1}$]	0.72 ± 0.23	1.64 ± 0.21	1.80 ± 0.38

Table 13

The endophyte metabolic state in three different leaf tissues (means ± standard deviation, values were pooled from the two plant genotypes Nui UIII and Nui UIV, except for the emerging leaf, which was from Nui UIII only). In the first column, the EMS was calculated based on measured GUS activities and hyphal biovolume (Section 3.2.3). In the second column, the metabolic activity in fungal mycelium was calculated with measured GUS in a leaf section and estimated endophyte biomass concentration in the same section (see text, this section).

The range for the estimated metabolic activity concentration in endophyte mycelium was 1.53 - 6.23 x 10^5 (pmol MU) min^{-1} (mg DW)$^{-1}$.

Leaf section	EMS x 10^5 [(pmol MU) min^{-1} $(mm^3)^{-1}$]	Estimated metabolic activity concentration x 10^5 [(pmol MU) min^{-1} $(mgDW)^{-1}$]
emerging leaf sheath (n=3)	0.72 ± 0.23	2.17 ± 0.68
first mature leaf sheath (n=8)	1.59 ± 0.23	4.77 ± 0.67
first mature leaf blade (n=8)	0.99 ± 0.18	2.96 ± 0.54
third mature leaf sheath (n=4)	1.70 ± 0.25	5.12 ± 0.75
third mature leaf blade (n=4)	1.08 ± 0.27	3.23 ± 0.82
average ± std dev.	1.22 ± 0.42	3.65 ± 1.25

(Tables 10 and 11). The lower EMS in leaf blades relative to leaf sheaths may be due to the difference in hyphal diameter between the two leaf parts (Section 3.1.6). As the average hyphal diameter in the blade was greater, the biovolume per hypha was larger in the blade (Section 2.7), contributing to the lower EMS. However, endophyte metabolic activity concentration per hyphal number in a leaf section was also slightly lower in leaf blades than in leaf sheaths (data not shown). Unexpectedly, the EMS in the emerging leaf (immature leaf sheath) was on average less than 50 per cent of the EMS in mature tissues and the difference between the two was also statistical significant (Table 12). The reason(s) for the lower EMS in the emerging leaf was not further investigated.

These findings indicated that the endophyte was metabolically active throughout the whole plant. It was also of interest to determine how the *in planta* EMS corresponds to previously measured metabolic activity in endophyte mycelium in culture (Section 3.2.1.). As the EMS *in planta* was calculated based on the biovolume of the endophyte mycelium, it could not be directly compared with the *in vitro* metabolic activity, which had been determined based on the dry weight of the fungus. Therefore, to allow for direct comparison, the endophyte biovolume in the leaf tissues was converted to dry weight (assuming a buoyant density of 1.1 g per cm^3 of the endophyte mycelium, Kubitschek, 1987; and a dry matter content of 30 per cent, Woldringh *et al.*, 1993). GUS activity in a section was divided by the estimated endophyte dry weight in the same section to calculate the EMS based on endophyte biomass in the different plant tissues (Table 13).

The *in planta* EMS based on the dry weight of endophyte mycelium was on average more than three-fold greater than endophyte metabolic activity in exponentially growing mycelium in culture [3.65×10^5 (pmol MU) min^{-1} (mg DW) $^{-1}$ in the plant *versus* 0.99×10^5 (pmol MU) min^{-1} (mg DW) $^{-1}$ in culture, Tables 9 and 13]. Assuming that most hyphae in a plant tissue are metabolically active, the difference in metabolic activity between mycelium *in vivo* and *in vitro* is not surprising, since in culture parts of the mycelium may undergo senescence, even if other parts sustain active growth, due to physiological unfavourable conditions (as for example, limiting oxygen concentrations, Righelato, 1979).

In summary, the EMS in the plant was overall very similar in different tissues, despite of substantial and significant differences in endophyte biovolume (= biomass) concentrations between plant genotypes and plant tissues. No decline in the EMS was observed in older tissues, on the contrary, the EMS was greater in the mature tissues. The EMS in all plant tissues was apparently greater than in exponentially growing mycelium from cultures, indicating high levels of metabolic activity in endophyte mycelium throughout the plant.

3.2.4 *In Planta* Alkaloid Distributions and Their Relation to Endophyte Metabolic Activity – None of the Alkaloids is distributed in Exact Proportion to Metabolically Active Endophyte Mycelium

With improved methods for quantitative alkaloid determination (Section 3.1.4), it was now possible to map the distribution of the alkaloids ergovaline, peramine, and lolitrem B with the same resolution and sensitivity as endophyte metabolic activity with the GUS system. This enabled to determine the *in planta* distribution of the alkaloids in relation to the distribution of metabolically active endophyte mycelium. To avoid any influence of temporal variation on the results, in these experiments all plants were planted on the same day (Section 2.4.4). Two weeks prior to this experiment, four tillers per plant genotype had been randomly sampled and assessed for GUS activity. No GUS-negative tillers were found (data not shown).

Sampling from replicate plants was performed alternating between genotypes within three weeks. Plant age at sampling was 60–80 days (from planting). Per plant, 10–12 tillers of similar size and that possessed three mature leaves were selected and dissected into 19 plant-tissue fractions. To obtain sufficient material for the analyses, mature tissues were pooled from five tillers, and immature tissues and stem tissues from 10–12 tillers of each plant. Endophyte metabolic activity and the three alkaloids were determined each on subsamples from the sampled tissue material (Section 2.5.2).

As endophyte metabolic activity and alkaloids were assessed on the same sample material, ratios between the two were determined for each tissue section. Since levels of metabolic activity in endophyte mycelium were similar throughout the plant and between plant genotypes (see above, previous section), its concentration largely corresponds to the concentration of endophyte mycelium. The ratio alkaloid to endophyte metabolic activity was therefore used to approximate the concentrations of the alkaloids per endophyte mycelium in a plant tissue. To test whether within a plant genotype overall concentrations of the three alkaloids were determined by overall endophyte metabolic activity

concentration, the ratio values determined for each plant tissue were pooled for each plant genotype.

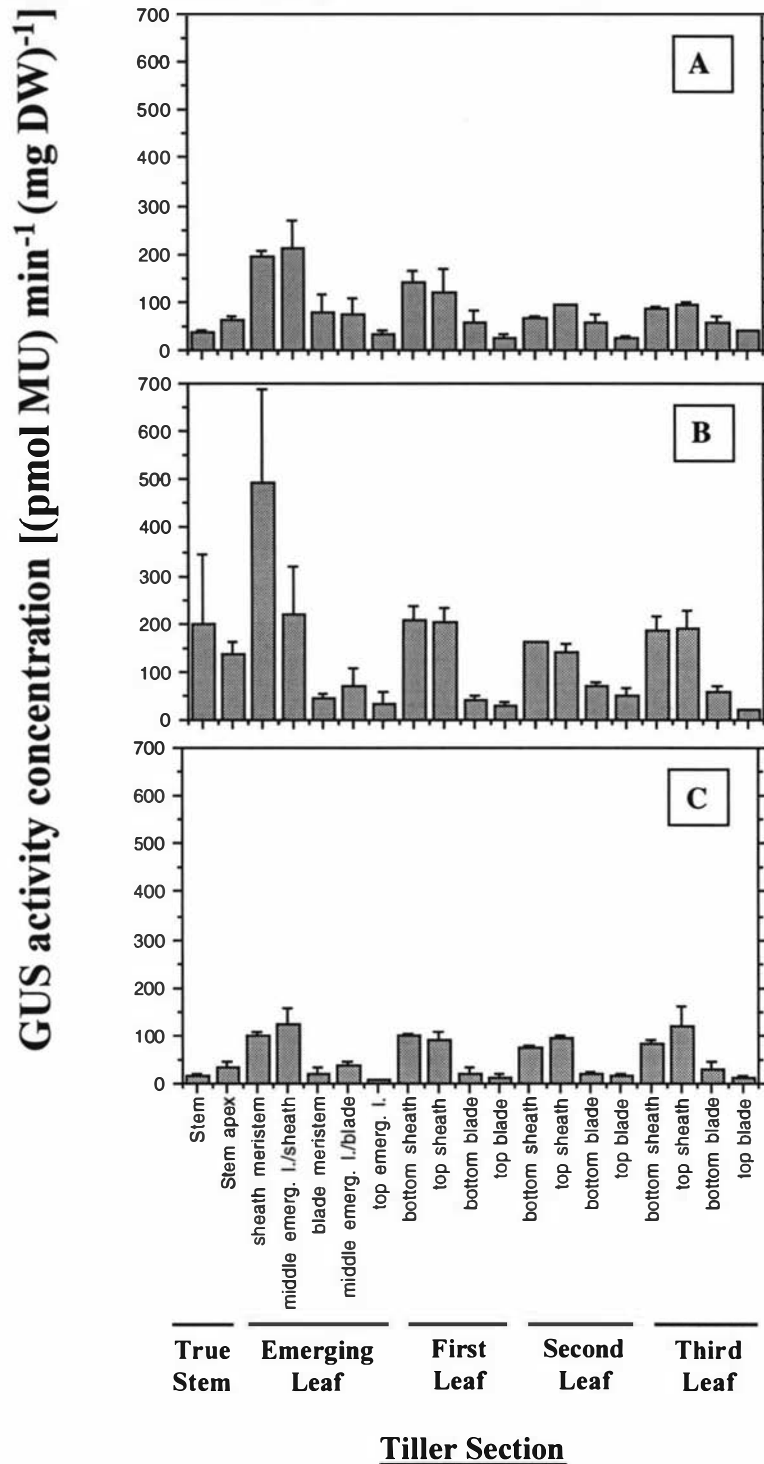
3.2.4.1 Within-Tiller Distribution of Endophyte Metabolic Activity in Three Plant Genotypes

In all plant genotypes leaf sheaths had higher concentrations of endophyte metabolic activity than leaf blades (Fig. 19). In leaf sheaths, lower and upper sections had very similar concentrations, whereas in leaf blades endophyte metabolic activity was consistently lower in the upper sections (Fig. 19). The difference in endophyte metabolic activity concentration between sheath and blade, and also between upper and lower blade, was statistical highly significant ($P < 0.0001$, t-tests, values were paired within each sample-replicate leaf and all leaves included for the two analyses). Leaves of different age did not significantly differ in overall endophyte metabolic activity concentration ($P > 0.05$, t-test, leaves were paired within sample replicates). Consistent with previous investigations in this study (Sections 3.2.2 and 3.2.3), plant genotypes varied measurably in endophyte metabolic activity concentration. Notably in the genotype Nui UIII the concentration was greater than in Nui UIV and Nui D (Fig. 19). The difference between Nui UIII and Nui UIV was highly significant ($P< 0.001$), and the difference between Nui UIII and Nui D was also significant ($P< 0.01$, both analyses were performed on the pooled data including all plant tissue sections by Scheffé's test for comparison of means).

These results indicate that the distribution of endophyte metabolic activity within a tiller is largely similar in all of the plant genotypes, despite of significant variation in overall concentration of endophyte metabolic activity between the genotypes.

Figure 19

GUS activity (endophyte metabolic activity) concentration in 19 sections of a tiller of the three plant genotypes Nui D (**A**), Nui UIII (**B**) and Nui UIV (**C**) infected by the strain Lp 19 (GUS-transformant KS1).
GUS activity was determined on subsamples from tissues pooled from five (mature tissues) or 10-12 (immature tissues and true stem) tillers.
Each bar represents the mean from measurements on two plants.
Error bars = 1 standard deviation.



3.2.4.2 Levels of Endophyte Metabolic Activity in Leaf Meristematic Regions Provide Evidence for Synchronous Growth of Endophyte and Plant

Based on the pattern of the *in planta* distribution of metabolically active mycelium, it has been proposed that growth of plant and endophyte occur synchronously (Schmid *et al*., 2000), but this hypothesis has not been tested previously. Given synchronous growth, the concentration of the endophyte in immature tissues should be similar to the concentration in the mature tissues. Since variation in concentration between tissues occur, of particular interest is the level of endophyte metabolic activity in immature tissues relative to the corresponding mature tissues (for example, emerging leaf sheath sections *versus* mature leaf sheaths). In all plant genotypes, the concentrations of endophyte metabolic activity in immature tissues were similar to, or higher than the concentrations in the corresponding mature tissues (Fig. 19). The occasionally greater concentrations of endophyte metabolic activity in immature tissues were probably due to the lower accumulation of plant biomass in these tissues.

Since the EMS (= endophyte metabolic activity per endophyte biomass) was very similar in all tissues (Section 3.2.3), the *in planta* distribution of endophyte metabolic activity can be assumed to correspond to the distribution of endophyte biomass. Thus, the similar concentrations of endophyte metabolic activity and, hence, endophyte biomass in mature and immature leaf tissues indicate that growth of the plant and endophyte co-occur. A further prediction of this hypothesis is that the ratio between apical and basal leaf tissues should remain constant through the development of the leaf. The pooled data from three genotypes (Table 14) show only a small and non-significant increase in the endophyte metabolic activity concentration ratio between blade and sheath as leaves develop and age, indicating that the ratio is already established in the emerging leaf.

Table 14

Pooled ratios (of the three genotypes Nui D, Nui UIII and Nui UIV, n=6) of endophyte metabolic activity concentration between blade and sheath in meristematic and mature leaves. No significant difference in ratios was detected between the different leaves ($P > 0.05$, ANOVA).

Ratio	Value for ratio	Standard deviation
blade meristem : sheath meristem	0.27	0.16
emerging blade : emerging sheath	0.27	0.23
1st leaf blade : 1st leaf sheath	0.29	0.15
2nd leaf blade : 2nd leaf sheath	0.44	0.18
3rd leaf blade : 3rd leaf sheath	0.39	0.20

3.2.4.3 Distribution of the Alkaloid Ergovaline in Plant Tissues, and Its Relationship to Endophyte Metabolic Activity

Plant genotypes varied noticeably in overall concentration of the alkaloid ergovaline: Nui UIV and Nui D had overall lower concentrations than Nui UIII (Table 15A). The difference between Nui D and Nui UIII was statistically highly significant ($P < 0.001$). The difference between Nui UIV and Nui UIII was also significant ($P < 0.01$), and no significant difference was detected between Nui D and Nui UIV ($P > 0.05$). Comparisons were performed with one-way ANOVA and Scheffé's test for comparison of means. However, differences in the means for the ergovaline : endophyte metabolic activity-ratio between the genotypes were smaller than differences in the means for ergovaline concentration (Table 15A). Only between the genotypes Nui D and Nui UIII a significant difference in the ratio existed ($P < 0.001$, Scheffé's test). This may indicate that the overall levels of ergovaline in the plant are to some degree determined by the overall amount of metabolically active mycelium, but that the plant genotype may also control ergovaline production in the endophyte mycelium.

In all plant genotypes, ergovaline was mostly concentrated in basal tissues, notably the true stem and lower leaf sheath (Fig. 20). A steep basal-apical concentration gradient existed between lower and upper sheath, especially of leaf 2 and 3 in all genotypes (Fig. 20), and the difference between these two sections was statistically highly significant ($P < 0.0001$, t-test including all leaves from all genotypes, data were paired for each leaf). Ergovaline concentrations in the leaf blade were much lower than in the leaf sheath (Fig. 20). In the emerging leaf ergovaline was more evenly distributed (Fig. 20).

The values for the ratio ergovaline : endophyte metabolic activity determined on all plant tissues are presented for each plant genotype (Fig. 21). In all of the plant genotypes, the distribution of the ratio ergovaline : endophyte metabolic activity was similar to the distribution of ergovaline concentration (Fig. 20). The ratio was generally greater in the

Table 15
Mean endophyte metabolic activity and concentrations of ergovaline (**A**), peramine (**B**) and lolitrem (**B**) in the plant genotypes Nui D, UIII, and UIV (values determined on plant tissue sections were pooled, giving each tissue the same weight; all tissue sections of a tiller were included). The alkaloid : endophyte metabolic activity ratios were determined on each tissue and tissues were then pooled, giving seach tissue the same weight.

A

Plant genotype	endophyte metabolic activity [(pmol MU) min^{-1} (mg DW)$^{-1}$]	Total ergovaline [µg g^{-1}]	ergovaline/ endophyte metabolic activity x 10^{-6} [µg (pmol MU)$^{-1}$ min^{-1}]
Nui D	82	0.13	2.17
Nui UIII	134	0.73	5.73
Nui UIV	53	0.23	4.76

B

Plant genotype	endophyte metabolic activity [(pmol MU) min^{-1} (mg DW)$^{-1}$]	peramine [µg g^{-1}]	peramine/ endophyte metabolic activity x 10^{-4} [µg (pmol MU)$^{-1}$ min^{-1}]
Nui D	82	24.5	3.96
Nui UIII	134	21.0	2.81
Nui UIV	53	12.3	4.01

C

Plant genotype	endophyte metabolic activity [(pmol MU) min^{-1} (mg DW)$^{-1}$]	lolitrem B [µg g^{-1}]	lolitrem B/ endophyte metabolic activity x 10^{-5} [µg (pmol MU)$^{-1}$ min^{-1}]
Nui D	82	2.19	3.47
Nui UIII	134	0.88	0.87
Nui UIV	53	0.77	1.86

Figure 20

Distribution of the alkaloid ergovaline within a tiller of each of the three plant genotypes Nui D (**A**), Nui UIII (**B**) and NUI UIV (**C**) infected by the strain Lp 19 (GUS-transformant KS1).

Each bar represents mean of measurements on two replicate samples..
Error bars = 1 standard deviation. nd=not detected.

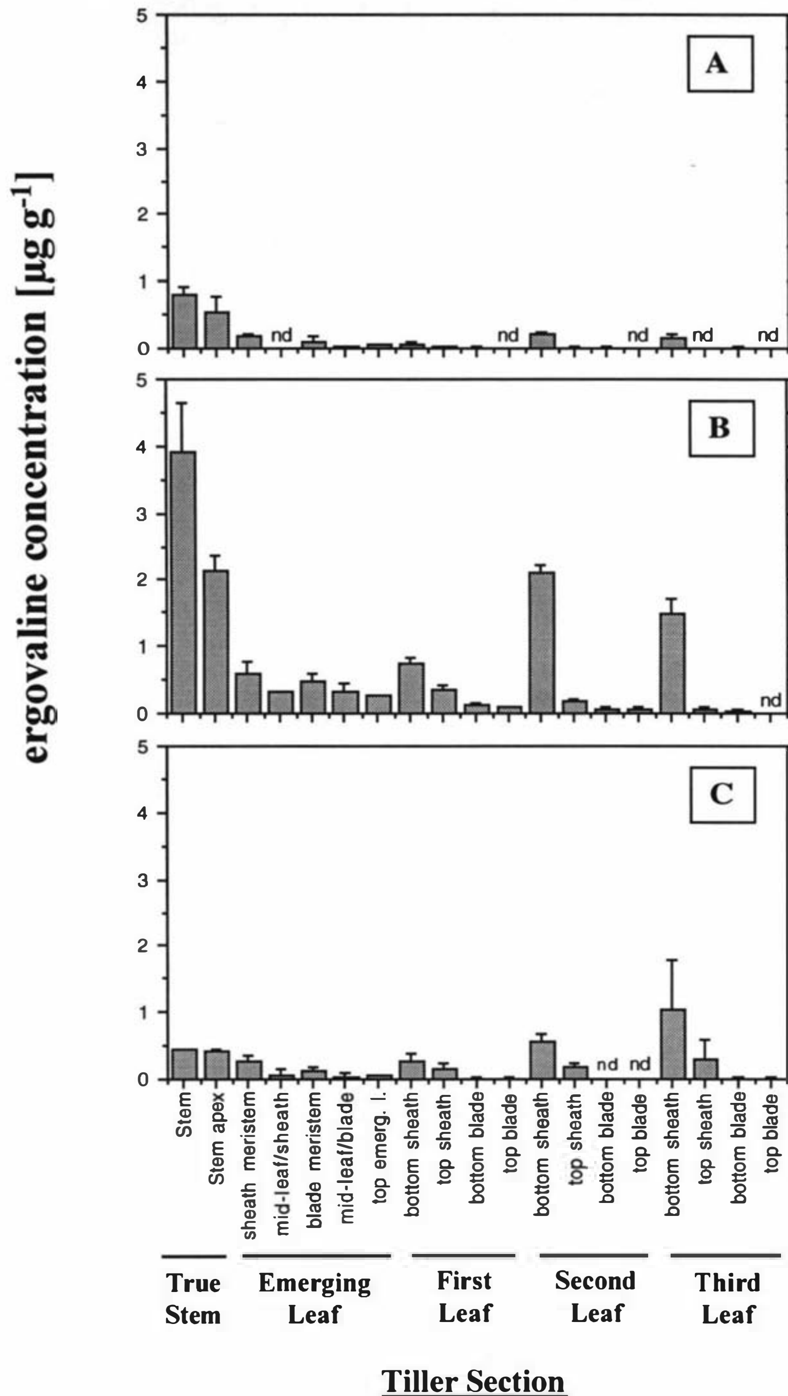
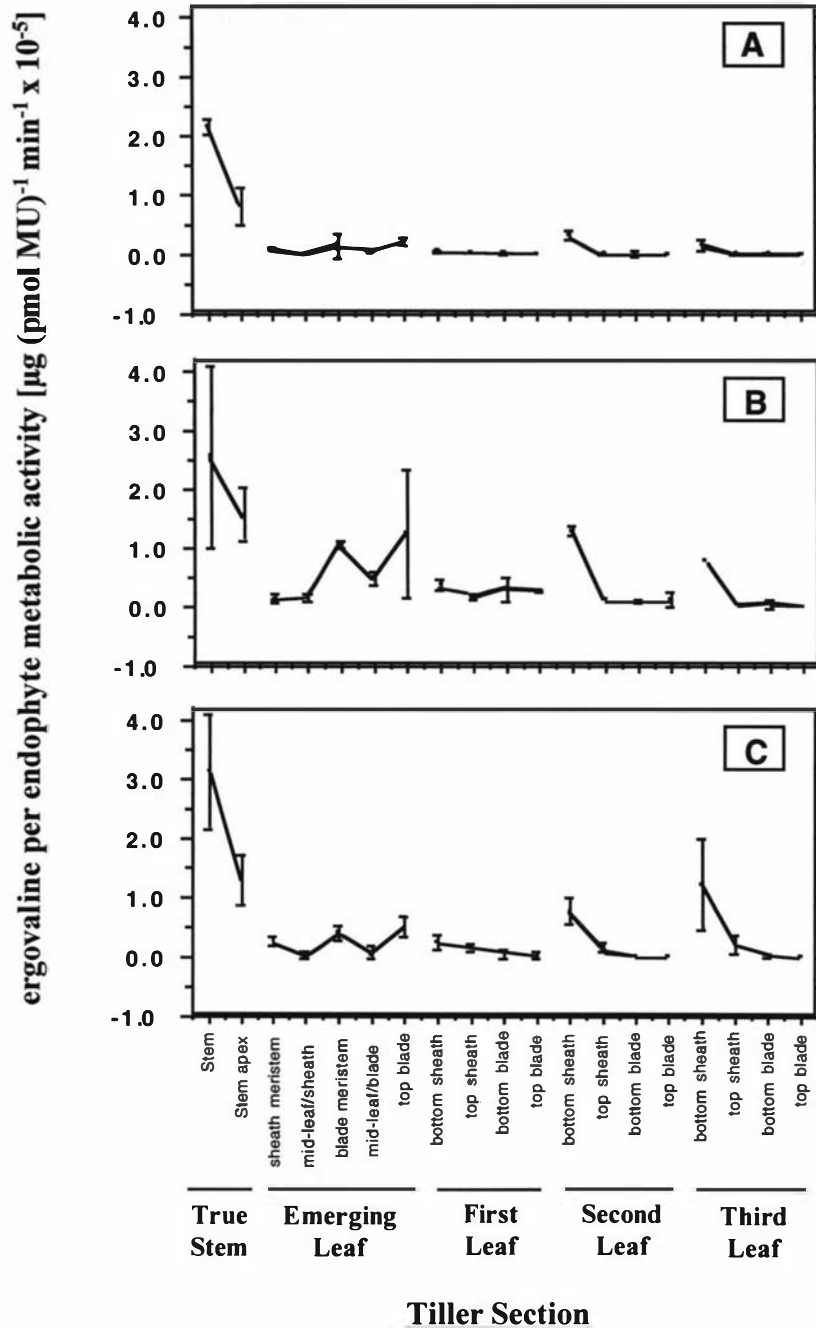


Figure 21

Ergovaline-GUS ratios in 19 stem and leaf sections of a tiller of the plant genotypes Nui D (**A**), Nui UIII (**B**) and Nui UIV (**C**) infected by Lp 19 (GUS-transformant KS1). Ratios were determined using endophyte metabolic activity and ergovaline concentrations (shown in Figs. 19 and 20) assessed on the same sample material. Each data point represents mean of two replicates. Error bars = ±1 standard deviation.



true stem and lower sheaths of mature leaves than in mature leaf tissues further removed from the leaf base. Most differences between the lower (true stem and lower sheaths) and upper tiller sections (upper sheaths and blade sections) were statistical significant ($P < 0.05$, Scheffé's test, including all genotypes). In the emerging leaves, as ergovaline was relatively evenly distributed between tissues (Fig. 20), the ratios were evidently more strongly influenced by the metabolic activity concentration (Fig. 21).

These results indicate for a strong localisation of the alkaloid ergovaline within a tiller. Moreover, the quantitative distribution of this alkaloid appears not exactly proportional with the quantitative distribution of endophyte metabolic activity, indicating that, although showing significant basal-apical gradients somewhat similar to endophyte metabolic activity, the distribution of this alkaloid is not strictly following the distribution of the endophyte. Therefore, *in planta* synthesis of ergovaline may be differentially regulated in endophyte mycelium, with greater production in true stem and lower leaf sheath sections.

3.2.4.4 Distribution of the Alkaloid Peramine in Plant Tissues, and Its Relationship to Endophyte Metabolic Activity

Plant genotypes varied in absolute concentration of peramine (Table 15B), but differences in overall concentrations were smaller than differences observed for the alkaloid ergovaline (Table 15A). A statistical significant difference in overall concentration was found only between Nui UIV and the other two genotypes ($P < 0.001$, ANOVA, Scheffé's test). Between genotypes, differences in the peramine : endophyte metabolic activity-ratio were smaller than differences in overall GUS activity and peramine concentration (Table 15B). A significant difference in the ratio was detected only between Nui D and Nui UIII ($P < 0.05$ ANOVA, Scheffé's test). As previously with ergovaline, this suggests that overall levels of peramine may be influenced by the overall amount of endophyte metabolic activity in the plant, but some control of the plant genotype over production in mycelium may exist.

Figure 22

Distribution of the alkaloid peramine within a tiller of each of the plant genotypes Nui D (**A**), Nui UIII (**B**) and NUI UIV (**C**) infected by the strain Lp 19 (GUS-transformant KS1).
Each bar represents mean of measurements on two replicate samples.
Error bars = 1 standard deviation.

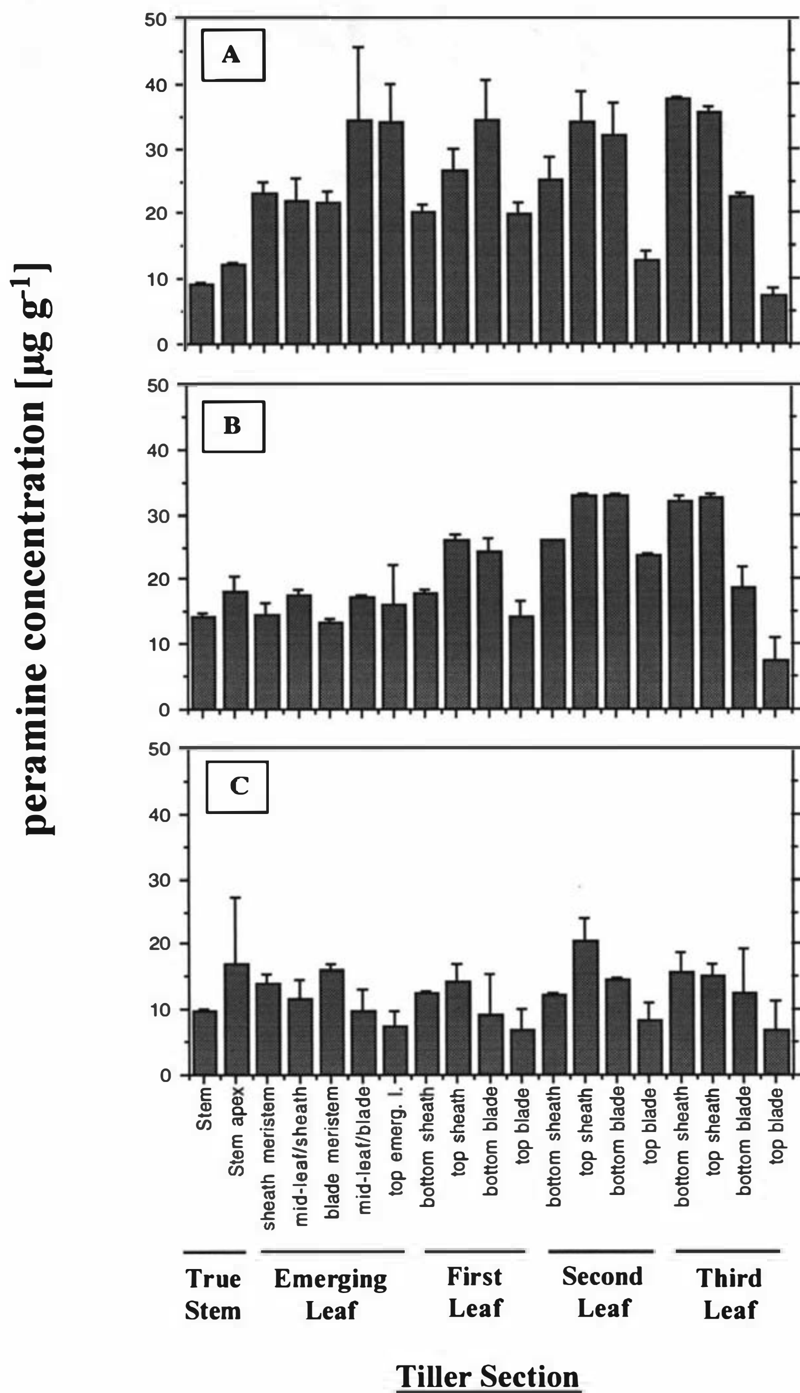
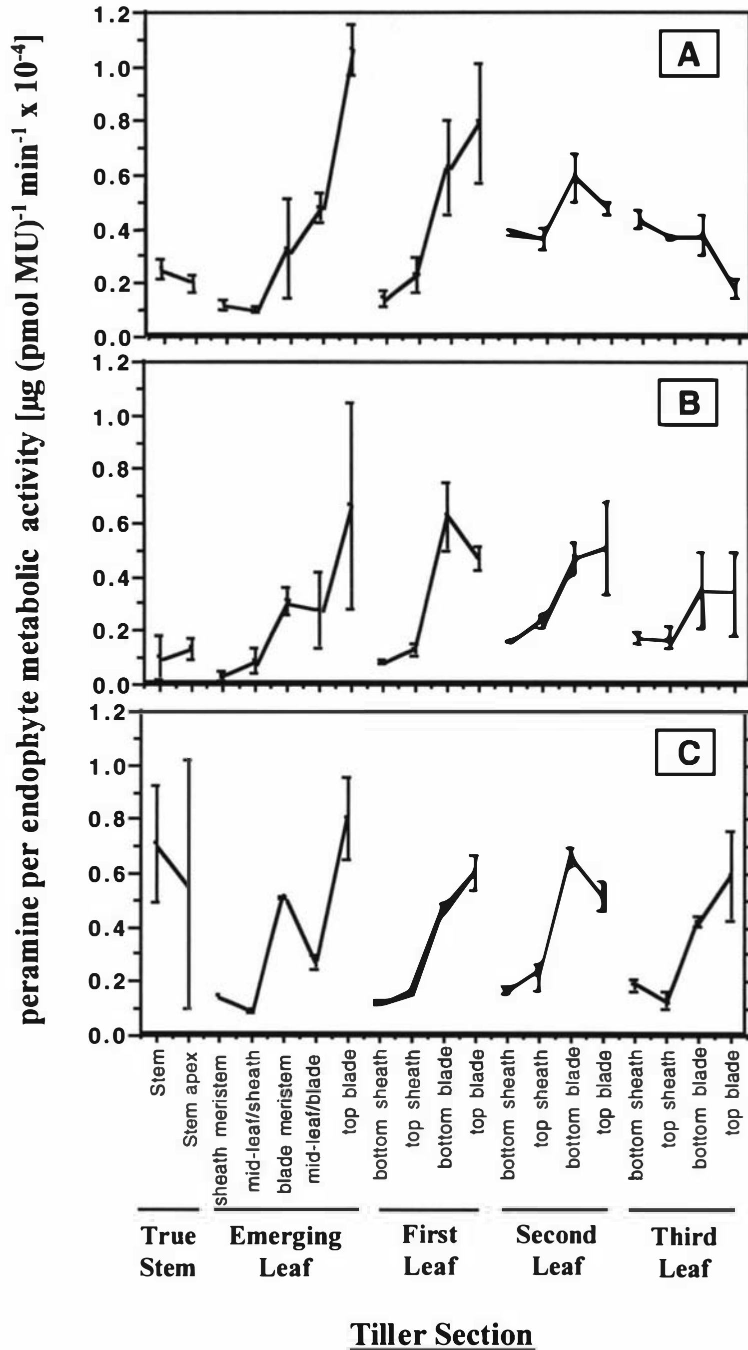


Figure 23

Peramine-endophyte metabolic activity ratios in 19 tissue sections of a tiller of the plant genotypes Nui D (**A**), Nui UIII (**B**) and Nui UIV(**C**) infected by Lp 19 (GUS-transformant KS1). Ratios were determined using endophyte metabolic activity and peramine concentrations (shown in Figs. 19 and 22) assessed on the same sample material.

Each data point represents mean of two replicates. Error bars = ±1 standard deviation.



Peramine showed little localisation in plant tillers (Fig. 22), contrasting the distribution of ergovaline (Fig. 20). In the emerging leaf and true stem tissues, distribution of this alkaloid was relatively even. However, with increasing leaf age, variation in the peramine concentration became larger between leaf sections, with lower concentration in the upper leaf blade of older leaves (Fig. 22). In all genotypes, peramine was more concentrated in the leaf sheaths and lower blades (Fig. 22).

The distribution of the ratio peramine : endophyte metabolic activity within tillers was largely influenced by the distribution of endophyte metabolic activity. The ratio was consistently greater in leaf blades, in which endophyte metabolic activity concentration was consistently lower (Fig. 19). A decrease in the ratio in leaf blades with increasing leaf age was seen in two plant genotypes, while at the same time, the ratio increased in the leaf sheaths (Fig. 23), indicating that the distribution of peramine came closer to that of endophyte metabolic activity as the leaf aged. This suggests that peramine concentration is regulated with leaf age, may be due to differential synthesis and/or translocation within and/or between leaves.

3.2.4.5 Distribution of the Alkaloid Lolitrem B in Plant Tissues and Its Relationship to Endophyte Metabolic Activity

The concentration of lolitrem B was different between some plant genotypes. Nui UIII and Nui UIV had similar overall concentrations, whereas in the genotype Nui D lolitrem B concentration was on average greater (Table 15C) and significantly different from the other two genotypes ($P < 0.001$, ANOVA, Scheffé's test). The lolitrem B : endophyte metabolic activity-ratio mean for Nui D (Table 15C) was significantly different from the corresponding means for Nui UIII and NUI UIV ($P < 0.001$, ANOVA, Scheffé's test). Ratio means for Nui UIII and Nui UIV were also significantly different ($P < 0.05$, ANOVA, Scheffé's test). This supports a relatively strong effect of the plant genotype on accumulation of the alkaloid lolitrem B per metabolically active endophyte mycelium. For this alkaloid, the effect of the plant genotype on accumulation per mycelium was the strongest (about four-fold difference between genotypes, Table 15C).

Figure 24

Distribution of the alkaloid lolitrem B within a tiller of each of the plant genotypes Nui D (**A**), Nui UIII (**B**) and NUI UIV(**C**) infected by the endophyte strain Lp 19 (GUS-transformant KS1).
Each bar represents measurements on two replicate samples (plants).
Error bars = 1 standard deviation. nd = not determined.

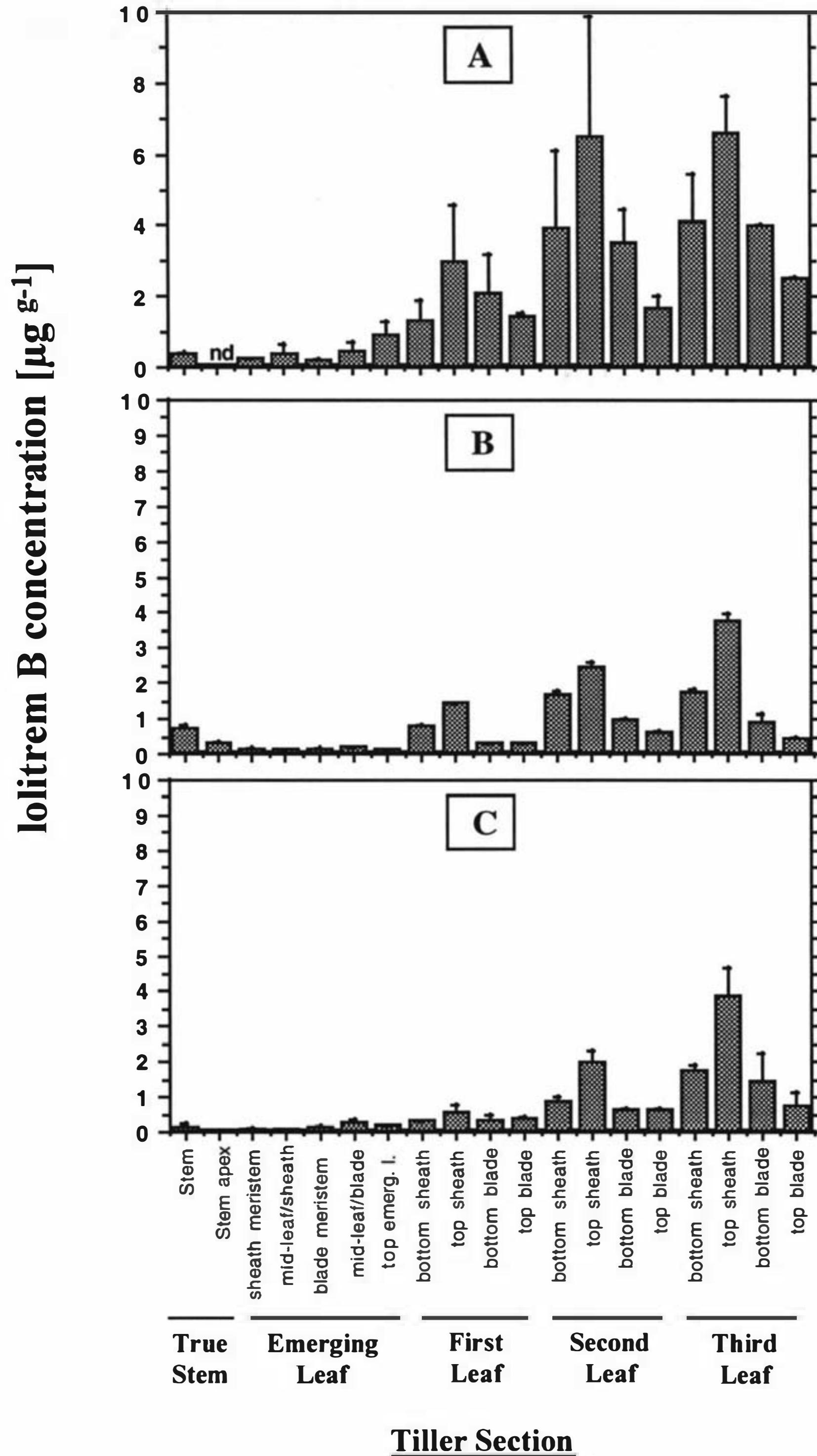
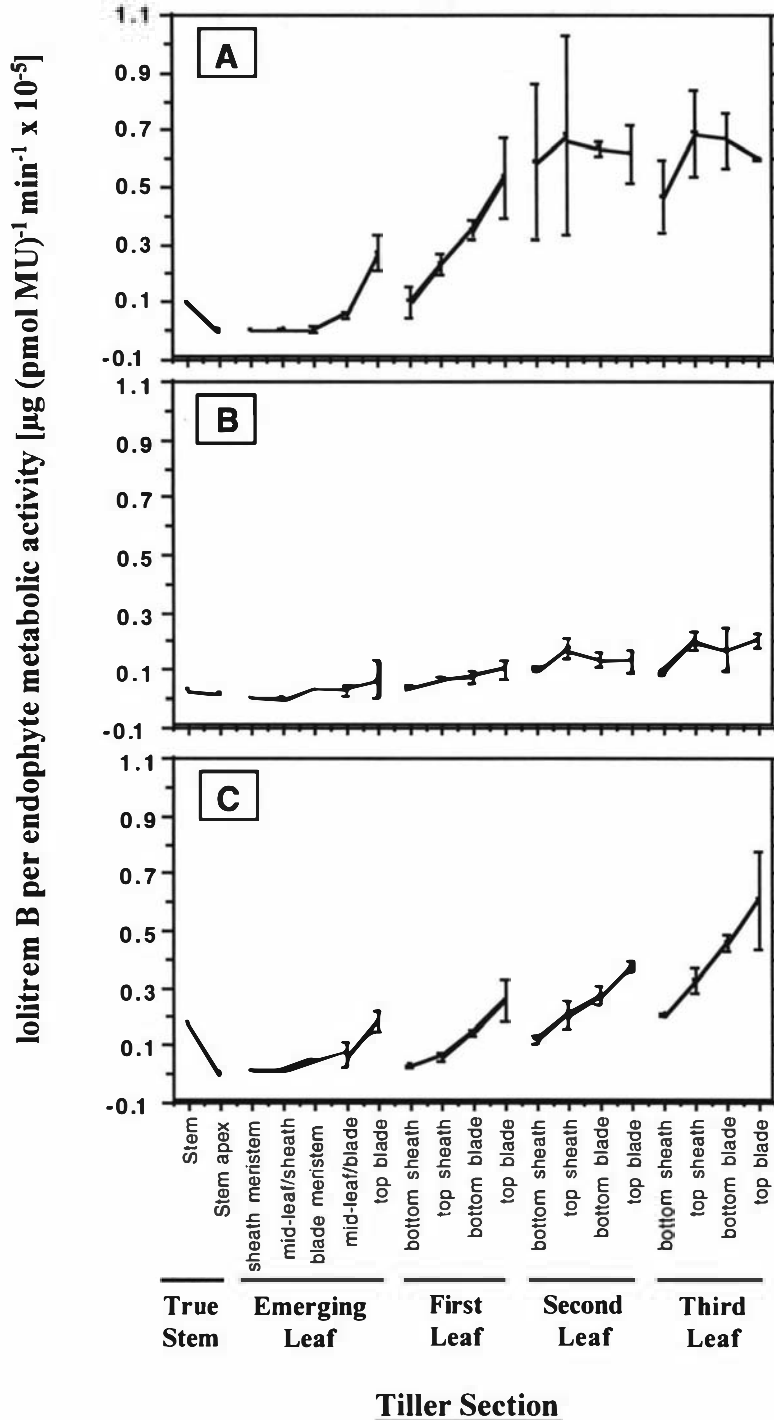


Figure 25

Lolitrem B-endophyte metabolic activity ratios in 19 tiller sections of a tiller of the plant genotypes Nui D(**A**), Nui UIII (**B**) and Nui UIV(**C**) infected by Lp 19 (GUS-transformant KS1). Ratios were determined using endophyte metabolic activities and lolitrem B concentrations (shown in Figs. 19 and 24) assessed on the same sample material.
Each data point represents mean of two replicates. Error bars = ± 1 standard deviation.



Lolitrem B concentration increased markedly with increasing leaf age. In emerging leaf sections, lolitrem B was relatively evenly distributed (Fig. 24), as previously observed for ergovaline and peramine (Figs. 20, 22). The true stem contained lolitrem B at levels comparable to emerging leaf or first leaf sections (Fig. 24), as seen before for peramine (Fig. 22), but contrasting ergovaline, which was more highly concentrated in the stem (Fig. 20). In all plant genotypes, the increase in lolitrem B concentration in tissues with increasing leaf age (Fig. 24) was due to an increase in lolitrem B per metabolically active mycelium, as indicated by the distribution of the lolitrem B : endophyte metabolic activity ratio (Fig. 25). This suggests leaf age-dependent accumulation of lolitrem B per mycelium.

In the genotype Nui UIV, all leaves exhibited an increase in the lolitrem B : endophyte metabolic activity ratio with increasing distance from the leaf base (Fig. 25). For Nui D and Nui UIII there is less of a basal : apical gradient in leaves two and three, indicating that the lolitrem B distribution more closely approaches that of endophyte metabolic activity as the leaf ages.

3.2.4.6 Levels of the Alkaloid Lolitrem B are Affected by Plant Age

During preliminary samplings some variation was observed for the concentration of lolitrem B if plants differed in age (from last planting); the concentration increased with increasing age as indicated for one plant genotype in Table 16. A similar increase was seen for the other two genotypes (data not shown). Since environmental conditions were kept stable during all measurements, this suggests overall accumulation of this alkaloid with plant age within the plant. However, no increase was noticeable for peramine, whereas ergovaline tended to be lower in older plants (data not shown).

Table 16
Concentration of the alkaloid lolitrem B in leaf tissues of plants varying in age and grown environmentally controlled (Section 2.4.4). Tissue material analysed was pooled from 3-5 tillers, harvested from two plants at each sampling. Three samplings were carried out from the younger plants (65 – 78 days) and two samplings from the older plants (110-116 days).

Plants were of genotype Nui D. The difference in lolitrem B concentration in tissues from plants differing in age was statistical significant ($P < 0.01$, t-test, data were paired according to tissue type, *i.e.* sheath or blade, and leaf age).

	Lolitrem B concentration ± standard deviation [$\mu g\ g^{-1}$]	
Plant age [days] / plant tissue	65 – 78	110 – 116
1st sheath	2.19 ± 0.77	6.85 ± 1.85
1st blade	1.75 ± 0.44	3.74 ± 0.16
2nd sheath	6.56 ± 3.11	11.33 ± 1.25
2nd blade	2.49 ± 0.49	3.78 ± 0.41
3rd sheath	6.93 ± 2.92	13.39 ± 3.22
3rd blade	2.87 ± 0.60	4.78 ± 0.67
mean all	3.80 ± 2.31	7.31 ± 4.12

3.2.4.7 Summary and Conclusions

The *in planta* endophyte metabolic activity distribution showed a stable basal-apical gradient in all leaves of all genotypes that was already established in the emerging leaf. The alkaloids ergovaline, peramine, and lolitrem B showed each a characteristic pattern of distribution generally conserved among the three plant genotypes. None of the alkaloids was distributed in exact proportion to endophyte metabolic activity. Ergovaline was largely confined to the very basal sections of a tiller; peramine concentration was greater in leaf regions further removed from the base; and lolitrem B increased measurably and significantly with leaf age. Higher concentrations of the alkaloids, especially of ergovaline, in lower tiller regions indicate that these regions essential for growth and survival of the tiller have greater protection against herbivores. Plant genotypes influenced overall levels of the alkaloids, but the alkaloids were not affected in the same way. Concentrations of peramine and ergovaline were to some degree positively correlated with the overall concentration of metabolically active endophyte, whereas no such correlation was seen for lolitrem B. Some overall variation in lolitrem B concentration occurred due to plant age.

3.3 EFFECTS OF ENDOPHYTE-INFECTION ON PLANT GROWTH AND PHOTOSYNTHESIS

Under environmental conditions favourable for plant growth, some positive effects of infection by *N. lolii* on growth of perennial ryegrass plants have been reported (Cheplick *et al.*, 1989; Latch *et al.*, 1985). However, little is known about the physiological basis for the enhancement in growth rate in the endophyte-infected plants. In order to assess whether plant growth and physiology were also affected by the endophyte-infection in the associations previously characterised for endophyte concentration and alkaloid content, plant growth rates and photosynthesis were examined.

Plant growth and net photosynthesis in leaves was assessed on endophyte-infected plants and endophyte-free plants. As grass plant genotypes occasionally significantly differ in growth parameters such as leaf extension rates (Volenec and Nelson, 1984a), it was important to compare clonal plants in this experiment. To remove the endophyte out of tillers, for all plant genotypes a fungicide treatment on tillers was performed as described (Section 2.4.6). In this way, a possible confounding effect of the plant genotype on the results could be excluded, thus the influence of the endophyte on plant growth and physiology directly determined.

Plant growth was assessed as increase in tiller number per plant, and as leaf extension rates as described (Sections 2.10.1 and 2.10.2). Plants were planted on the same day and grown environmentally controlled as described (Section 2.4.4). Assessments of tiller number, and leaf extension rate, were performed on all plants at the same time. Three plants per genotype were assessed.

3.3.1 Some Effects of Endophyte-Infection on Plant Growth

Endophyte-infected and uninfected plant differed in leaf extension rates; infected plants of all three genotypes had lower elongation rates than the corresponding uninfected plants, and the difference was statistical significant (Table 17). However, final tiller

Table 17

Leaf extension rates of plants infected by the endophyte strain Lp 19 (GUS-transformant KS1) and uninfected.

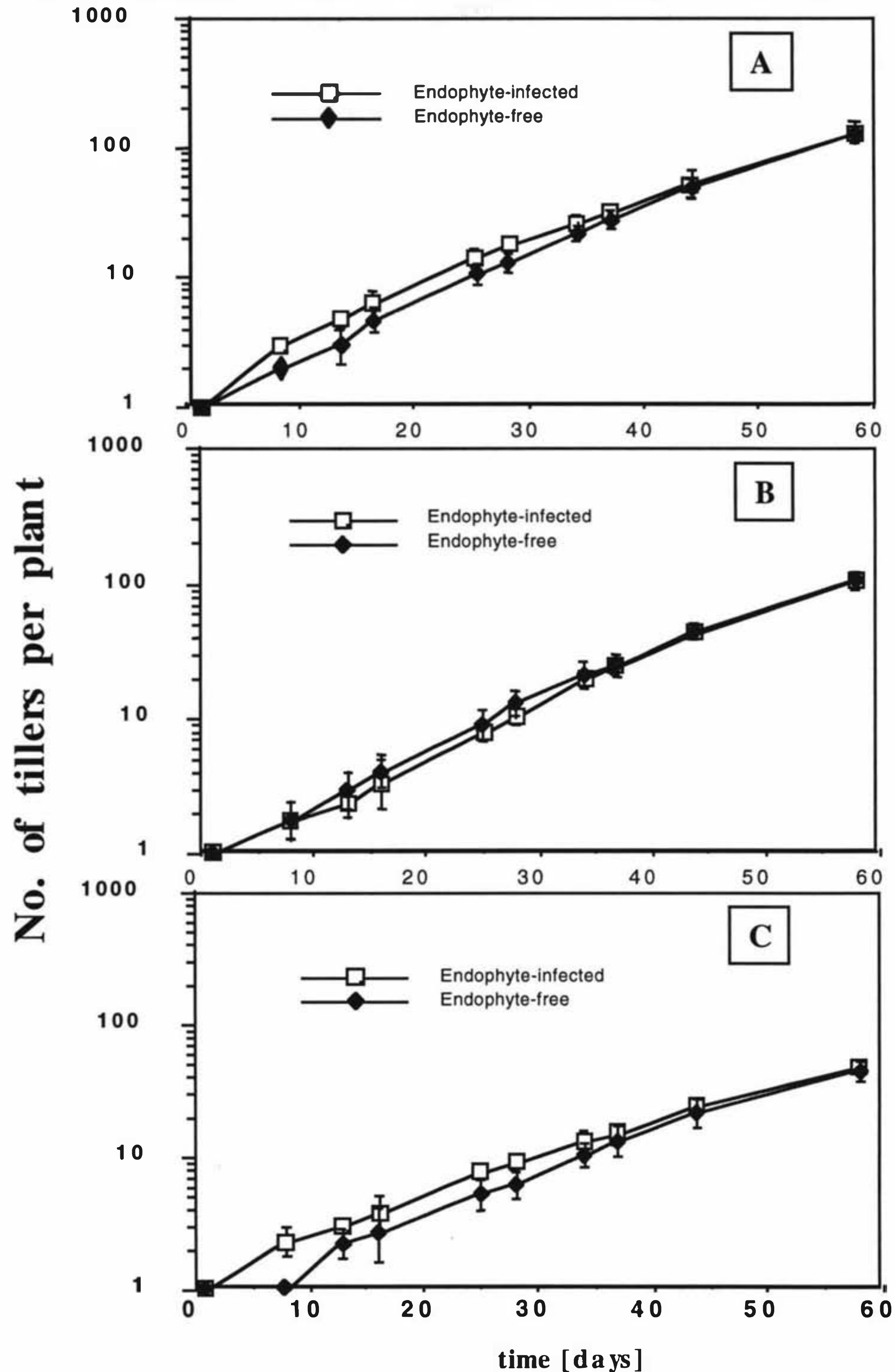
Rates were assessed within 24 h (Section 2.10.2) on all plants at three different dates (age of plants at measurements was 22, 26 and 36 days, respectively) on three replicate plants (8-10 leaves per plant) per genotype, respectively. The difference between infected and uninfected plants was statistical significant ($P < 0.01$, GLM-ANOVA, including plant genotype and plant age at sampling).

Plant genotype/infection status	Leaf extension rate ± std. err. [mm h^{-1}]
Nui D infected	0.92 ± 0.13
Nui D uninfected	0.94 ± 0.07
Nui UIII infected	0.92 ± 0.09
Nui UIII uninfected	0.95 ± 0.14
Nui UIV infected	1.10 ± 0.14
Nui UIV uninfected	1.28 ± 0.13
average infected	0.98 ± 0.03
average uninfected	1.05 ± 0.03

Figure 26

Plant growth assessed as tiller number per plant of plants infected by the endophyte strain Lp 19 (GUS-transformant KS1), and endophyte-free controls of the genotypes Nui D (**A**), Nui UIII (**B**), and Nui UIV (**C**). All plants were grown in a controlled environment (Section 2.4.4).

Error bars = ± 1 standard deviation of the mean (n=3). No significant differences in tiller number between infected and uninfected plants were detected after completion of the experiment (growth for 58 days, $P > 0.05$, t-test, plants were paired within genotypes).



numbers were not significantly different between plants of different infection status (Fig. 26). Tillering and leaf extension rates were relatively similar between the two genotypes Nui D and Nui UIII. The genotype Nui UIV had an on average greater elongation rate and a lower number of tillers per plant. Infected plants of two plant genotypes (Nui D and Nui UIV) tended to have slightly greater tiller numbers per plant during early growth (Fig. 26 A, C). The slightly lower leaf elongation rate in endophyte-infected plants may be suggestive of an endophyte effect on overall herbage production, since it has been suggested that leaf elongation would be a useful selection criterion for yield (Horst *et al.*, 1978). However, in a separate experiment (same experimental conditions) the herbage yield, assessed as average dry weight per tiller (= dry weight per plant divided by number of tillers per plant), was not consistently higher in uninfected plants. Only in one plant genotype (Nui UIII) the dry weight per tiller was about 15% lower in infected plants (data not shown).

3.3.2 Leaf-Net Photosynthesis in Response to Light Intensity and Temperature in Endophyte-Infected and Endophyte-Free Plants

Since infection by the endophyte decreased leaf elongation rates measurably, photosynthesis in infected and uninfected plants was also assessed. Measurements of leaf-photosynthetic rates as exchange rates of CO_2 were performed as described (Section 2.11). These investigations were carried out with the genotypes Nui D and UIV only, to allow for more replication on each.

3.3.2.1 Endophyte-Infection Reduces Net Photosynthesis Under High Light Intensities and Moderate Leaf-Temperature in Young, Actively Growing Plants

Plant age (from planting) when experiments were conducted was between 1-2 months. To account for possible effects of the plant age on photosynthesis, measurements were carried out alternating between infected and uninfected plants: on each day an infected plant and an uninfected plant were measured. Ten tillers (two per measurement) of each infected and uninfected Nui D, and six tillers of each infected and uninfected Nui

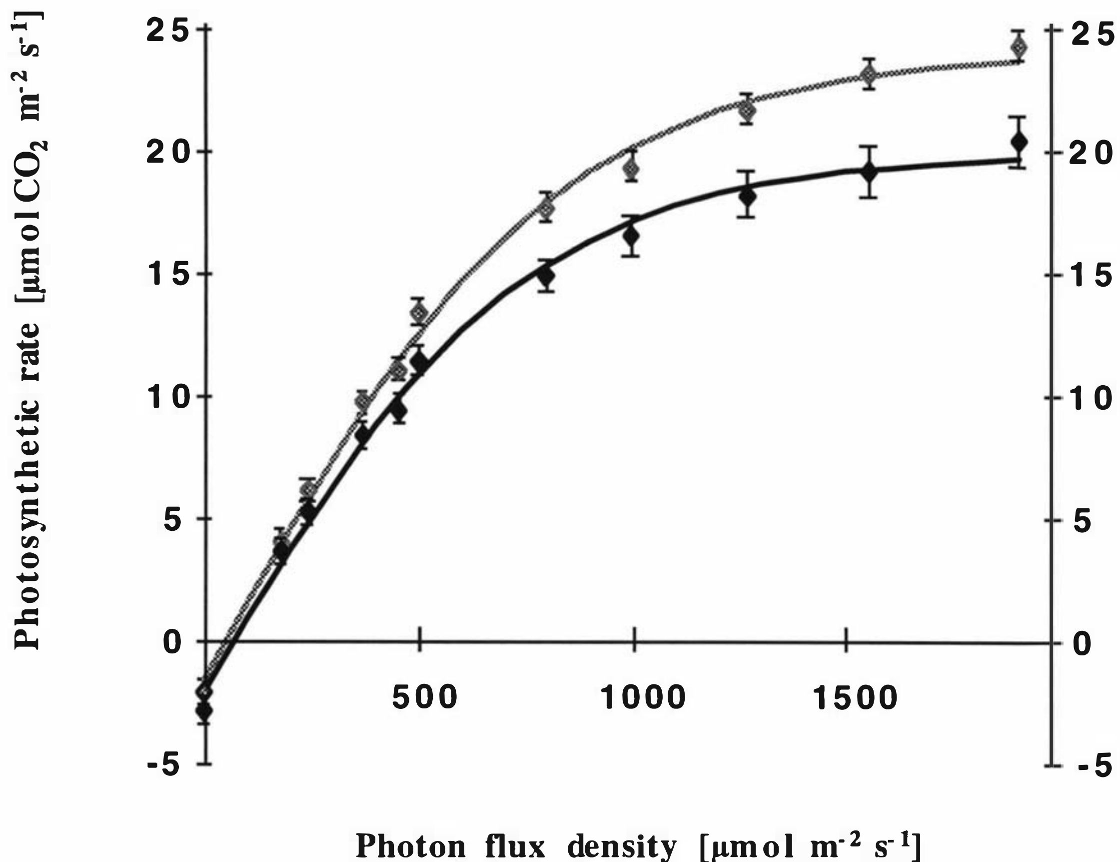
UIV plants were used for assessing rates of net photosynthesis in leaves as described (Section 2.11.3.1).

Plants of the two genotypes achieved very similar rates of photosynthesis (not shown). Therefore, the data from the two genotypes were pooled for infected and uninfected plants, respectively. In darkness, evolution of CO_2 was measured in all plants, probably due to respiration in the leaf. No significant difference in respiration was detected between infected and uninfected plants (Fig. 27). An increase in light intensity led to an increase in leaf-photosynthetic activities (Fig. 27). At greater light intensities (500-1900 μmol m^{-2} s^{-1}), the rate of increase in photosynthetic activities with increasing light intensity became less, which indicated for saturation at the higher light levels (Fig. 27). The rates of photosynthesis at intensities approaching saturation (> 1000 μmol m^{-2} s^{-1}) were significantly lower in infected plants (Fig. 27). At the highest measured light intensity (1900 μmol m^{-2} s^{-1}) photosynthesis in endophyte-infected plants was on average 84 per cent of the photosynthesis in uninfected plants.

Figure 27

Net photosynthesis in perennial ryegrass leaves in response to light intensity. Plants infected (dark squares) by the strain Lp 19 (GUS-transformant KS1) and endophyte-free (grey squares) were measured. Non-linear regression was used to fit least square lines ($r^2 > 0.99$ for both lines) to the data for each infected and uninfected plants.
Error bars = ± standard error (n=8).

Regression equation used: $PS = PS_{max} \times TANH(PFD \times \alpha / PS_{max}) - Resp.$
PS_{max} = maximum photosynthesis, PFD = photon flux density, α= photon yield, Resp.= respiration. No significant differences in respiration ($P > 0.05$, t-test on actual values) and in initial increase in photosynthesis (photon yield, $P > 0.05$, t-test on estimated values from regression) were found between infected and uninfected plants. Differences in photosynthesis at higher light intensities (> 1000 μmol photons m^{-2} s^{-1}) were statistical significant ($P < 0.05$, t-test on actual values and estimated maximum photosynthesis from the regression).



The results of this experiment indicated that the net photosynthesis in leaves at high light intensities under the chosen experimental conditions (moderate temperature during measurements and young, actively growing plants) was significantly reduced by endophyte-infection.

Stomatal conductances, known to affect net photosynthesis (Mohr & Schopfer, 1992) were estimated from measured rates of transpiration and leaf temperature. Estimates for leaf stomatal conductance were highly similar in endophyte-infected and uninfected plants (data not shown). For identification of the physiological basis of the observed changes in photosynthesis in infected plants on the enzymatic level, attempts were made to quantify D-ribulose 1,5-bisphosphate carboxylase-oxygenase (rubisco), a key enzyme in photosynthesis (Andrews & Lorimer, 1987; Salvucci, 1989; Woodrow & Berry, 1988). Therefore, denaturing polyacrylamide gel electrophoreses (PAGE; Laemmli, 1970) were performed on protein extracts from endophyte-infected and uninfected leaf blades of the youngest mature leaf. Slightly lower amounts of rubisco were determined in endophyte-infected leaf material (as assessed by densitometry on bands after staining with Coomassie brilliant blue). However, some degradation of proteins in both infected and uninfected plant materials, probably due to storage conditions prior to extractions, occurred that may have impacted on this determination (not shown).

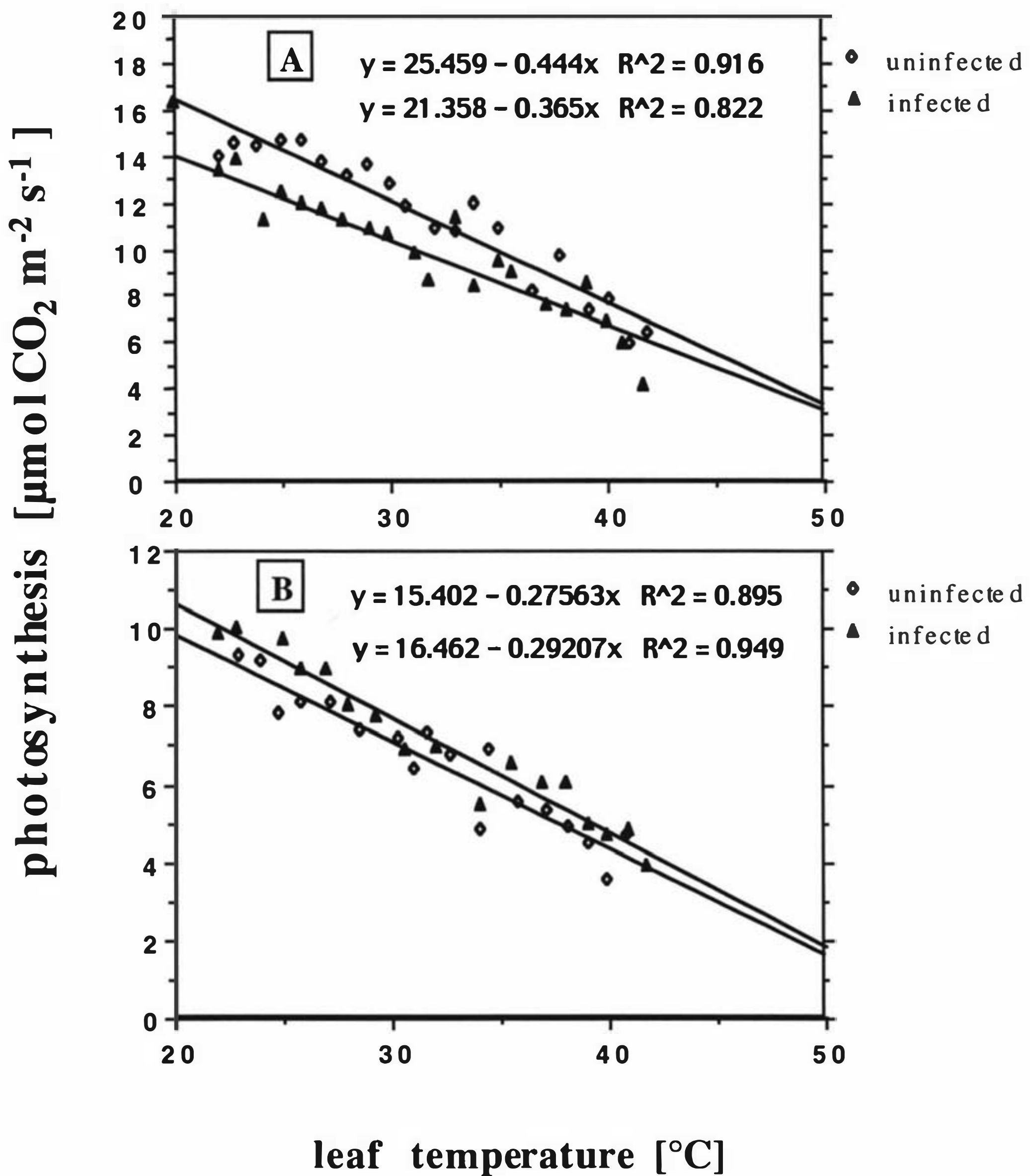
3.3.2.2 Net Photosynthesis Assessed at Different Leaf-Temperatures is not affected by Endophyte-Infection in Older Plants

Results of a recent glasshouse study indicated that tall fescue plants infected by *N. coenophialum* achieve greater net photosynthesis at higher environmental temperatures than uninfected plants (Marks & Clay, 1996). As differences in photosynthesis in response to light between infected and uninfected plants were found (previous section), the photosynthetic response to temperature was also examined (Section 2.11.3.2).

Plants used in these experiments were 3-4 months old (from planting). Measurements were performed as previously described (previous section). In all plants, the increase in leaf temperature led to a decline in photosynthesis (Fig. 28). The decline

Figure 28

Net photosynthesis in endophyte-infected and endophyte-free plants of the genotypes Nui D (**A**) and Nui UIV (**B**) in response to leaf temperature. Each data point represents the mean of 2-4 measurements. Regression on data pooled for infected and uninfected plants gave R^2 values of 0.787 (Nui D) and 0.904 (Nui UIV).



was presumably caused by an increase in photorespiration, for it is known that photorespiration is enhanced at higher temperatures (Mohr & Schopfer, 1992). At a leaf temperature of 40 °C, in all plants photosynthesis was reduced to less than 50 per cent of the activity at 22 °C (Fig. 28). The decline, and the level of photosynthesis at high temperatures (>35 °C), were very similar in infected and uninfected plants.

However, as shown in Figure 28, differences in slope and intercept of linear regressions between infected and uninfected plants existed. The R square values of the regressions however indicated that appreciable variation in the data also existed. When, within genotypes, regressions were performed on the data for infected and uninfected plants, the R squared values were similar to the R squared values obtained from the regressions for infected and uninfected plants done separately (Fig. 28). This suggested that the separate regressions did not significantly remove variation in the data. This was tested by determining the residuals in the regressions on data separated according to infection status and, conversely, on all data combined within genotypes. A t-test was then used for comparing the values for the residuals from each of the separate regressions by pairing them with the corresponding values from the regression on the combined data. For each genotype, no significant difference between the residuals was detected ($P > 0.05$), indicating that although some differences in temperature response exist between infected and uninfected plants, these are small and non-significant.

The rates of net photosynthesis were overall lower in these plants than in younger plants of the same genotypes, previously assessed under the same conditions (previous section, Fig. 27). Plants of the genotype Nui D had higher levels of photosynthesis at 22 °C than plants of Nui UIV (Fig. 28). Moreover, in contrast to the previous measurements (previous section), no significant difference in photosynthetic rate at a leaf temperature of 22 °C (2 °C higher than in the experiments described in the previous section) was found between endophyte-infected and endophyte-free plants ($P > 0.05$, t-test, plants were paired according to the date of measurements).

3.3.3 Summary and Conclusions

The plant growth in the selected plant-endophyte associations was not strongly affected by endophyte-infection under the chosen environmental conditions. However, endophyte-infected plants had slightly lower leaf extension rates than uninfected controls of the same plant genotypes. In endophyte-infected plants, leaf-net photosynthesis was significantly lower under light intensities near saturation of photosynthesis than in endophyte-free plants. However, in older plants no differences in net photosynthesis at saturating light, and also in response to temperature were observed between endophyte-infected and uninfected plants. These results demonstrated that endophyte-infection affects elongation of grass leaves and rates of net photosynthesis. Moreover, they showed that the effect of the endophyte on photosynthesis apparently depends on the age/developmental stage of the whole plant.

4.0 DISCUSSION

In this study, the *N. lolii* endophyte strain Lp 19 was used that had been transformed previously with a construct comprised of a constitutive fungal promoter and the ß-glucuronidase (GUS) gene of *E. coli*. With this transformant, the following aspects of the symbiosis between *N. lolii* and selected genotypes of the grass *L. perenne* were investigated: (i) the physiological state of the endophyte in the plant, (ii) its quantitative distribution within a grass tiller, (iii) the quantitative distribution of the three alkaloids ergovaline, peramine and lolitrem B, and (iv) the effects of the endophyte on plant growth and photosynthesis.

Initially, it was verified that GUS expression in culture varied significantly with growth phase, hence, with levels of metabolic activity of the endophyte-transformant (Section 3.2.1.1), demonstrating that constitutive GUS expression allows for the determination of the metabolic state of the endophyte. At the beginning of this study, it was hypothesised that in the mature parts of the plant the endophyte might be in a physiological state similar to a stationary phase state in culture (Section 1.3.4). To test this hypothesis, the metabolic state of endophyte mycelium in the plant was determined with the GUS system, and the results of these experiments demonstrated that the endophyte is metabolically highly active in all plant tissues examined (Section 3.2.3). Thus, contrary to the initial hypothesis, in the plant, the fungus is not in a physiological state similar to a stationary growth phase state in culture. As noted (Section 1.3.1), in culture-synthesis of alkaloids in fungal mycelium commonly takes place in the stationary growth phase. As the *in planta* endophyte metabolic state was determined high, and at the same time considerable amounts of alkaloids were present in the plants investigated (Section 3.2.4), this suggests that regulation of alkaloid production in the plant differs from regulation in culture.

Investigations into the distribution of metabolically active endophyte within the grass tiller, in particular with regard to concentrations in young growing tissues, provided evidence that growth of the endophyte is largely synchronous with growth of the plant.

Although endophyte mycelium was metabolically highly active in the plant, as indicated by the EMS, the endophyte largely ceases to proliferate in the mature tissues.

As a further objective of this work, the *in planta* relationship between the alkaloids ergovaline, peramine, and lolitrem B and endophyte metabolic activity in selected *N. lolii*-perennial ryegrass symbioses was determined. In this investigation, it was discovered that none of the alkaloids was distributed in exact proportion to metabolically active endophyte mycelium (Section 3.2.4). Each alkaloid followed a characteristic distribution that was distinctively different from the distributions of the other alkaloids. This characteristic distribution was maintained, notwithstanding substantial differences in alkaloid concentration between the plant genotypes assessed. The stability of the alkaloid distributions across plant genotypes indicates for tissue specificity of alkaloid accumulation. The plant genotype affected absolute levels of accumulation, and some of the alkaloid levels in plant genotypes were correlated with the overall concentration of endophyte mycelium in a tiller, suggesting that some regulation of *in planta* alkaloid levels occurs *via* regulation of the amount of endophyte in grass tillers. However, some control of the plant genotype on alkaloid production in endophyte mycelium was also evident.

The detailed analyses for determining the *in planta* alkaloid distribution was made possible by the development of a quantitative assay from small plant tissue quantities for the alkaloid ergovaline, carried out in this study (Section 3.1.4). In addition, also part of this work was the implementation of a controlled environment for plant growth (Section 3.1.3), that allowed for the characterisation of alkaloid production in the plant-endophyte symbioses under constant and defined environmental conditions.

Results in this study provided, for the first time, information about the effects of *N. lolii* on photosynthesis in perennial ryegrass. In some plants, net photosynthesis in leaves was significantly lower in endophyte-infected plants than in clonally identical endophyte-free plants (Section 3.3.2). Plant growth rates were examined and found not strongly affected by the endophyte under the chosen environmental conditions. However, infected plants of all plant genotypes exhibited significantly lower leaf elongation rates than

uninfected plants of the same clonal lines (Section 3.3.1). These findings indicate that the infection by *N. lolii* leads to changes in growth and physiology of perennial ryegrass plants.

4.1 GUS REPORTER GENE EXPRESSION IN CULTURE – CONSTITUTIVE EXPRESSION INDICATES FOR THE METABOLIC STATE OF ENDOPHYTE MYCELIUM

In vitro, expression of the GUS reporter gene in the *N. lolii*-transformant strain KS1 (Lp19) was strongly and positively correlated with active growth of the endophyte: in exponentially growing mycelium, initiated with an inoculum from a stationary phase culture GUS activity expression showed a marked increase (Section 3.2.1.1). As synthesis of proteins is a necessary prerequisite for growth, the co-occurrence of active growth and high levels of GUS expression confirmed the proposed correlation between constitutive GUS expression and the rate of overall protein biosynthesis in the endophyte mycelium (Section 1.4). The rate of GUS synthesis was presumably largely constant, resulting in a constant rate of increase of GUS activity in mycelium growing in the exponential phase. GUS activity reached then a steady state (= balanced rate of synthesis and degradation) as determined by culture of mycelium at non-limiting growth conditions (Section 3.2.1.2). The constant GUS level attained in the continuously growing mycelium thus indicated for a level of metabolic activity (= metabolic state) in the mycelium that allowed active growth of the fungus in culture.

GUS activity concentration in mycelium was much (> 30-fold) lower in stationary phase and in mycelium incubated with a metabolic inhibitor (Sections 3.2.1.1 and 3.2.1.3) than in exponentially growing mycelium. The GUS enzyme is known to be a relatively stable enzyme with an *in vitro* half-life of about two hours at 55 °C (Jefferson, 1987). However, little appears to be known whether and how it is degraded in organisms that have been transformed with the GUS gene. The results presented here have demonstrated that GUS activity in mycelium of the GUS-transformed endophyte decreases measurably under physiological unfavourable conditions for the fungus.

The observed decline of GUS activity in stationary phase was probably compounded by a reduction in expression and an onset of lytic processes for protein recycling within mycelium that are known to occur under culture conditions unfavourable to fungal growth (Didek-Brumec *et al.*, 1996; Greasham, 1992; Prosser, 1995). The half-life of the enzyme determined under the physiological unfavourable conditions (Sections

3.2.1.1 and 3.2.1.3) provided information about how fast degradation of the enzyme probably occurs in the plant, as this information was required for evaluating the measured endophyte metabolic state in the plant (Section 3.2.3).

4.2 Plant Growth in a Controlled Environment – A Necessary Pre-Requisite for Studying Alkaloid Production in Endophyte-Grass Associations

A focus of the present study was the production of the agriculturally important alkaloids in endophyte-infected grass plants. As known from previous studies, levels of the alkaloids in the plants are significantly affected by environmental conditions and by the developmental status of the plant (Section 1.3.4). Control over the environmental conditions for plant growth was therefore a major goal for the experiments in this study.

For control over the environment, and to allow maintenance under conditions favourable for vegetative growth, plants were grown in a growth cabinet (Section 3.1.3). The environmental conditions in this cabinet were constant throughout all experiments, therefore, the plant-endophyte symbioses, well characterised for alkaloid production, endophyte distribution, and growth in this environment, are now available as model symbioses for further studies. For example, future studies could involve defined alteration of environmental parameters, such as light, temperature, and water status in the growth cabinet. Using the GUS system and the newly developed alkaloid assays, endophyte metabolic activity and alkaloids in the symbioses in response to these changes could be assessed simultaneously, as performed in the present study. In this way, the effect(s) of the environment on colonisation of the plant by the endophyte and alkaloid production could be determined with far greater accuracy and reliability than previously in other, less controlled environments.

In all experiments, the growth cabinet conditions were similar to natural environment conditions in spring or autumn. Hunt and Halligan (Hunt & Halligan, 1981) assessed growth rates of perennial ryegrass over a range of different temperatures, and determined that the optimum temperature for maximum growth was at 20 °C. In the present study, plants in the growth cabinet were grown at 15 °C; however, even under these conditions plant growth was rapid and uniform among genotypes (Sections 3.3.1), indicating that the growth of plants was at near optimum. Moreover, by adjustment of the light period to 12 hours plants could be maintained in a vegetative state.

The use of the growth cabinet had the advantage of a low incidence of fungal and insect diseases, frequently encountered in other environments, as for example in the glasshouse. The containment of the plants in the cabinet provided physical protection, and the lower temperature in this environment probably prevented fungal and other pathogens from thriving. Only occasionally spraying of pesticides was required the application of which was tested as part of this study, and found to have no negative effect on viability and growth of the endophyte (Section 3.1.3.2). Presumably because of the constant conditions within the cabinet, significant seasonal effects on GUS activity expression and alkaloid concentration were never apparent (not shown).

However, technical malfunctions, as defective light bulbs or some deviation from the set temperature occasionally occurred. These were generally quickly noticeable and rectifiable, but some undesirable short-term environmental variations ensued. Therefore, care was taken to eliminate these variations, especially at times between samplings, which required frequent monitoring of light and temperature in the cabinet. Some variation in alkaloid concentration was observed in preliminary experiments in the growth cabinet. For example, a noticeable and significant effect was observed for the alkaloid lolitrem B, if plants differed in age (Section 3.2.4.6). However, when plants of similar age were compared, even when sampling dates were separated by a year, measurements gave highly similar estimates for endophyte metabolic activity and all three alkaloids (not shown).

4.3 ENDOPHYTE DISTRIBUTION AND ITS METABOLIC STATE *IN PLANTA*

4.3.1 Evidence that the Endophyte remains in a Highly Active Metabolic State throughout the Plant

The production of the alkaloids by the endophyte and its lack of measurable growth in mature plant tissues suggested that in these tissues the endophyte is in a physiological state similar to stationary phase in culture (Section 1.3.4). However, contrary to this initial hypothesis, the results of the study presented here indicate that the level of metabolic activity in the endophyte mycelium, the EMS, is significantly and consistently higher in the plant than in mycelium in culture (Section 3.2.3). *In planta,* the EMS was on average nearly four-fold greater than in endophyte mycelium growing exponentially in culture; in none of the grass tissues examined, the metabolic activity per mycelium was lower than in exponentially growing cultures (Table 13).

GUS activity levels in mycelium in response to growth conditions showed some time delay in culture (Sections 3.2.1.1 and 3.2.1.3); thus a similar behaviour of the EMS in the plant was conceivable. The half-life of GUS in stationary culture varied between eight and twelve days, and was reduced to three days in metabolically inhibited mycelium. Given a similar decline of GUS in the plant and that the oldest leaf tissues were presumably older than 30-40 days (Durand *et al.*, 1999; Robson *et al.*, 1988), the measured level of the EMS in old (= third) leaves indicated (Table 12) that no decrease in the *in planta* EMS occurred.

Unexpectedly, the EMS was significantly lower in the young emerging (= elongating) leaves than in mature leaves (Section 3.2.3). As shown in this work, leaf elongation and growth of the endophyte appear synchronised (Section 3.2.4.2). Thus, active hyphal growth in the emerging leaf has to occur for colonisation of the leaf, and it seems evident that for this growth high metabolic activity in the mycelium is required. The lower measured EMS in this leaf may therefore be due to other reason(s), perhaps

including rapid hyphal extension during leaf elongation; it is possible that in the growing leaf rates of GUS expression cannot suffice to prevent measurable dilution of the enzyme in the growing hyphae. It is also a possibility that in this tissue some consistent error(s) in the measurements for the EMS occurred.

The finding of a high and relatively uniform EMS in the plant was surprising. The previously noted down-regulation of endophyte growth in mature tissues (Section 1.2.1) also found in this study (Section 3.2.4.2), and the production of secondary metabolites in the plant had suggested that the physiological state of the endophyte in these tissues is similar to a stationary growth phase in culture. Moreover, the physiological state of the endophyte mycelium - high metabolic activity (= high protein biosynthesis) in the absence of growth - has apparently never been observed in other fungi previously. However, a biological reason for this apparent deviation from the expected relationship between growth and metabolic activity can be hypothesised: in the grass plant, high levels of metabolic activity in endophyte mycelium in the absence of mycelial proliferation may be a necessary prerequisite for maintaining high alkaloid levels in plant tissues.

In some plant tissues, the sum of the concentrations of all measured alkaloids exceeded 20 $\mu g\ g^{-1}$ (Figs. 20, 22, and 24). Based on the endophyte biovolume concentrations in plant tissues (Tables 10, 11, and 12), and by using appropriate conversion factors to dry weight (Section 3.2.3), the endophyte dry weight concentration ranges approximately between 200 to 600 μg per gram dry weight of plant tissue. Other alkaloids, not included in this study, are also produced in this symbiosis (for example, paxilline at quantities equivalent to lolitrem B, Rowan, 1993). Therefore, in many of the plant tissues the bulk concentration of the alkaloids reaches more than ten per cent of the dry weight of the fungus. This concentration per mycelium in the plant is significantly greater than in culture, where alkaloids seemingly accumulate to less than one per cent of the fungal biomass (for example, 4 mg paxilline were purified from a 200 ml culture, lacking measurable production of lolitrem B, Weedon & Mantle, 1987). The high metabolic activity of the endophyte in the plant may reflect high metabolic rates of the

fungus, required to maintain a high equilibrium concentration for each of the alkaloids, as some of these compounds (*e.g.* ergovaline) may also have a short half-life in the plant.

Because of the importance of alkaloid synthesis for the persistence of the grass and, thus, for the whole symbiosis, and therefore maintained at levels allowing effective protection against biotic insult, high metabolic activities in non-growing hyphae may be an adaptation of the fungus unique to this symbiosis. However, it is a distinct possibility that in other symbiotic relationships, as well as in solitary fungi, high metabolic activities in the absence of growth are common, but may have not been previously observable, due to the lack of a suitable marker for metabolic activities in these organisms. Moreover, it is probably not possible to stop hyphal extension in laboratory cultures without inhibiting the metabolism of the fungus. Thus, results from studies in cultures may be often misleading, as it is difficult *in vitro* to appropriately mimic the situation *in vivo*.

A significant difference in the EMS was observed between leaf sheath and leaf blade (Section 3.2.3). This difference was largely due to a difference in hyphal diameter between the two leaf parts. In the leaf blade, the diameter of endophyte hyphae was on average 20 per cent greater than the hyphal diameter in the leaf sheath (Section 3.1.6). In the calculation of hyphal biovolume in plant tissues (Section 2.7) the greater diameter in the blade led to a higher hyphal biovolume per hypha, and, hence biovolume concentration in this leaf part. The greater diameter being a factor for biovolume caused the EMS calculated based on endophyte metabolic activity and biovolume (Section 3.2.3) to be consistently lower in the leaf blade. With the light microscopy method used it was not possible to assess whether the difference in diameter between the tissues was due to a true variation or may be caused by a consistent artefact in the measurements of the diameter.

Assuming that the measured difference in hyphal diameter reflected a true variation, this difference may be due to changes in hyphal structure. For example, changes in fungal cell wall structure and dimension are common in symbiotic relationships and occur in response to nutritional exchange as well as for protection against plant defences (Gooday, 1995). Therefore, if the difference in diameter between sheath and blade was due

to an alteration in the cell wall, this may indicate that the two plant tissues have different physiological qualities for the endophyte. Alternatively, hyphae that grow with higher extension rates commonly have larger diameters than hyphae extending with lower rates (Prosser, 1993). As discussed below (Section 4.3.3), the elongation rate of the leaf blade is faster than that of the leaf sheath, and as found in this study, growth of leaves and endophyte hyphae appears synchronised. Thus, hyphae in the leaf blade have to grow faster in this leaf portion, to keep growth in pace with the elongation of the leaf. Therefore, the larger diameter measured in the leaf blade may indicate for a more rapid hyphal extension in this leaf part.

4.3.2 Endophyte Growth and Proliferation in the Plant is not Controlled *via* Regulation of the EMS

As discussed in the previous sections, the EMS was very similar in different plant tissues and also plant genotypes. The uniformity in the EMS was contrasted by occasionally marked variations in hyphal biomass concentration between plant genotypes and plant tissues (Tables 10, 11, and 12). If the EMS were unknown, these variations would have obviously suggested that proliferation of the endophyte be controlled by factors impacting on its metabolic activity, as for example, short supply of nutrients by the plant.

The hyphae of *Neotyphodium* endophytes occur intercellularly in the plant, which means that the endophyte has no direct access to host nutrients, and consequently the lack of fungal growth in mature tissues has been interpreted previously as being the result of nutrient deprivation (Kirby, 1961). The high and uniform EMS in the plant found in the present study (Section 3.2.3) disproves this hypothesis, for it indicates that the endophyte is not starved in the plant. The *in planta* EMS reveals that the concentration of the endophyte in the plant is apparently not controlled *via* a regulation of endophyte metabolic activity in mycelium. The endophyte is highly metabolically active in different tissues and plant genotypes, but notwithstanding its high level of metabolic activity, the fungus does

not invest this activity into further hyphal extension past a certain limit, predetermined by plant tissue and genotype.

To date, no firm knowledge about nutrient relations and fungal growth in the intercellular spaces seems to exist. However, the assumption that the apparent inhibition of endophyte growth in the mature plant tissues is caused by a lack of nutrients is not supported by previous reports on fungal growth in the apoplast of the grass plant. An endophyte species, belonging to the genus *Phialophora* also grows solely intercellularly in perennial ryegrass, yet this fungus shows vigorous and extensive growth, including sporulation in mature leaves (Philipson, 1989). Accordingly, it has been proposed that the levels of photosynthates, such as sugars and other metabolites in the apoplast are sufficiently high to allow for fungal growth (Hancock & Huisman, 1981). For example, it could be demonstrated that carbohydrate efflux occurs relatively readily from plant protoplasts (Huber & Moreland, 1980), and that solutes diffuse into the apoplast of leaves (Canny, 1990).

The principal carbohydrate transferred to a closely related *Epichloë* endophyte is sucrose (Thrower & Lewis, 1973), and a *Neotyphodium* endophyte seems to possess a specific uptake mechanism for this solute (Lam *et al.*, 1994), suggesting that this important nutrient can be accumulated by the fungus against a concentration gradient. Moreover, *N. coenophialum*, a close relative of *N. lolii*, produces indole acetic acid (IAA) in culture (De Battista *et al.*, 1990a). If these fungi produce IAA also in the plant, this may allow them to gain additional access to nutrients, as IAA potentially induces the leakage of metabolites from plant cells (Beattie & Lindow, 1999). *N. lolii* is capable of producing a proteinase, which perhaps facilitates the degradation of apoplastic proteins for uptake and utilisation by the fungus (Reddy *et al.*, 1996). Hence, it appears that primary nutritional requirements for growth of the endophyte are met, and that they are efficiently complemented by specific uptake mechanisms of the fungus, thus probably allowing for the high metabolic activity of the endophyte in the plant.

However, the question remains how growth of the endophyte is restricted in the mature plant tissues. A confinement of growth within the plant could conceivably occur by regulation *via* plant compounds specifically required for hyphal growth. Thiamine, an essential vitamin for growth of the endophyte *N. coenophialum* (Kulkarni & Nielsen, 1986), is found in herbage in concentrations of 2.5 to 10 $\mu g\ g^{-1}$(Butler, 1973), thus significantly exceeding the amounts required for growth of the fungus in culture (0.2 $\mu g\ g^{-1}$, Kulkarni & Nielsen, 1986). However, nothing appears to be known about the availability of this and perhaps other fungal micronutrients in the apoplast. Thus, although most nutrients are probably in ample supply, very specific requirements for growth might not be met. However, it can be expected that the absence of certain growth factors, probably needed for appropriate functioning of the fungal cell, should also lead to a lower metabolic activity of the fungus; a lack of certain micronutrients in the apoplast seems therefore also not probable.

As nutrient supply to the endophyte is probably sufficient, certain signals required for apical extension of endophyte hyphae might be lacking, or growth may be actively suppressed in the mature leaves. In another plant-fungus symbiosis, the mycorrhiza, hyphal growth prior to direct contact with the plant appears to be positively and significantly affected by metabolites exudated from plant cells (Gianinazzi-Pearson, 1996). Thus, it may be possible that similar factor(s), stimulating hyphal branching and extension of the endophyte are released from plant cells in young emerging tissues, but not from matured plant cells. Conversely, a specific repressor of endophyte growth may be present in tissues of mature tissues.

It is conceivable that the plant reacts towards the infection, to keep the proliferation of the endophyte in check. For example, infection by symbiotic nitrogen-fixing bacteria, and by mycorrhizal fungi, triggers plant defence responses which may be modulated between the symbionts, and the further development of the symbiotic structures appears to be then regulated by the plant (Jackson, 1996). However, at present not much information seems to exist about how the plant achieves this regulation.

Notwithstanding this present lack of knowledge, it is possible that regulatory mechanism(s) similar to the mycorrhiza symbiosis may be also involved in the endophyte grass symbiosis. For instance, as the leaf develops growth of the endophyte is induced to proceed, and as a certain leaf developmental stage is completed, fungal growth is countered by the plant in some, yet unknown ways. Growth of *Neotyphodium* endophytes can be readily induced from infected leaf tissue pieces placed on agar (Latch & Christensen, 1985, Christensen *et al.*, 1998). This may indicate that the plant controls the proliferation of the endophyte, and that deregulation occurs when the viability of the plant tissue is compromised. This tentative hypothesis is also supported by the finding that the related *Epichloë* endophytes show noticeable growth on senescent leaf tissues (Kirby, 1961).

In regards to regulation of endophyte growth by the grass host, plant compounds have been identified in this symbiosis that have effects on fungal growth. Some endophyte-infected grasses appear to have higher levels of chitinases (Roberts *et al.*, 1992), which are potent inhibitors of fungal growth (Schlumbaum *et al.*, 1986). However, in the former study (Roberts *et al.*, 1992) it was not determined whether the measured *in planta* levels of chitinases were due to production by the plant or by the endophyte, as fungi also possess endogenous chitinase activities (Gooday, 1977).

Chitinases, commonly elicited in the plant in response to infection, act by interfering with growth of the fungus at the hyphal tip (Schlumbaum *et al.*, 1986), often, depending on the fungal species, having little effect on the integrity of the hyphal wall (Gooday, 1977). Therefore, for understanding the role of plant defence mechanisms in regulating endophyte growth, it would be important to determine the origin of the chitinases previously measured by Roberts *et al.* (1992). The action of antifungal compound(s) produced by the plant could be inhibitory to hyphal extension at the tip, but at the same time may have no negative effect on the metabolic activity of the fungus. If it could be confirmed that the plant produces chitinases in response to the endophyte-infection, the possible role of these for the restriction of endophyte proliferation in mature tissues could be further tested.

Specific inhibition of extension at the hyphal tip, due to the action of certain antifungal compounds produced by the plant may, at a glance, explain lack of growth of the endophyte. Moreover, it could be hypothesised that these inhibitors may take time to accumulate in plant tissues, therefore allowing for growth of the endophyte in young, expanding leaves. However, in this model it is difficult to accommodate the development of the choke disease (Section 1.2) caused, for example, by the endophyte *Epichloë typhina* (Pers.: Fr.) Tul. Similarly to *Neotyphodium* endophytes, the mycelium of this fungus normally appears sparsely in above ground parts of the plant. Rapid fungal growth, leading to formation of a stroma is then incited by an unknown trigger at a defined stage during inflorescence development (Kirby, 1961). The reason(s) for this localised stimulation of growth of the fungus, contrasting to its inhibition in vegetative tissues, are not known.

The identification of factor(s) responsible for the lack of measurable growth of the endophyte in mature tissues could be an important step in understanding cessation of hyphal extension without measurable reduction in endophyte metabolic activity. Processes and factors involved in hyphal apical extension in fungal mycelium have been discussed previously (Prosser, 1993; Heath, 1997; Harold, 1997; Sietsma & Wessels, 1997); however, little appears to be known how regulation, and integration, of these processes is achieved in hyphae. *Neotyphodium* endophytes could provide an experimental system for studying hyphal extension because of its, at present, unique growth behaviour (= high metabolic activity in the absence of measurable growth). With this system, it may be possible to determine how hyphal extension is abolished in vegetative plant tissues, and how it is therefore uncoupled from levels of metabolically activity that otherwise would allow for appreciable growth of the endophyte mycelium.

An approach, aimed at identifying genes involved in apical extension could be the application of subtractive hybridisation techniques, that could be used for comparing plant tissues with growing and non-growing endophyte mycelium. With this method, based on subtraction of RNA, it could be selected for messenger RNA expressed only in plant tissues with growing endophyte mycelium, such as emerging leaves. To discriminate for

fungal rather than plant messenger RNA, it would be necessary to also subtract with endophyte-free plant tissue of the same type (that is, emerging or mature), that is already available as material clonal to infected plant material (Section 3.3). If differentially expressed messenger(s) that are present only in growing endophyte hyphae could be successfully isolated, the cDNA(s) derived from these messengers could be used for isolation of the gene(s) involved in apical extension of endophyte hyphae.

More detailed studies would be needed to determine, for example, branching rates of hyphae in meristematic tissues, as these are evidently important in determining the endophyte concentration in mature tissues. Confocal and/or electron microscopy methods could be valuable tools for examining branching in endophyte mycelium colonising leaf and apical meristems.

4.3.3 The *In Planta* Distribution of the Endophyte Suggests Plant-Regulated Proliferation of Mycelium

To date, detailed studies of growth and proliferation of the endophyte during growth and development of the grass tiller are lacking. Thus far, methods were of limited power for detailed investigations into endophyte growth and distribution within the grass plant. The use of the GUS system has now significantly enhanced the detection and resolution for mapping of the endophyte, particularly in growing tissues such as emerging leaf sections, providing information about endophyte growth during leaf development (Section 3.2.4.2). Some problems with the quantitative detection of GUS activity in certain plant tissues have been detected (Section 3.1.9) indicating that compounds (probably leaf pigments) in extracts can interfere with the measurements. However, a quantitative estimation of this inhibition allowed accounting for this in measurements.

The results of the present study have shown that *N. lolii* is substantially higher concentrated in leaf sheaths than leaf blades of perennial ryegrass (Section 3.2.4.1). This is in agreement with previous studies concerning the *in planta* distribution of *N. lolii*, or endophytes closely related to this fungus that infect perennial ryegrass or tall fescue (Herd

et al., 1997; Hinton & Bacon, 1985; Keogh *et al.*, 1996). Hence, it appears that this distribution pattern is a common feature in endophyte-grass associations.

It has been proposed that availability of nutrients may be the reason for the observed pattern of colonisation by *Neotyphodium* endophytes. Hinton & Bacon (1985) suggested that the relative abundance of endophyte mycelium in the leaf sheath might be due to a greater concentration of sugars in this leaf part. However, firstly, the results in the present study are consistent with synchronous growth of endophyte and leaf (Section 3.2.4.2), therefore, differences in endophyte concentration between sheath and blade are apparently established in the emerging leaf. Consequently, the levels of sugars in mature tissues should hardly have effects on the concentration of endophyte mycelium. Secondly, as discussed previously (Sections 4.3.1 and 4.3.2), the endophyte metabolic state (EMS) was uniformly high in plant tissues also arguing against regulation of growth of the endophyte by a curtailed nutrient supply.

The observed gradient in endophyte concentration between blade and sheath of a leaf may be due to an increase of the number of hyphae over time, during leaf expansion from the stem apex. Leaf blade and sheath are derived from an intercalary meristem at the base of the elongating leaf (Soper & Mitchell, 1956), and elongation of the leaf blade precedes the elongation of the leaf sheath (Durand *et al.*, 1999; Schnyder *et al.*, 1990). It could thus be proposed that initially, during formation of the blade, only a small number of hyphae invade the extending leaf. Subsequently, with progression of leaf development, more hyphae are inserted at the leaf base that later constitutes the leaf sheath. In this model, it is assumed that branching in mycelium is largely restricted to the base of the leaf. However, some branching also seems to occur in leaf sections distal to the leaf base (personal observations, not shown).

The gradient in endophyte concentration between sheath and blade could yet also reflect differences in growth rates during leaf elongation between the two leaf sections. Indeed, differences in growth rates of elongating leaves in perennial ryegrass have been reported, depending on whether sheath or blade are formed as the leaf expands (Schnyder

et al., 1990). Elongation of the leaf blade was determined to be about 2-3 times more rapid than that of leaf sheath (Schnyder *et al.*, 1990). Hence, given synchronous growth of endophyte and plant, for colonising the developing leaf as it expands, the endophyte growing into the leaf blade has to achieve a faster hyphal extension. Faster extension of hyphae could potentially affect the concentration of endophyte mycelium in the mature blade.

To explain this proposed interrelationship of concentration with growth rate, hyphal extension has to be briefly addressed. The extension of hyphae occurs in a defined zone at the hyphal tip (Prosser, 1993; Heath, 1997; Sietsma & Wessels, 1997). Faster extension rates at hyphal tips can be attained by reduced branching in mycelium (Prosser, 1993, Trinci *et al.*, 1997), that is, an area or volume is colonised by fewer hyphae that extend with a faster rate. Therefore, assuming that the rate of hyphal extension is kept equivalent to the elongation rate of the leaf part in which hyphae are growing, branching in mycelium would be expected less in the leaf blade than in the leaf sheath, because of the faster elongation of the former. In this context it has to be noted that leaf elongation is largely continuous, since growth of grass leaves is not interrupted by the light changes occurring in the daily cycle (Volenec and Nelson, 1982), which otherwise would allow hyphae to "catch up" with the expanding leaf section.

Similar to the question how the *in planta* distribution of the endophyte may be regulated is the question how hyphal growth is controlled in different plant genotypes. Up to five-fold differences in endophyte metabolic activity concentration between plant genotypes were observed (Section 3.2.2). For three plant genotypes, it was shown that differences in metabolic activity concentration were due to a corresponding variation in concentration of endophyte mycelium (Section 3.2.3). Because of the observed similarity of the EMS for these three associations, it appears probable that the same is true in the other associations. Since a single endophyte strain was used to infect the plants, this indicated that the plant genotype in the *N. lolii*-perennial ryegrass symbiosis is important in determining the levels of colonisation by the endophyte, but not the metabolic state of the fungus.

It is conceivable that differences in the response to endophyte-infection between plant genotypes exist. For example, assuming that the proliferation of the endophyte is controlled by specific factors that affect fungal growth and are released by the plant (see above, this section), the quantity and/or quality of these factors may be different between plant genotypes. Critical parameters that determine growth in fungi are specific growth rate and branching (Prosser, 1993, Righelato, 1979, Trinci, 1979), both of which are significantly influenced by environmental factors (Trinci *et al.*, 1997). The specific growth rate is usually not affected if nutrients are present in excess and conditions such as the pH are favourable for growth (Trinci *et al.*, 1997). However, branching in mycelium is regulated by multiple factors, such as calcium, calmodulin, phosphoinositides, and cyclic nucleotides (Trinci *et al.*, 1997). In addition, branching can be stimulated by certain compounds called "paramorphogens" (Tatum *et al.*, 1949).

The variation in the concentration of endophyte mycelium between genotypes may be due to genotype-specific differences in the concentration of compounds affecting branching, such as for example calcium and the paramorphogens (for example, cellobiose and sorbose, Trinci *et al.*, 1997, which may also occur in plant tissues). Genotypes of perennial ryegrass vary, occasionally markedly, in tissue concentrations of carbohydrates and proteins (Cooper, 1973), and genotypes of tall fescue showed on average three to four-fold differences, for instance, in glucose and fructose in leaf meristematic regions (Volenec & Nelson, 1984a). Hence, significant variation also in compounds that impact on branching in endophyte mycelium might occur.

Besides, differences in the rates of leaf elongation between plant genotypes may also contribute to the differences in endophyte concentration. The rate of leaf elongation depends on environmental factors, but is also genetically determined in grasses; up to two-fold differences in leaf extension rates existed between plant genotypes of the grass tall fescue (Volenec & Nelson, 1984a). The plant genotypes in the present study also differed slightly in leaf extension, and, incidentally, the plant genotype with the highest leaf

extension rate had the lowest overall concentration of endophyte mycelium, notably in the leaf blade (Table 14).

A putative relation particularly of hyphal branching with the concentration of the endophyte in plant tissues poses the question how branching in endophyte mycelium may be regulated in these tissues. As discussed, a number factors have been identified that affect branching in mycelium. However, is not yet known how these factors precisely regulate the branching (Trinci *et al.*, 1997). The very intimate relationship between plant and endophyte suggests that a number of signals be mutually exchanged, which are necessary for the co-ordinated development of the symbiosis. Therefore, more detailed investigations aimed at identifying the mechanisms controlling branching and, hence, proliferation of the endophyte in the grass plant could lead to a more sound and general understanding of mycelial growth not only in this symbiosis but of fungal growth in general.

In conclusion, differences in concentration of endophyte mycelium between plant genotypes and plant tissues are probably not the result of a limited nutrient availability. Rather, they probably reflect differences in a number of factors, such as the developmental time course of leaf expansion and fungal proliferation during invasion of the leaf, and tissue-specific growth and branching rates. Differentially expressed plant-derived factors that affect branching and proliferation in the endophyte mycelium may exist in different tissues, leading to the observed differences in mycelial concentration. The factor(s) responsible for the suppression in mature tissues and for growth regulation in emerging tissues remains to be discovered.

4.5 ALKALOID ACCUMULATION AND ITS RELATIONSHIP TO ENDOPHYTE METABOLIC ACTIVITY

4.5.1 An Optimised Extraction Method for the Alkaloid Ergovaline Allows for Quantitation from Small Sample Quantities

For studies presented in this work procedures for reliable extraction of the alkaloids lolitrem B, ergovaline, and peramine from plant tissues were needed to accommodate smaller sample amounts, allowing for mapping of the *in planta* distribution of these compounds in fine detail. The newly developed method for quantitative extraction of the alkaloid ergovaline meant a significant improvement in terms of sample quantities, required for determination (Section 3.1.4). In addition, the behaviour of ergovaline and internal standard in extractions was characterised in greater detail than in previous studies.

As aimed, sample quantities were successfully reduced with the new method. Whereas in previous protocols at least 50 mg samples were needed, extractions using the new method could be performed from only 2 mg dry weight plant tissues (Section 3.1.4.5), allowing for detection of ergovaline with high sensitivity. In addition, time and work requirements for sample preparation were reduced; samples could be processed and analysed within one day. This significantly reduced the exposure of analyte and internal standard to the extractive environment, which, although carefully selected for retaining stability of analyte and internal standard, inevitably affects the integrity of these compounds (Garner *et al.*, 1993; Yates & Powell, 1988)

Extractions during optimisation were carried out with an isopropanol-lactic acid solvent. Isopropanol has the advantage over the previously used chloroform in that it has a lower volatility; thus, volumetric variation caused by evaporative losses impacting on accuracy in the determination of ergovaline was reduced. Lactic acid was included into the solvent to provide a weak acidic environment, which is required for extraction of ergovaline. In addition, this organic acid had been previously found to have good qualities for extraction and stabilisation of this alkaloid (Moubarak *et al.*, 1993; Testereci *et al.*,

1990). This was confirmed by assessment of isomerisation, an indicator of possible degradation of the added internal standard ergotamine. Isomerisation was much lower in this solvent than in an acetic acid solvent of a recently published method (Shelby & Flieger, 1997) that was also investigated for its usefulness in extractions (Section 3.1.4.3).

In the initial experiments, variation was observed in recovery of ergovaline and internal standard depending on the proportion of the organic solvent isopropanol in extractions (Section 3.1.4.2). As noted in several studies (Hill *et al.*, 1993; Rottinghaus *et al.*, 1991; Shelby & Flieger, 1997; Yates & Powell, 1988), recovery of the alkaloid analysed and internal standard in extractions is never 100 per cent. An internal standard can compensate for this incomplete recovery, but only if analyte and standard are recovered from the sample with the same rate, that is, in proportion to their concentration in the sample. The isopropanol – lactic acid system fulfils this requirement, as closely similar proportions of ergotamine and ergovaline were recovered, demonstrated by re-extraction experiments (Section 3.1.4.2 and 3.1.4.3), which indicated that with the newly developed method both compounds were recovered with the same efficiency.

The importance of consistency in recovery of analyte and internal standard was demonstrated by comparison of the newly developed isopropanol-lactic acid method with a protocol involving extraction with acetic acid (Shelby & Flieger, 1997). Extraction with acetic acid gave high estimates of ergovaline, initially suggesting a higher efficiency of extraction (Table 6). However, when absolute chromatographic peak areas were examined, these showed that the higher estimates were not due to higher recoveries of ergovaline, but to lower recoveries of the internal standard ergotamine.

Differences in the recovery of the two ergopeptides depending on the solvent system used were revealed by re-extraction experiments. In the re-extractions, the proportion of residual ergotamine was 66 per cent higher than that of ergovaline in the acetic acid solvent (Section 3.1.4.3). This comparative study demonstrated that in applying internal standardisation methods, failure to examine the recovery of the internal standard as well as the analyte could result in strongly biased results.

Moreover, the consistency of the results obtained with this solvent system also indicated for the higher accuracy of this method. As shown in extraction experiments with different proportions of isopropanol-lactic acid (Section 3.1.4.2, Table 4 and 5), variations both between and within ergovaline estimates with different solvent ratios were considerably smaller than variations in the ergovaline peak areas. This also demonstrated the robustness of the isopropanol-lactic acid method. The selected conditions of 50% isopropanol, 1% lactic acid extraction provide high and consistent recovery of both analyte and internal standard.

The experiments during optimisation of the extraction method revealed that the internal standard as well as the analyte might bind to plant material in the extraction mixture and can suffer losses due to degradation. It is evident from the time course experiment (3.1.4.4) that equilibration of the standard with the matrix (= plant sample material) takes some time. Extraction for two hours has been shown to be sufficient to permit equilibration of the internal standard with the sample, without excessive degradative losses.

The 50% isopropanol 1% lactic acid solvent procedure adopted provides high and comparable recovery of the standard and analyte, and adequate time for standard equilibration with the sample. Moreover, it is compatible with polyethylene laboratory equipment that allows greatly simplified handling procedures. In addition, it permits the measurement of peramine and ergovaline in a single extract; therefore, significantly reducing sample amounts for analyses aimed at the determination of all of the major alkaloids in a single tissue. Thus the suitability of this procedure for accurate quantitation of ergovaline and peramine in very small samples of plant material (< 5 mg dry weight) has been successfully demonstrated.

4.5.2 Each Alkaloid Exhibits a Characteristic Distribution in Grass Tillers

In the present study, the quantitative distribution of each of the alkaloids within the grass tiller was determined with a significantly greater resolution than in previous studies. An experiment was designed for analysis of the spatial and temporal aspects of alkaloid production. Firstly, a dissection scheme was devised in which plant tissues were fractionated, according to the anatomy of the tiller, with far greater precision than done previously in other studies. In this way, alkaloid production was examined in fine detail within the grass tiller. Secondly, analyses for all three alkaloids, and for endophyte metabolic activity were performed on the same fractionated sample material, which permitted direct and meaningful correlation of these parameters. Thirdly, these experiments were carried out on three plant endophyte associations differing in plant genotype that were maintained in a controlled environment, therefore allowing effects of the plant genotype on alkaloid and endophyte metabolic activity distribution to be isolated.

The main finding of these experiments concerning the *in planta* relationship between alkaloids and endophyte indicated that none of the alkaloids was distributed in exact proportion to the distribution of the fungus (Section 3.2.4). This indicates that differential synthesis and/or degradation in mycelium, and probably translocation within the plant may be involved in determining the concentration of the alkaloids within the grass tiller. The alkaloid ergovaline was strongly localised to basal plant tissues (Fig. 20). Lolitrem B tended to be higher concentrated in lower leaf sections, but appreciable amounts were also present in the upper leaf (Fig. 24). Peramine showed the least localised distribution within a tiller (Figs. 22). These findings are consistent with previous observations on the distribution of these compounds within perennial ryegrass infected by *N. lolii* (Ball *et al.,* 1997a; Ball *et al.,* 1997b; Keogh *et al.*, 1996; Lane *et al.*, 1997c). However, in none of these studies all alkaloids were examined at the same time.

The localised accumulation of the alkaloid ergovaline in the true stem and lower sheath tissues, both in relationship to plant biomass and endophyte metabolic activity in all plant genotypes may be taken as an indicator for tissue specific synthesis of ergovaline.

Hence, the inner-plant environment might significantly regulate the synthesis of ergovaline in endophyte mycelium. However, little is known about the stability of ergovaline in the plant, thus, it is also possible that synthesis in the other plant sections occurs at similar rates, but that rates of degradation/metabolisation are higher in these tissues.

The *in planta* distribution of ergovaline could also reflect unidirectional translocation of ergovaline to basal tissues. Because of their lipophilic properties, ergot alkaloids can accumulate in storage lipids as observed in the related ergot fungus *Claviceps purpurea* (Neumann, 1985). A dependence of ergot alkaloids solubility on pH and the retention in lipids however exists (Neumann, 1985), hence, situations may arise in the plant where this alkaloid gains a higher mobility. The very even distribution of ergovaline in the emerging leaf (Fig. 20) is suggestive of some transfer of ergovaline, presumably *via* diffusion from the stem regions in which ergovaline concentration is high. Notwithstanding this probable indication for some transfer of ergovaline within the plant, no evidence was found in this or other studies supporting strong unidirectional transport of this alkaloid.

The finding of ergovaline in greater concentrations in basal tissues of the second mature leaf of some genotypes (Fig. 20A, 20B) suggests varying rates of synthesis and degradation of ergovaline as the leaf ages. Differential synthesis of ergot alkaloids has been previously demonstrated. *In planta* ergot alkaloid production in *Claviceps purpurea* takes place during a defined phase in its life cycle (Didek-Brumec *et al.*, 1996; Tudzynski *et al.*, 1997). *In vitro,* ergot alkaloid production in *C. purpurea* is also temporally regulated; the synthesis of enzymes required for production occurs at a certain growth phase of the fungus (Floss & Anderson, 1980).

This raises the question about the factors that may play a role in production in the perennial ryegrass plant. For example, ergot alkaloid formation is known to be favoured by higher concentrations of sugars (Didek-Brumec *et al.*, 1996; Gröger, 1985). The greater concentrations of ergovaline in the lower leaf sheath and true stem regions in *N. lolii*-infected ryegrass could thus be the result of greater availability of carbohydrates in these

regions. Indeed, in grasses, the major site of sugar accumulation for storage is mainly the pseudostem (Parsons, 1988). This tissue is comprised mostly of leaf sheaths, and greater amounts of assimilates are also partitioned into the meristematic regions, such as leaf primordia and axillary buds located on the true stem (Robson *et al.*, 1988). Thus, leaf sheaths and true stems seem to fulfil an important requirement for the synthesis of this alkaloid.

Other factors are also important for ergot alkaloid production in *Claviceps* species: the availability of the precursor tryptophan (Krupinski *et al.*, 1976) and of divalent ions, notably calcium that stimulates the activity of the DMAT synthetase (Cress *et al.*, 1981), the first enzyme in ergot alkaloid biosynthesis (Section 1.3), are important determinants for ergot alkaloid synthesis. In culture, ergot alkaloid synthesis is often associated with depletion of nutrients such as nitrogen and phosphate (Vining, 1973; Didek-Brumec *et al.*, 1996). As shown in this work, the high *in planta* EMS indicates that the endophyte is not exposed to a nutrient-depleted environment. Therefore, this suggests that the regulation of alkaloid synthesis in the plant be significantly different from regulation in culture. On the other hand, it is known that organs allocated to storage (such as leaf sheaths and stems) lose total nitrogen during rapid herbage growth (Sheard, 1973); thus, changes seem to occur in the plant, possibly having an effect on alkaloid production without affecting the metabolic state of the endophyte.

Less is known about the regulation of the synthesis of the other two alkaloids lolitrem B and peramine, unique to the *Neotyphodium/Epichloë* endophytes. The very even *in planta* distribution of peramine (Section 3.11.3) suggests synthesis throughout the plant, and some translocation may be involved in determining the observed distribution. For both peramine and lolitrem B translocation within developing grass seedlings has been previously reported. Experiments with seeds containing non-viable endophyte showed that the two compounds are transferred by the plant (Ball *et al.*, 1993). However, since the endophyte was non-viable in these experiments, it is not yet established that in a functional symbiosis the plant carries out the translocation solely. In these seeds with the non-viable

endophyte, substantial loss of integrity of the dead endophyte mycelium and uncontrolled release of peramine and lolitrem B into the plant tissues may have occurred.

Transport of alkaloids within plants can involve transfer over long distances: In tobacco, alkaloids are produced in the roots and translocated *via* the xylem into the leaves (Mothes & Romeike, 1958). In the *N. lolii*-perennial ryegrass situation, the distribution of peramine relative to the distribution of endophyte metabolic activity suggests translocation within and also between leaves. Translocation between leaves may be the result of transport processes within the plant, for example, assimilates are commonly transported from older, assimilating into young, growing leaves (Robson *et al.*, 1988). Given its relatively high mobility (Rowan, 1993), peramine may co-migrate with photosynthates into growing tissues explaining its relatively high concentrations in the emerging leaf.

However, the results presented here do not preclude that some differential synthesis may also be a factor for the distribution of peramine in leaf tissues, as previously implicated for ergovaline. It is conceivable that peramine is synthesised in greater quantities in leaf parts further removed from the base. The relatively consistent concentrations as leaves age thereby suggest that synthesis, putative export, and degradation remains largely in balance.

Lolitrem B exhibits a rather more regular distribution pattern in relation to endophyte metabolic activity (Fig. 25) than peramine. The increase of lolitrem B in older tissue suggests constitutive expression and also higher stability. The observed increase of the lolitrem B : endophyte metabolic activity ratio along the leaf, with increasing distance from the base may indicate higher rates of synthesis in leaf parts further removed from the base. As noted, in contrast to lolitrem B the alkaloid ergovaline accumulates more strongly at the tiller base. This may indicate that in the production of lolitrem B and ergovaline some competition occurs between the biosynthetic pathways of the two alkaloids, as these both use the common precursor tryptophan (Fig. 2).

On the other hand, the distribution of lolitrem B may also be explained by within-leaf translocation of this alkaloid towards distal regions. Examples for this mode of transfer in related plants exist: the compound 3-di-methylaminomethyl-indole (gramine) is produced in the basal region of leaves of barley seedlings and is then transported to the leaf tip where it accumulates (Gröger, 1985). Perhaps a similar process occurs in endophyte-infected grasses; localised production of lolitrem B in lower-leaf regions by the fungus and subsequent transport by the plant to distal leaf parts.

Whether the observed apical-basal pattern of lolitrem B accumulation per mycelium is the result of differential synthesis and/or unidirectional translocation remains to be established. Further studies, for example with targeted labelling of these compounds in the symbiosis would be required to elucidate possible mechanisms and routes of transfer of peramine and lolitrem B within the plant. Also, using molecular biology-based techniques, genes putatively involved in alkaloid biosynthesis in fungi closely related to the *Neotyphodium* endophytes have successfully been isolated (Tsai *et al.*, 1995; Tudzynski *et al.*, 1999). Hence, the time seems close where the corresponding genes in endophytes may be found. Once identified, it may be possible to monitor the expression of these genes in the plant.

Recently, the gene for 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase of the endophyte strain Lp 19 has been cloned (Dobson, 1997). This enzyme contributes to the formation of mevalonic acid, an intermediate compound in the biosynthesis of paxilline, a precursor in the biosynthesis of lolitrem B (Section 1.3.1). By using the promoter of this or other, yet to be isolated, genes involved in alkaloid biosynthesis in *Neotyphodium* endophytes in genetic constructs with a reporter gene such as GUS or green fluorescent protein (GFP), the regulation of alkaloid biosynthetic genes in the plant could be investigated. The analysis of reporter gene expression from a promoter that normally regulates the expression of an alkaloid-biosynthetic gene could permit precise mapping of the sites of alkaloid synthesis in the plant.

In conclusion, the findings in this study have shown that none of the alkaloids is distributed in exact proportion to the amount of endophyte mycelium within the grass tiller. The differences in distribution between alkaloids seem to reflect their variation in localised synthesis and degradation and/or translocation. Ergovaline accumulation appears to be regulated by the inner-plant environment, suggesting that the physiological conditions in some plant tissues are particularly favourable for production. The more even distribution notably of peramine than the fungus may indicate for less control of the plant environment on production and perhaps a greater influence of transport processes within and between leaves on peramine distribution. The accumulation of lolitrem B with leaf age is indicative for constitutive synthesis of this alkaloid, and its within-leaf distribution suggests variation in concentration per fungal mycelium due to differential synthesis and/or apical translocation of this compound.

4.5.3 The *in Planta* Concentrations of the Alkaloids are influenced by Plant Genotype

In an initial study, variation in concentration of the alkaloids, notably of lolitrem B and ergovaline, between perennial ryegrass genotypes was found, despite very similar plant growth conditions, sampling on one day and from the same tissue (Section 3.2.2). All plants were colonised by the same endophyte strain, therefore indicating that the plant genotype significantly affects the production of the alkaloids.

A more detailed experiment on alkaloid distribution (Section 3.2.4) confirmed the earlier findings of consistent differences in alkaloid accumulation between plant genotypes. A noticeable difference existed between the two plant genotypes Nui D and Nui UIII. These genotypes varied significantly and conversely in ergovaline and lolitrem B concentration: Nui D had a significantly lower concentration of ergovaline and a higher concentration of lolitrem B in tissues and also per metabolically active mycelium than Nui UIII (Table 15A, 15C). Thus, as noted in the previous section, as both alkaloids require tryptophan as a precursor, the inverse relationship between ergovaline and lolitrem B especially per endophyte mycelium, seen for different plant genotypes and also plant

tissues may indicate for some competition between the biosynthetic pathways of these two alkaloids.

Mean endophyte metabolic activity concentrations, alkaloid concentrations, and ratios of alkaloids to endophyte metabolic activity are summarised as relative rank numbers in Table 18. As indicated by the listed ranks, there is no simple relationship between amounts of endophyte metabolic activity and any of the alkaloids; hence, alkaloid production per metabolically active endophyte mycelium is variable between the different plant genotypes. However, differences in plant tissue concentration of the alkaloids ergovaline and peramine between genotypes were apparently also influenced by corresponding differences in endophyte concentration (Sections 3.2.4.3 and 3.2.4.4). Hence, overall levels of these alkaloids are to some degree determined by the overall levels of endophyte mycelium.

As with differences in alkaloid concentration per endophyte mycelium between plant tissues, the variation in production per mycelium between plant genotypes found for lolitrem B in particular (Section 3.2.4.5) suggests that the alkaloid synthesis be affected by the availability of certain compounds to the mycelium. As discussed previously (Section 4.3.2), plant genotypes of perennial ryegrass differ significantly in the amount and composition of important metabolites such as carbohydrates and proteins. Thus, it is possible that differences in the production of the alkaloids between genotypes are determined by plant-genotypic differences in the availability of specific compounds required for alkaloid synthesis by the fungus. Moreover, as the alkaloid content is also influenced by the amount of endophyte mycelium in the plant, specific putative regulators of hyphal proliferation (see above, section 4.3.3) possibly differing between plant genotypes may be important in determining alkaloid levels. The identification of these compounds and there potential utility as criteria for selecting genotypes with more desirable alkaloid profiles remains to be established.

Table 18

Rank numbers for the three plant genotypes used in this study, depending on the concentration of endophyte metabolic activity, each of the alkaloids and ratios alkaloid to endophyte metabolic activity.

Rank "1" relative highest mean concentration or ratio, rank "3" relative lowest mean concentration or ratio.
ev = ergovaline, per = peramine, lol = lolitrem, EMA = endophyte metabolic activity

Plant genotype	endophyte metabolic activity	Total ergovaline	Total peramine	Total lolitrem B	Ratio ev : EMA	Ratio per : EMA	Ratio lol : EMA
Nui D	2	3	1	1	3	2	1
Nui UIII	1	1	2	2	1	3	3
Nui UIV	3	2	3	3	2	1	2

4.6 *SOME EVIDENCE FOR DEFINED SEGREGATION OF GUS-EXPRESSING AND NON-EXPRESSING HYPHAE INTO DEVELOPING TILLERS*

Unexpectedly, the GUS system provided some information as to how endophyte mycelium may be segregating into single tillers of a whole plant. The high sensitivity of the method (Section 1.4) allows for detection of very small amounts of the GUS enzyme in plant tissues, thus, activity in only a few endophyte hyphae in plant tissues can still be detected. Therefore, complete absence of GUS expressing endophyte hyphae from plant tissues became noticeable quickly (Section 3.1.8).

If two genetically distinct populations, for example, GUS-expressing and non-expressing, of endophyte hyphae are growing in a single tiller, the question arises whether hyphae of these populations will be proportionally distributed on tillers newly developing from this tiller, or whether they will be segregating in a defined manner into these daughter tillers. This question was important especially for the maintenance and purity of the GUS gene marker in plants infected by the GUS-transformed endophyte. Mixtures of GUS-expressing and non-expressing hyphae in tillers would have caused confounding effects in the determination of endophyte metabolic activity, and methods for maintaining the genetic purity of the marker were therefore developed and employed (Sections 3.1.2 and 3.1.8).

A loss of GUS activity from the transformed endophyte due to a mutation was a likely possibility. In culture no loss was detected in mycelium, despite extended subculture (Section 3.1.2), which suggests that the mutation rate at the GUS gene was low under these conditions. However, loss of GUS activity expression from mycelium in plants was observed repeatedly (Section 3.1.8). The reasons for the apparently increased mutation rate at the GUS gene in the plant were not known, but it may be due to the different growth conditions and higher selective pressure in the inner-plant environment.

The GUS loss was characterised by non-expression in endophyte-infected tillers among GUS-expressing tillers of a plant, and the lack of expression was maintained in

cultured mycelium, as determined for a fungal isolate from a single tiller that had been previously identified as GUS-negative (Section 3.1.8). This indicated that the loss was due to a mutation at the GUS gene, and because of the variation in GUS expression at the tiller level suggested that GUS-expressing and non-expressing endophyte hyphae segregate when tillers develop. Therefore, the endophyte hyphae within a tiller appear to be clonal. That clonal segregation is possible in mycelium of *N. lolii* was demonstrated by investigation of the nuclear distribution within apical compartments of growing hyphae, which were shown to be monokaryotic in this fungal species (Section 3.1.2).

Recent studies, reported contemporaneously with this work, have also provided evidence for segregation within endophyte mycelium at the level of the tiller. Meijer and Leuchtmann (1999) investigating the transmission of *Epichloë* endophytes between grass plants found evidence for dual infection of grass plants with endophyte strains differing in allozyme profiles. A single grass clump contained tillers that were infected either by one allozyme type or the other type; both types together in one tiller were not detected. A more detailed investigation by Wille *et al.* (1999), utilising endophyte genotype-specific primers for polymerase chain reaction (PCR) that allows for sensitive detection, revealed that in plants infected previously with two different endophyte genotypes, tillers contained each only one endophyte genotype, but mixed infection at the level of the whole plant was maintained.

Wille *et al.* (1999) speculated that a potential physiological reason for mutual exclusion of strains might be intra-specific competition for nutrients within host tissues, and differences in growth rates between endophyte strains. In the present study, although only one endophyte strain was used, it is a possibility that in the plant significant variation in growth rate between GUS-expressing and non-expressing mycelium exists, due to the additional metabolic cost, originating in the production of the GUS enzyme, for the GUS expressing mycelium. However, comparisons of growth rates under varying conditions (growth media, temperature, and growth media x temperature) carried out in culture did not support consistent differences in rates between GUS-expressing and non-expressing Lp 19 (not shown).

Aside from possible differences in growth rates, it is conceivable that the segregation of hyphae into different tillers is similar to the formation of sectors in a fungal colony. Vegetatively formed grass tillers develop from axillary buds located on the stem apex of previously existing tillers. Initially, the axillary buds probably contain only a small number of hyphae, perhaps originating from a single hyphal strand grown from the true stem towards the meristematic region. These founder hyphae may then exclusively colonise the newly developing tiller. As demonstrated in this study (Section 3.1.2), hyphae of Lp 19 contain only a single nucleus per apical compartment; therefore clear-cut segregation of genetically different hyphae was expected to occur. Endophyte mycelium, constituting in different genotypes, colonising the stem apex of a tiller could thus segregate in the defined way as has been observed here and by others.

The defined segregation of the *N. lolii* endophyte into plant tillers may be an important requisite for the persistence and survival of the fungus and thus for the symbiosis as a whole. *N. lolii* is an asexual fungus (Section 1.2), and, therefore, is likely to experience a loss of biological fitness, commonly referred to as "Muller's ratchet" (Felsenstein, 1974), which is, in the absence of recombination, caused by the continuous accumulation of deleterious mutations within organisms. For example, if a "mixed" population of hyphae regularly infected tillers, deleterious mutations would tend to accumulate, since in the absence of segregation these mutations can be complemented by hyphae that have not undergone the same mutations but may have also acquired deleterious mutations.

The sum of all hyphae constituting the mycelium in a tiller could thus temporarily retain the fitness of the symbiosis. However, an overall decline in the number of functional gene products would be the result. Ultimately, the process of Muller's ratchet is thought to lead to a "mutational meltdown" and to the extinction of lineages (Gabriel *et al*., 1993). Schardl *et al.* (1991) postulated that this process might diminish the survival of an asexual endophyte and its benefits conveyed to the symbiosis. It has been therefore proposed that interspecific hybridisation, that is, parasexual events involving fusion of hyphae and nuclei

of two endophyte species co-existing in one tiller, of sexual *Epichloë* with their asexual descendants may mitigate the effects of this process on the latter (Schardl, 1996). This hypothesis has been further substantiated by findings, which indicate that many asexual endophytes have undergone interspecific hybridisation (Tsai *et al.*, 1994).

However, interspecific hybridisation apparently has no implication for the long-term survival of some *N. lolii* strains, as this species occurs also in the Southern hemisphere which is devoid of sexually reproducing and thus infective endophytes (White, 1997). Since other mechanisms are seemingly not available to rescue *N. lolii* strains from accumulation of deleterious mutations, clonal segregation, for which some evidence in the *N. lolii* strain Lp 19 was found in the present study, may therefore be a mode for overcoming, or alleviating the negative effects of Muller's ratchet as discussed in the following.

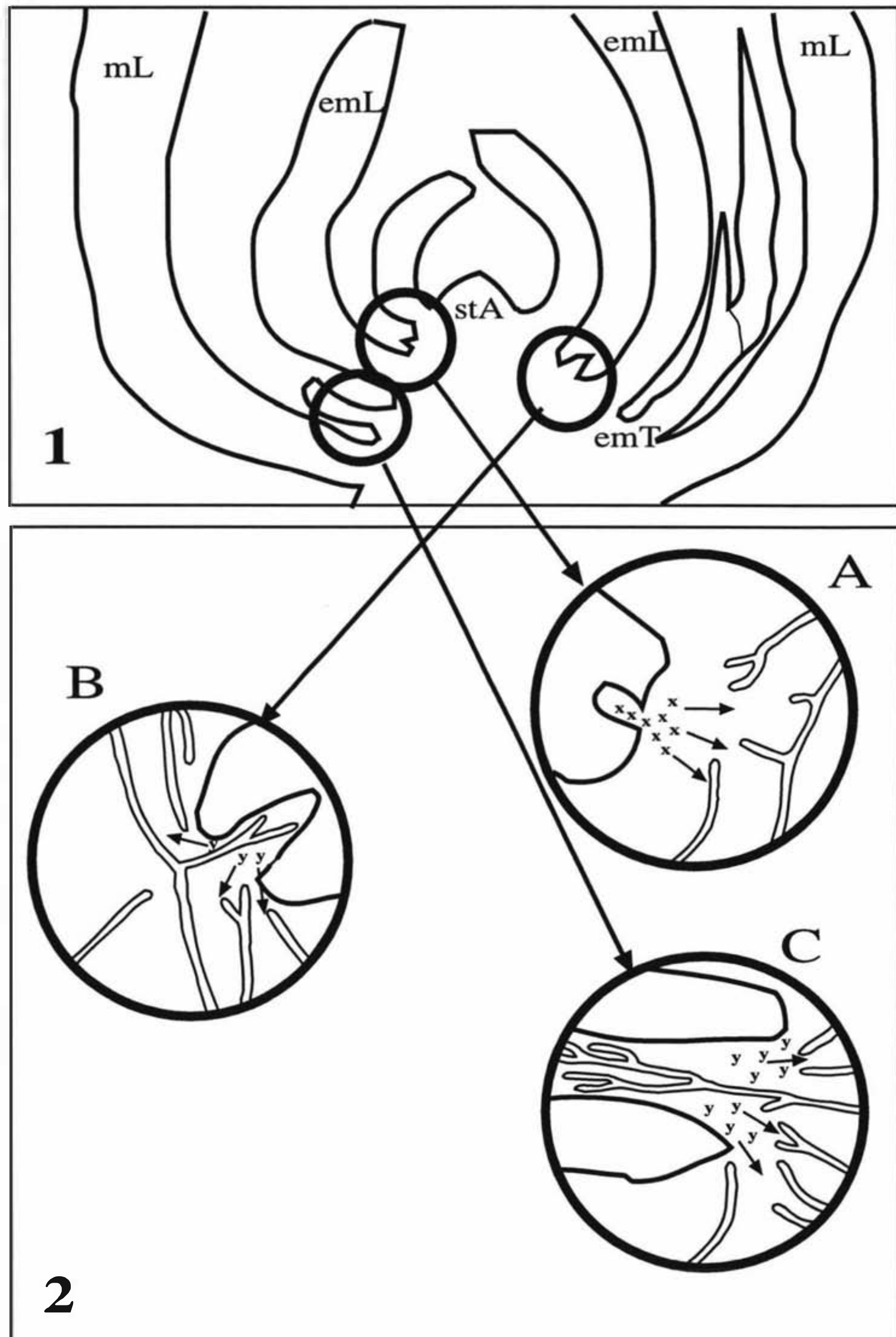
Examples for long-term survival of asexual organisms and organelles have been reported. A model for the evolution of mitochondria, also lacking recombination and having high mutation rates, has recently been proposed (Bergstrom & Pritchard, 1998). In this model, a mitochondrial "bottleneck", that is, transmission of a very small number of mitochondria from one generation to the next, ensures the maintenance of mitochondrial gene quality in lineages. After genetically different mitochondria have segregated into lineages, those lineages that have not experienced deleterious mutations, or have even acquired beneficial mutations will have a selective advantage over lineages which have experienced deleterious or not acquired beneficial mutations in the mitochondrial genome, respectively (Bergstrom & Pritchard, 1998).

Thus, a similar bottleneck may exist in the vegetative transmission of endophyte hyphae into new tillers. If a small, number of clonal hyphae colonises each new tiller, this would lead to a greater variance in fitness among tillers (for example, if alkaloid production is affected, enhanced or decreased protection of tillers against herbivores may result), hence, allowing for immediate selection against tillers that contain endophyte hyphae carrying a negative mutation, which impacts on the viability and persistence of the

tiller. Clonal segregation of the endophyte might be a way by which the detrimental effects of Muller's ratchet on the fitness of the symbiosis may be overcome or at least slowed down (Schmid *et al.*, 2000).

Given the implications of clonal segregation for the long-term survival of the endophyte and thus for the symbiosis as a whole, it is possible that aside from segregation due to spatial separation of genetically different hyphae within a tiller (see above, this section), the colonisation of the tiller may be somehow regulated to safeguard infection by clonal hyphae. A hypothetical model as outlined in Figure 29 may explain the observed defined segregation of hyphae into tillers. In this model, attracted by a factor "x" released locally in the stem of a tiller, endophyte hyphae grow into the direction of a meristematic region within the stem, which later will give rise to a new tiller. As one or very few hyphae have entered the tiller meristem, factor "y" is released which suppresses growth of hyphae from the stem into the newly forming tiller. In this way, control over the number of hyphae colonising the tiller during its early development may be efficiently controlled. However, this model is merely speculative, as at present no more evidence for the proposed regulation of tiller colonisation by plant (and/or fungal)-derived factors has been reported.

Figure 29
Model outlining a hypothetical mechanism by which regulated colonisation of tillers by clonal hyphae could occur. Panel **1** shows a schematic view of a tiller, cut longitudinally,along the direction of the stem axis. Circled areas indicate regions within the tiller where new tillers emerge (emL=emerging leaf, emT=emerging tiller, mL=mature leaf, stA=stem apex). Panel **2** shows the circled stem regions enlarged. In circle **A**, from meristematic activity in an axillary bud a factor "x" is released, attracting hyphae to grow towards this region. Circle **B** shows an axillary bud of a more advanced developmental stage; a hypha has entered this region and a factor "y" is released. In circle **C**, showing further development of an axillary bud, hyphae that have already entered the tiller primordium continue growing, whereas growth of hyphae in the adjacent stem region is suppressed by factor "y".



4.7 PHOTOSYNTHESIS AND PLANT GROWTH IN PERENNIAL RYEGRASS ARE AFFECTED BY INFECTION WITH *N. LOLII*

4.7.1 Rates of Leaf Photosynthesis and Elongation could indicate for an Effect of the Endophyte on the Nitrogen Metabolism of Its Host

It has been previously established that the greater persistence of endophyte-infected grass plants is to some extent due to effects of the endophyte on growth and physiology of its plant host. For assessing the effects on plant physiology a small number of studies has previously addressed photosynthesis in endophyte-infected plants. All of these studies were performed with tall fescue infected by *N. coenophialum* (Section 1.2.2), and so far, only few studies (Latch *et al.*, 1985; Cheplick *et al.*, 1989) have addressed plant growth in response to endophyte-infection in perennial ryegrass infected by *N. lolii*.

In the study presented, plant growth rates were assessed as tillering and leaf extension rates, and in addition, photosynthesis was assessed in response to light and temperature. In experiments, all plants were grown environmentally controlled allowing for comparisons between the experiments. The results of this study have shown that plants infected by *N. lolii* had on average lower net photosynthetic rates in leaves than uninfected plants. This effect was strongest under saturating light intensities, where uninfected plants achieved more than 15 per cent higher levels of net photosynthesis compared with their endophyte-infected counterparts (Section 3.3.2.1), indicating for an effect of the endophyte on leaf-photosynthetic capacity.

Belesky *et al.* (Belesky *et al.*, 1987), investigating tall fescue infected by the endophyte *N. coenophialum,* likewise found that net photosynthesis was lower in endophyte-infected plants. The data of this study also supported an effect of endophyte-infection on photosynthesis under higher light intensities. This suggests that in both symbioses a common mechanism is responsible for the decrease in photosynthesis.

However, in the present study it was found that the difference in photosynthesis between infected and uninfected plants was present only in younger plants (Section

3.3.2.1). Older plants had generally lower rates of photosynthesis and no significant variation was observed between plants differing in infection status (Section 3.3.2.2). The difference in photosynthetic rates between younger and older plants was perhaps caused by variation in tiller density due to plant age. Younger plants consisted in a lower number of tillers, therefore less mutual shading occurred, and more nutrients per tiller were available. Shaded leaves have frequently a lower photosynthetic capacity (= maximum rate of photosynthesis under saturating light) than unshaded leaves, and a lower availability of the plant nutrient nitrogen also reduces the photosynthetic capacity (Robson *et al.*, 1988).

The significant reduction in photosynthetic capacity observed in leaves of younger endophyte-infected perennial ryegrass plants may argue for an effect of *N. lolii* on nitrogen availability or allocation within the plant. Lyons *et al.* (Lyons *et al.*, 1990) reported that concentrations of organic (amino acids) and inorganic (ammonia) nitrogen were higher in leaves of tall fescue grass infected by *N. coenophialum*. Leaf photosynthesis is correlated with leaf nitrogen as nitrogen is comprised of 70 – 80 per cent protein, much of which is part of the photosynthetic apparatus (Ryle *et al.*, 1992). Hence, given that during the experiments endophyte-infected and uninfected plants were fertilised with equal amounts of nitrogen (Section 2.4.4), in infected plants more of this nutrient may have been allocated into non-protein pools.

Cheplick *et al.* (1989) determined that at low nutrient levels infected plants exhibited significantly reduced growth, indicating for a metabolic cost to the host plant due to competition for nutrients (Cheplick *et al.*, 1989). As the endophyte possesses mechanisms by which it could access host nitrogen *via* proteolytic enzymes (Reddy *et al.*, 1996), it is thus probable that significant and measurable competition for this nutrient occurs between plant and endophyte. The presence of the endophyte might place a significant nutritional demand on the plant. For instance, it probably requires appreciable amounts of the amino acid tryptophan for the synthesis of the alkaloids ergovaline and lolitrem B (Section 1.3.1). This amino acid is one of the most expensive, in terms of energy, to produce of the standard amino acids (Radwanski & Last, 1995). Therefore, it is a possibility that the production of the alkaloids in the symbiosis has a direct and

measurable impact on photosynthesis, as this process depends on levels of available nitrogen.

However, other mechanisms leading to a reduction in photosynthetic capacity are also conceivable. An enzyme important for photosynthesis is D-ribulose 1,5-bisphosphate carboxylase-oxygenase (rubisco), as it is a rate-limiting factor in this process (Andrews & Lorimer, 1987; Salvucci, 1989; Woodrow & Berry, 1988). Rubisco content in leaves is often variable and can be influenced, for example, by levels of carbohydrates in leaves. For instance, accumulation of sugars can lead to a decline of rubisco, therefore causing a decrease in leaf photosynthesis (Moore *et al.*, 1998). Scholes *et al.* (1994) demonstrated that barley leaves infected by the powdery mildew fungus had a lower photosynthetic capacity than uninfected leaves. This coincided with a lower amount of rubisco and other enzymes of the Calvin cycle in the powdery mildew-infected leaves (Scholes *et al.*, 1994), which constitute the "dark reactions" of photosynthesis (Mohr & Schopfer, 1992).

The decreasing enzyme activities in leaves infected by powdery mildew were correlated with increasing levels of carbohydrates in leaf tissues, the latter increase being probably due to the activity of an acidic invertase, and presumably produced by the fungus (Scholes *et al.*, 1994). Endophytes also elevate levels of invertases in the plant (Lam *et al.*, 1994), and Richardson *et al.* (Richardson *et al.*, 1992) reported higher concentrations of sugars in endophyte-infected than in uninfected plants. The reduced photosynthetic capacities in endophyte-infected plants found in the present study may therefore indicate that the infection by the endophyte decreases levels of photosynthetic enzymes *via* increasing the levels of soluble carbohydrates in the plant.

Leaf photosynthesis, for instance in the grass tall fescue, is not positively correlated with forage yield (Nelson *et al.*, 1975). This may explain that aside from differences in photosynthesis between endophyte-infected and endophyte-free plants no significant differences where found in plant growth, assessed by tiller number per plant (Section 3.3.1). However, in all plant genotypes the presence of the endophyte slightly reduced the rate of leaf elongation. As photosynthetic capacity, leaf elongation rates are affected by

nitrogen; a lower availability of this plant nutrient leads to a decrease in the rate of leaf extension (Volenec & Nelson, 1984b).

The lower leaf extension rates of endophyte-infected plants may thus also indicate that the *N. lolii*-endophyte has an effect on the nitrogen metabolism of its host that is similar to the proposed effect on the photosynthesis in infected plants. To test this hypothesis, it would be necessary to determine activities of key enzymes in the nitrogen metabolism of perennial ryegrass as previously accomplished for tall fescue infected by *N. coenophialum* (Lyons *et al.*, 1990). In addition, it would be of interest to assess quantities and/or activities of enzymes of the photosynthetic apparatus; as part of this study attempts were made to quantify rubisco (Section 3.3.2.1) and some lower amounts of this enzyme were determined in endophyte-infected leaf blade material. However, since these results were influenced by technical difficulties (Section 3.3.2.1) it may be necessary to confirm this assessment.

Marks and Clay (Marks & Clay, 1996) investigating photosynthesis in the *N. coenophialum*-tall fescue symbiosis reported higher photosynthetic rates of infected than in uninfected plants at higher (>35 °C) temperatures. However, in the present study, no differential response of photosynthesis to temperature between infected and uninfected plants was found (section 3.3.2.2). This disparity may be due to the different fungal and plant species under investigation and/or the different growth conditions prior to the experiments.

In the study by Marks and Clay, plants were grown at higher (> 20 °C) and more variable temperatures in the glasshouse (Marks & Clay, 1996). Plants in the present study were grown at a lower and constant temperature (Section 2.4.3); thus, differences in adaptations of plants to the environment may have occurred. Moreover, the plants used for the experiment in the present study did not show measurable variation in photosynthesis between infected and uninfected plants at moderate temperatures (Section 3.3.2.2). Therefore, as photosynthetic rates at high temperatures are also determined by the activity of enzymes in the photosynthetic apparatus (Mohr & Schopfer, 1992) such as rubisco (see

above, this section), the lack of variation at these temperatures might be due to the similar composition of enzymes in the plants of this growth stage.

In conclusion, the studies concerning plant growth and photosynthesis have shown that the endophyte measurably affects both parameters, but with little effects on the overall plant performance under the chosen environmental conditions. The results suggest that the endophyte impacts on the nitrogen metabolism of its host, but elucidating the underlying mechanisms would require further investigations. For assessing photosynthesis, the plant-developmental status (= age from last potting) is of importance, since differences in photosynthesis between endophyte-infected and endophyte-free plants evidently depend on the plant age.

4.7.2 Altered Regulation of Nitrogen Allocation in Endophyte-Infected Plants may improve Plant Persistence

A possible effect of the endophyte on nitrogen utilisation and allocation in its host deserves attention, since effects on growth performance of the grass may become visible in some environments only. For example, growth of plants in the present study was only slightly and perhaps negatively affected by the endophyte under the chosen environmental conditions (Section 3.3.1). The lower rates of photosynthesis in endophyte-infected plants suggested that less nitrogen had been allocated into the photosynthetic apparatus of the plant, rather, it may have been made available to the endophyte for production of the alkaloids (see above, previous section).

Under the environmental conditions chosen for this study, in many plant tissues of all plant genotypes the alkaloid lolitrem B reached levels (Section 3.2.4.5) that had been determined previously to induce ryegrass staggers in mammalian herbivores (Section 1.2.3). Thus, the higher levels of production of the alkaloids required for protection against herbivores may lead to a measurable alteration of plant growth rates and photosynthesis. It may be possible that the light intensity and/or temperature in the controlled environment

were conducive to production of lolitrem B, at the same time decreasing the photosynthetic capacity of infected plants.

The light intensities in the plant-growth environment were below levels where differences in photosynthesis between infected and uninfected plants became apparent and significant (Sections 2.4.4 and 3.3.2.1). Thus, under these conditions, uninfected plants had invested an excess of nitrogen into the photosynthetic apparatus, as indicated by the higher rates under greater light intensities under the experimental conditions, without receiving an apparent benefit from this investment. By contrast, the infected plants had apparently invested less nitrogen into photosynthesis, and therefore lower rates under saturating light, but had made nitrogen available for alkaloid production by the endophyte, earning higher protection against potential herbivores. Therefore, in a natural environment with conditions similar to the conditions in the growth cabinet, endophyte-infected plants would have a higher competitive edge because of their greater protection against herbivores, likely to occur in this environment.

5.0 SUMMARY AND CONCLUSIONS

This study has demonstrated the usefulness of constitutive GUS expression in an endophyte transformant for determining the metabolic state of the endophyte. In utilising this transformant for *in planta* studies, it was shown that the *N. lolii* endophyte strain Lp 19 infecting different genotypes of perennial ryegrass maintains high and uniform metabolic activities within the grass plant. The finding of a high *in planta* EMS has significant implications for the regulation of alkaloid production; alkaloids were present in all leaf tissues examined and determined under the same plant growth conditions when the endophyte metabolic state was assessed. Thus, in the plant, production of the alkaloids by the fungus is evidently not the result of a metabolic state similar to a stationary phase state in culture.

Moreover, in this work, it was shown that growth of the plant and endophyte appears highly synchronised; the concentration of endophyte mycelium does not further increase with leaf age in mature leaves. Therefore, growth of the endophyte largely ceases in mature leaf tissues, despite the high EMS. This finding is a novelty, since cessation of fungal growth is usually accompanied by a decline in metabolic activity; it indicates that the different levels of the endophyte in different plant tissues and plant genotypes are not due to regulation by the plant *via* the EMS. The mechanism(s) by which endophyte concentration is controlled to certain levels, predetermined by tissue type and plant genotype remain to be established.

Each of the alkaloids followed a distinct pattern of distribution. None of the three alkaloids ergovaline, peramine, and lolitrem B was distributed in exactly the same proportion to metabolically active mycelium within the plant. Differential synthesis and/or degradation and translocation in the plant are therefore important determinants for the observed distributions of the alkaloids. The levels of these compounds are substantial relative to the amount of the endophyte in the plant, probably reaching ten per cent or more of endophyte biomass; the high EMS also found here might be essential for maintaining these levels.

Plant genotypes varied in overall alkaloid content, and this variation often coincided with a corresponding variation in the concentration of the endophyte. This indicated that some of the overall alkaloid concentration is determined by the overall amount of endophyte mycelium in the plant, suggesting some control of the plant genotype on overall amounts of alkaloids *via* regulation of endophyte colonisation. However, some differential production of alkaloids per endophyte mycelium in plant genotypes was also observed that indicated for regulation of synthesis and/or degradation in the mycelium by the plant.

Endophyte-infection had some measurable effects on plant growth and photosynthesis. Infected plants had significantly lower leaf extension rates than uninfected plants. In addition, in infected plants, leaf net photosynthesis under high light intensities was significantly lower than in uninfected plants, which indicated for an impact of the endophyte on photosynthetic capacity. As nitrogen is a strong determinant for leaf extension and also photosynthetic capacity in leaves, these findings suggest an influence of the *N. lolii*-endophyte on the nitrogen metabolism of its host. The effect of the endophyte on photosynthesis was however influenced by plant age, indicating that the latter is an important parameter in determining effects of the endophyte on the physiology of its host. The lower rates in some of the infected plants may indicate for a better adaptation of these to the experimental growth conditions. Further studies will be required to identify the physiological reason(s) of the effect of the endophyte on photosynthesis and leaf elongation.

These previous investigations were made possible by the establishment of a controlled environment for growth of the selected plant-endophyte associations in experiments, as it allowed for obtaining results, especially for the alkaloids, that were reproducible between samplings. The development of a method for quantitative extraction of ergovaline from plant tissue quantities of less than five milligram dry weight was a significant step for defining the alkaloid distribution in a single tiller. The developed method for ergovaline extraction from plant tissue was tested against two published protocols, and was found to have a significantly enhanced sensitivity and reliability.

Some instability of GUS expression in the endophyte was observed in the plant. However, this loss of GUS activity was readily apparent as it affected whole tillers. Defined segregation of GUS-expressing and non-expressing endophyte mycelium into tillers suggests that tillers are colonised by clonal hyphae. Colonisation of tillers by clonal hyphae may be a mechanism by which the quality of the genes and, therefore, fitness is maintained in this asexual fungus. Future studies could address how this defined segregation into tillers might occur and whether the plant or the endophyte significantly regulates it.

In conclusion, the results of these study have answered a number of questions regarding the interactions between fungal and grass symbionts and implications of these interactions for alkaloid synthesis in the agriculturally important symbiosis of the endophytic fungus *Neotyphodium lolii* and perennial ryegrass. The methods developed and described in here, and well-characterised plant-endophyte associations are now available for further studies. In addition, research is now under way to identify genes involved in the production of the alkaloids, which could ultimately be used, for example, with the GUS reporter-gene system to investigate regulation of alkaloid synthesis in the plant in fine detail. These studies could help identifying further interesting and important characteristics and peculiarities in this unique plant-fungus symbiosis.

APPENDIX

Manufacturer's References

List of manufacturers used in this study

Company	Address
Aalborg Instruments	Monsey, NY, USA
Advanced Technology Comp.	Vista, CA, USA
AgResearch Grasslands	Palmerston North, New Zealand
Alpha Innotech Corporation	San Leandro, CA, USA
Alltech Associates	Derfield, IL, USA
BDH	Poole, England, UK
BIO 101 Inc.	La Jolla, CA, USA
Coherent Scientific Pty, Ltd.	Unley, Australia
Difco Laboratories	Detroit, MI, USA
DuPont	Wilmington, Delaware, USA
Eppendorff	Hamburg, Germany
Fuji Film Company	Tokyo, Japan
General Eastern Instr. Corp.	Watertown, MA, USA
Grant Instruments Ltd.	Cambridge, England, UK
Griffiths Engineering	Palmerston North, New Zealand
Hoefer Scientific Instruments	San Francisco, CA, USA
Heraeus Sepatech GmbH.	Osterode, Germany
Jasco Corporation	Tokyo, Japan
Labline Instruments Inc.	Melrose Park, IL, USA
Levingston Bros. Ltd.	Penrose, New Zealand
Leybold Heraeus	Hanau, Germany

List of manufacturers continued:

Michrome	London, England, UK
Mettler	Zürich, Switzerland
Molecular Probes	Eugene, Oregon, USA
MSE Scientific Instruments	Sussex, England, UK
National Instruments Corp.	Austin, Texas, USA
Nikon	Tokyo, Japan
Nunclon	Denmark
Olympus	Tokyo, Japan
Osmocote	Grace Sierra, Australia
Perkin Elmer Corporation	Norwark, CT, USA
Phenomenex	Torrance, CA, USA
Philips	NY, USA
Radiometer Copenhagen	Copenhagen, Denmark
Rhom and Haas	Philadelphia, Pennsylvania, USA
Sarstedt	Nürnbrecht, Germany
Sigma Chemical Corporation	St. Louis, MO, USA
Shimadzu	Kyoto, Japan
Sophora Products Ltd.	New Zealand
Techne Corporation	Cambridge, England, UK
Temperzone Ltd.	Otahuhu, New Zealand
Tropicool	Christchurch, New Zealand
Uniroyal	Middlebury, Connecticut, USA
Valco Instruments	Houston, TX, USA
Waters Associates	Milford, MA, USA
Yates	New Zealand
Zeiss Ikon	Stuttgart, Germany

List of Abbreviations

ANOVA	Analysis of variance
°C	Degree Centigrade
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EMS	Endophyte metabolic state
FW	Fresh weight
g	Gram
GUS	ß-D-glucuronidase
h	Hour
HPLC	High Performance Liquid Chromatography
IAA	Indole acetic acid
IP	Isopropanol (Propan-2-ol)
IRGA	Infrared gas analyser
l	Litre
LA	Lactic acid
m	Metre
M	Molar
min	Minute
MU	4-methyl umbelliferone
MUG	4-methyl umbelliferyl glucuronide
MW	Molecular weight
PDA	Potato dextrose agar
PDB	Potato dextrose broth

List of abbreviations continued

Rubisco	D-ribulose 1,5-bisphosphate carboxylase-oxygenase
s	Second
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
YEG	Yeast extract glucose medium

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