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INTERRELATIONSHIPS BETWEEN PERENNIAL RYEGRASS (*LOLIUM PERENNE*), THE ENDOPHYTIC FUNGUS *ACREMONIUM LOLII* AND BLACK BEETLE (*HETERONYCHUS ARATOR*)

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ABSTRACT

Three broad, but related, categories of research were undertaken in this study. The first involved developing an enzyme-linked immunosorbent assay (ELISA) to quantify *Acremonium lolii* levels in perennial ryegrass. The second was aimed at investigating how concentrations of the alkaloids peramine and lolitrem B (measured using high performance liquid chromatography) and of *A. lolii* (measured using the ELISA), varied with factors such as plant/endophyte genotypes, season, plant component, plant age and plant management. The third was aimed at investigating the effect of endophyte-infection on adult black beetle, and once an effect had been established, the chemical mechanism of the resistance.

1. A quantitative ELISA to *A. lolii* was developed by inoculating rabbits with either the mycelial antigen fraction or the soluble antigen fraction of *A. lolii*. Both antigen types were effective in eliciting immune responses, and it was found that using two different antibodies in the assay, one derived from a rabbit injected with the mycelial antigen, the other derived from a rabbit injected with the soluble antigen, greatly enhanced detection of the endophyte. It was also found that grass samples had to be freeze-dried rather than oven-dried before detection of *A. lolii* was possible.

2. *A. lolii*, peramine and lolitrem B concentrations were lowest in winter. The increase in *A. lolii* content appeared to be correlated with reproductive development of the ryegrass host. Peramine and lolitrem B concentrations in the plants were largely determined by the concentration of *A. lolii*.

Peramine concentrations increased, and lolitrem B concentrations decreased as leaf age decreased. Also, lolitrem B and *A. lolii* concentrations were generally higher in leaf sheaths compared to leaf blades. Peramine was distributed more evenly between the leaf sheath and leaf blade. Compared to all other plant components *A. lolii*, peramine

and lolitrem B concentrations were highest in the seed. *A. lolii* growth in plants which were not regularly trimmed and therefore allowed to become reproductive, was significantly enhanced compared to the growth of *A. lolii* in trimmed plants. The opposite was true of peramine concentrations early in the reproductive phase of ryegrass.

The significance of these results, and those of an experiment investigating the effect of plant age on alkaloid concentration, from the perspectives of the plant, insect pests and livestock are discussed.

3. Adult black beetle was found to be deterred from feeding by the presence of *Acremonium* endophytes in perennial ryegrass. Peramine, lolitrem B and paxilline did not deter adult black beetle from feeding on artificial diet discs. Ergotamine significantly deterred black beetle from feeding on artificial diets at a concentration of 5μg/g or more. Choice tests using ryegrass plants infected with *Acremonium* endophytes producing different alkaloid spectra, confirmed that peramine and lolitrem B were not required for adult black beetle resistance. Ergovaline was also not necessary for adult black beetle resistance. The significance of these results with regard to using endophytic fungi as biocontrol agents of black beetle is discussed.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW.

1.1 INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.), is the predominant pasture grass in New Zealand (Belgrave *et al.* 1990) as it is easy to establish, is highly productive and is highly digestible. However, perennial ryegrass may be infected with the clavicipitaceous endophytic fungus *Acremonium lolii* Latch, Christensen and Samuels. In New Zealand, this fungus is commonly found in many commercial ryegrass cultivars as well as in 'wild type' ryegrass (Neill 1940, Lloyd 1959, Scott 1983). *A. lolii* cannot be transferred from an infected to an uninfected plant contagiously, transmission of the endophyte may only occur via the seed of an infected ryegrass plant (vertical transmission) (Sampson 1935, Neill 1941). Hyphae are intercellular and infected host plants show no external symptoms of infection (Neill 1940). *A. lolii* may however be isolated from its host grass, cultured in artificial media, and reinoculated into another grass plant of the same species (Latch and Christensen 1985). Its presence in seeds of perennial ryegrass was recorded as early as 1903 (Freeman 1903).

Compared to more recently sown pastures (less than 5 years old), it had been observed that long established pastures contained a disproportionately high number of ryegrass plants infected with *A. lolii* (Neill 1940, Lloyd 1959). This indicated that *A. lolii*-infected ryegrass plants were, in some way, at a selective advantage compared to their *A. lolii*-free counterparts. However, the biological significance of *A. lolii* infecting perennial ryegrass remained hidden until the early 1980's at which time two important discoveries, which have had considerable repercussions on New Zealands pastoral

agricultural industry, were made. The first discovery, made by Fletcher and Harvey (1981), established a link between the presence of *A. lolii* in perennial ryegrass and ryegrass staggers, a neurological disorder sometimes seen in livestock grazing ryegrass dominant pastures. For much of this century, ryegrass staggers in livestock has been one of the major management problems encountered by farmers in New Zealand. Although not usually fatal, serious outbreaks can result in severe animal production losses and the condition can make stock management virtually impossible (Mortimer *et al.* 1982). The discovery of its cause suddenly gave ryegrass staggers research the impetus and direction it needed. The most obvious solution to the problem of ryegrass staggers was to eliminate *A. lolii*-infected ryegrass from pastures, as the presence of the endophyte adversely affected the health of the grazing livestock. In the months following the discovery of the cause of ryegrass staggers, research into methods of eliminating *A. lolii* from infected seed and plants gained momentum (Harvey *et al.* 1982, Latch and Christensen 1982).

However, a second discovery soon after, established a link between the presence of *A. lolii* and resistance of perennial ryegrass to Argentine stem weevil (*Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae)) attack, infected plants being more resistant than non-infected plants (Mortimer *et al.* 1982, Prestidge *et al.* 1982). Argentine stem weevil is ranked as New Zealands most important pasture insect pest and infests all 7 million hectares of improved pasture. The total cost to New Zealand as a result of the effects of Argentine stem weevil is estimated at $78-251 million annually reflecting lost production opportunities and costs of renovating damaged pastures (Prestidge *et al.* 1991). Thus, in terms of Argentine stem weevil resistance, the presence of *A. lolii* in perennial ryegrass is agronomically highly desirable as ryegrass persistence is considerably improved.

The dilemma facing many New Zealand (and overseas) farmers therefore, was

whether to sow high endophyte pastures which are resistant to stem weevil but which increase the risk of ryegrass staggers, or to sow low endophyte pastures which are susceptible to stem weevil attack but which will not cause ryegrass staggers. A survey carried out in the central North Island in 1985 indicated that farmers preferred to sow high endophyte seed lines as they considered the advantages of increased ryegrass persistence and production to be more important than ryegrass staggers (Prestidge *et al.* 1985b).

Although *A. lolii* is at the centre of the dilemma, it has been seen as a potentially very effective biocontrol agent of Argentine stem weevil. Naturally occurring biological control agents, where they occur, are receiving increased attention and are often favoured, for several reasons, over their chemical counterparts (Waage and Greathead 1988). Firstly, there is a greater awareness of the importance of maintaining the balance of nature, and how easy it is for mankind to disrupt this. Chemical pesticides often have adverse effects on the environment causing ecosystem disruption, whereas biological control agents usually have little impact on the environment. Secondly, biological control agents exhibit greater specificity to their target organism and have little or no effect on other species. Chemical controls are usually non specific. Thirdly, chemical pesticides provide strong selective pressures favouring resistant mutants which can then dominate the pest population. Fourthly, repeat applications of chemical pesticides are usually necessary whereas biological control agents are usually self replicating and consequently need only be applied once. In this respect, *A. lolii* is well suited as a biocontrol agent as its transmission via the host seed means that only one 'application' is necessary. And lastly, in comparison with biocontrol agents, chemical pesticides are expensive.

However, if *A. lolii* is to be an effective biocontrol agent against Argentine stem weevil, the deleterious effects of the endophyte on livestock must be eliminated, or at

least reduced. This would remove the dilemma faced by the farmer of whether to sow endophyte-infected or endophyte-free seed.

Both ryegrass staggers and Argentine stem weevil resistance have been shown to be caused by several alkaloids which are only present in *A. lolii*-infected ryegrass plants. Gallagher *et al.* (1981, 1982a, b, 1984) found that a group of substituted indole-diterpenes, which were named lolitrems, were the likely cause of the ryegrass staggers syndrome. In particular, lolitrem B was implicated as the major alkaloid. However, Argentine stem weevil resistance was found to be due to an unrelated alkaloid peramine (Rowan and Gaynor 1986). The finding that different alkaloids were responsible for stem weevil resistance and ryegrass staggers indicated an even greater potential of *A. lolii* as a biocontrol agent, as the possibility existed that low or non lolitrem producing endophytes which produced peramine could be found or genetically engineered and introduced into a ryegrass cultivar.

Since the initial discoveries concerning the association between the presence of *A. lolii* and stem weevil resistance, ryegrass staggers, peramine and lolitrem B, the picture has become considerably more complex. For example, the number of different alkaloids found in *A. lolii*-infected perennial ryegrass has increased dramatically. Eight of the nine classes of ergopeptine alkaloids (known for their mammalian toxicity (Mantle 1969)) have been detected (Rowan and Shaw 1987) in addition to paxilline (Weedon and Mantle 1987), an indole-isoprenoid, and many more indole-diterpenes (Miles *et al.* 1992). Also, three more pasture dwelling insect species in New Zealand have been found to be adversely affected by the presence of *A. lolii* in ryegrass (see Prestidge and Ball 1993 for a review), and many more such species have been recorded in the United States (Clay 1989) and Australia (Quigley *et al.* 1993). In many of these cases, the active chemicals have not been determined and there is no guarantee that all insects are affected solely, or at all, by peramine. Also, in addition to suffering from ryegrass

staggers, it has become apparent that livestock may be afflicted by a number of subtle, but adverse, subclinical symptoms when ingesting endophyte-infected ryegrass material. For example sheep grazing endophyte-infected ryegrass show decreased serum prolactin levels and reduced live weight gains (Fletcher and Barrell 1984) as well as increased rectal temperatures and respiration rates (Fletcher 1993).

Other factors which complicate the situation even more and which should be taken into account when considering the agronomic worth of *A. lolii,* include the direct impact of the endophyte on the physiology and morphology, and therefore competitive ability (independent of the insect resistance advantage) of the host plant. Latch *et al.* (1985b) for example found that endophyte-infected ryegrass plants yielded significantly more dry matter than endophyte-free plants. Also, endophyte-infected plants showed significant increases in total leaf area, tiller numbers, and growth of leaves, pseudostems and roots (Latch *et al.* 1985). However, these phytotonic effects do not appear to be consistent, as other studies have shown that the performance of endophyte-free plants is similar (Hume *et al.* 1993), or superior to (Marks *et al.* 1991), endophyte-infected plants. Although uncertain, phytotonic or phytotoxic effects of *A. lolii* infection on the ryegrass host are another possible complication in the *A. lolii* dilemma which should not be ignored.

This introduction has served to illustrate how the many and varied effects on the host plant of the consequences of *A. lolii* infection arise, and are interrelated. Before an endophyte possessing desirable traits can be identified (ie: one which confers insect (and possibly nematode and pathogen) resistance on the host grass, maximises dry matter production under all possible environmental conditions and is non toxic to grazing mammals), the biology of the relationship between the endophyte and the host grass must be understood to the fullest possible extent, as a lack of understanding could have disastrous consequences. There are many aspects of the biology of the association about

which little is known and which require urgent study. Also, it is important to identify any other major invertebrate pasture pests which are adversely effected by *A. lolii* infection and understand the mechanism of the resistance, as resistance mechanisms may vary with each pasture pest. The current study was therefore undertaken to determine:

1. The temporal distribution of *A. lolii*, peramine and lolitrem B concentration (chapter 3).

2. The influence of some abiotic factors (ie temperature, rainfall and photoperiod) on *A. lolii*, peramine and lolitrem B concentration (chapter 3).

3. whether *A. lolii*, peramine and lolitrem B concentration varied in different ryegrass/*A. lolii* associations, and if so, what factors controlled this variation (chapter 3).

4. The effect of plant tissue on *A. lolii*, peramine and lolitrem B concentration (chapters 3, 4 and 5).

5. The effect of plant reproductive state and plant management on concentration and total production of *A. lolii*, peramine and lolitrem B (chapter 4).

6. The effect of plant age on peramine and lolitrem B concentration (chapter 5).

7. whether black beetle (*Heteronychus arator* (F.)) (Coleoptera:Scarabaeidae), a pest of northern North Island pastures, were adversely affected by the presence of *A. lolii* in perennial ryegrass, and if so, what was the mechanism of the resistance (chapter 6).

Although other alkaloids are present in *A. lolii*-infected perennial ryegrass, lolitrem B and peramine are still the most important. Monitoring lolitrem B, peramine and *A. lolii* may also help to answer questions concerning aspects of the nature of the other alkaloids.

Before details of the studies outlined above are given, a literature review is presented. In this review, it is shown that many grass species other than perennial

ryegrass may also be infected with *Acremonium* type endophytes. Like perennial ryegrass, some of these grasses are important pasture species and the endophytes which infect them often have effects on their host similar to those of *A. lolii* on *L. perenne*. They are therefore considered relevant in the context of the present study.

1.2 LITERATURE REVIEW

1.2.1 The evolution and taxonomy of the *Acremonium* endophytes with particular reference to *A. lolii*.

Acremonium-type endophytes were first studied in Europe at the turn of the century when Vogl (1898) revealed the presence of endophytic mycelium in the seeds of symptomless plants of darnel, *Lolium temulentum* (L.). *L. temulentum* was considered toxic, and it was suggested that the presence of this endophyte may be the cause of the toxicosis. Freeman (1903) conducted a detailed study of the endophyte of darnel and also noted the presence of endophyte mycelium in the seeds of *L. perenne*. The first comprehensive study of the endophyte of perennial ryegrass was conducted by McLennan (1920) who concluded that hyphae were often intracellular, and that all plants of *L. perenne* carried the endophyte. Both of these conclusions were later shown to be incorrect by Sampson (1935) and may have arisen through misidentification of the endophyte (Lloyd 1959). Incidentally, McLennan (1920) also concluded that the endophyte of *L. temulentum* was unlikely to be the cause of the darnel toxicosis as the fungus was also present in perennial ryegrass. Subsequent research has shown that the endophytes of *L. perenne* and *L. temulentum* are different and, incredibly, the endophyte of darnel has yet to be identified (Clay 1989).

The *Acremonium*-type endophytes are unusual in that none of them have a sexual stage making their classification difficult. However, Sampson (1933) noted that the endophytes of *L. perenne* and *L. temulentum* were very similar to the asexual stage of the fungus *Epichloe typhina* (Pers.:Fr.) Tul. infecting many species of Gramineae. The sexual stage of *E. typhina* involves the production of the well known conidial stroma in infected host plants. This is commonly referred to as 'choke' disease as stromal development often prevents the exsertion of the inflorescence (Sampson 1933,

Western and Cavett 1959). De Bary (1863) established a connection between mycelium in the host plant and the reproductive ectostromata (teleomorph) of *E. typhina* and Saccardo (1881) described what was thought to be the conidial state (anamorph) of *E. typhina* and named this *Sphacelia typhina* (Sacc.).

Sampson (1933) observed that for a large part of the year, *E. typhina* existed in the anamorphic state as a symptomless intercellular mycelium within the vegetative organs of the host plant. Thus the true endophytic nature of *E. typhina* was revealed. However, Sampson (1933) also noted that the expression of the teleomorph on the host was highly variable. For example, individual plants or even tillers on the same plant varied in the extent to which flowering was prevented. Infected reproductive tillers on some plants sometimes showed no evidence of the teleomorph even though mycelium was found to be present in the pith and seed components. Sampson (1933) termed this 'latent infection' and drew a comparison with the 'nonchoke-inducing' endophytes of darnel and perennial ryegrass. This was also noted by White (1988) who suggested that the symptomless mutualistic state of the *Acremonium*-type endophytes probably evolved from the choke-inducing pathogenic state of *E. typhina*-like ancestors.

A number of nonchoke-inducing *Acremonium*-type endophytes have been described and the close phylogenetic relationship between them and *E. typhina* has been inferred from the morphological similarities of the conidial states *in vitro* and on the common endophytic habit (Neill 1941, Bacon *et al.* 1977, Morgan-Jones and Gams 1982, White 1987, Christensen *et al.* 1991). Serological cross-reactivities (Johnson *et al.* 1985b, Musgrave *et al.* 1986), isozyme patterns (Leuchtmann and Clay 1990) and ribosomal RNA nucleotide sequences (Schardle *et al.* 1991) have confirmed this close phylogenetic relationship. The latter authors also presented evidence showing that in their evolutionary history, several of the *Acremonium*-type endophytes (including the endophyte of perennial ryegrass) have undergone hybridization with *E. typhina*. This

could have arisen through endophyte-infected plants becoming infected with *E. typhina* allowing an exchange of genetic material between the two. The formation of such hybrids would be expected to increase the fitness of the endophyte as it would counter the effects of the accumulation of marginally deleterious mutations. This would also help to explain the lack of a distinct branch separating *E. typhina* from the nonchoke-inducing *Acremonium* species (Leuchtmann and Clay 1990, Schardle *et al.* 1991). Schardle *et al.* (1991) also found no evidence for a coevolutionary interaction between the *Acremonium*-type endophytes and their grass hosts, leading to the conclusion that several non-pathogenic endophytes were separately derived from infections by *E. typhina* ancestors. There is also evidence indicating multiple origins for endophytes of individual grass species (An *et al.* 1992).

Species of *Epichloe* and the related *Acremonium*-type endophytes are classified in the tribe Balansiae of the family Clavicipitaceae of the division Ascomycota (Diehl 1950). Four other morphologically and biologically similar genera, *Atkinsonella*, *Balansia*, *Myriogenospora* and *Balansiopsis,* are also contained in the tribe Balansiae. *Epichloe* and the *Acremonium* endophytes primarily infect cool season turf and forage grasses in the subfamily Pooideae of the family Poaceae, (this commonality in host range further supports the hypothesis that the two groups of fungi are closely related) and surveys of field collections and herbarium specimens has revealed that such infections are both geographically widespread and common (White and Cole 1985a, Lewis and Clements 1986, Latch *et al.* 1987, Saha *et al.* 1987, Shelby and Dalrymple 1987, White 1987, Prestidge and Van de Zijpp 1988, Clay and Leuchtmann 1989, Wilson *et al.* 1991).

By convention, the nonchoke-inducing *Epichloe* related endophytes may only be classified in the Fungi Imperfecti as they have no perfect stage (in this respect, the taxon Deuteromycota is used as a 'class' of the division Ascomycota). Studying *E. typhina*

and the closely related endophyte of tall fescue (*Festuca arundinacea* Schreb.), Morgan-Jones and Gams (1982) observed that the conidiogenous cells of cultured isolates resembled those of the form genus *Acremonium* Link. However, differences in the number of conidia produced indicated that *E. typhina* and the symptomless endophyte were only distantly related to the members of this genus. To accommodate the *Epichloe* related tall fescue endophyte in the genus *Acremonium*, the section *Albo-lanosa* Morgan-Jones and Gams was erected (Morgan-Jones and Gams 1982) and the endophyte was named *Acremonium coenophialum* Morgan-Jones and Gams. This section also contained the anamorph of *E. typhina* which was renamed *Acremonium typhinum* Morgan-Jones and Gams. Latch *et al.* (1984) agreed with this classification and named the endophyte of perennial ryegrass *Acremonium loliae* Latch, Christensen and Samuels. This was later renamed *Acremonium lolii* Latch, Christensen and Samuels (Latch *et al.* 1987). Five additional *Acremonium* endophyte species have been described, *A. chilense* Morgan-Jones and White, *A. chisosum* White and Morgan-Jones, *A. huerfanum* White, Cole and Morgan-Jones, *A. starrii* White and Morgan-Jones and *A. uncinatum* Gams, Petrini and Schmidt (White *et al.* 1987, White and Morgan-Jones 1987a, c, Gams *et al.* 1990, Morgan-Jones and White 1990).

Acremonium endophytes have been largely identified according to the size and shape of the conidia, the length of the conidiogenous cells and the presence or absence of a septum at the base of the conidiogenous cell (Morgan-Jones and Gams 1982, White and Morgan-Jones 1987b), all of which may be observed in culture. However, the present classification of the *Acremonium* endophytes has recently been questioned. Christensen *et al.* (1991) found that endophytes from perennial ryegrass, identified as *A. lolii*, exhibited considerable variation in cultural, morphological and physiological characteristics. The variation was such that some of the isolates could not be accommodated in the taxon *A. lolii*. This was also the case with the endophyte of tall

fescue (Christensen and Latch 1991). A detailed investigation by Christensen *et al.* (1993) of the endophytes of perennial ryegrass, tall fescue and meadow fescue (*Festuca pratensis* Huds.) incorporating isozyme analyses, morphological characters, sensitivity to benomyl *in vitro* and production of alkaloids in the host grass, led to the identification of six taxonomic groupings. Two taxonomic groupings were found in the endophytes isolated from perennial ryegrass, only one of which fitted the current definition of *A. lolii*. Three taxonomic groupings occurred in endophytes from tall fescue, again only one of which fitted the description of *A. coenophialum*. Only one taxonomic grouping, which fitted into the *A. uncinatum* taxon, was found in endophytes isolated from meadow fescue. Christensen *et al.* (1993) concluded that the additional taxonomic groupings which did not fit into the accepted descriptions of *A. lolii* and *A. coenophialum* may deserve recognition at the species level. The diverse nature of *Acremonium* endophytes within a single host grass species has also been reported for a number of other grasses (Leuchtmann and Clay 1990, An *et al.* 1992).

1.2.2 Description of the *A. lolii* mycelium in perennial ryegrass.

Hyphae of *Acremonium* endophytes may be visually observed in leaf sheaths, culm piths and seed sections of infected ryegrass plants by examining stained tissue preparations under a compound microscope (Sampson 1935, Bacon *et al.* 1977, Clark *et al.* 1983, Hignight *et al.* 1993).

Hyphae of *A. lolii* and the *Acremonium* endophytes in general are intercellular, septate and rarely branched (Neill 1940, Latch *et al.* 1984, White 1987). Hyphae are also even in diameter and run parallel to the leaf axis (Neill 1940, Latch *et al.* 1984, White 1987). Although McLennan (1920) reported that the endophyte of perennial ryegrass entered some host cells in the mature plant (and seed), subsequent research showed this to be incorrect (Sampson 1935, 1937, Neill 1940). In vegetative host tissues at least, intracellular hyphae are never produced. It is important not to confuse

hyphae of *A. lolii* with those of the *Gliocladium*-like fungus which also commonly infects perennial ryegrass. The *Gliocladium*-like endophyte has septate, intercellular hyphae, but unlike *A. lolii*, the mycelium is strongly branched, and hyphae ramify throughout the leaf sheath (Latch *et al.* 1984). Hyphae of the *Gliocladium*-like endophyte also stain less strongly and evenly than those of *A. lolii.*

1.2.3 Dissemination of *A. lolii* and viability in host seed.

Seed transmission of endophytes was first observed by Freeman (1903). Although Freeman studied the endophyte of darnel, subsequent work showed *A. lolii* and the vast majority of the *Acremonium* endophytes could only be transmitted through maternal inheritance of infected seed (or by vegetative propagation of infected plants) (Sampson 1935, Neill 1940, Lloyd 1959, Latch and Christensen 1982, Siegel *et al.* 1984a, Clay 1988, White 1988). Transmission of the *Acremonium* endophytes does not occur via the pollen grain (Sampson 1935) and infection of endophyte-free plants by adjacent endophyte-infected plants has not been observed (Sampson 1933, Neill 1940 and 1941, Siegel *et al.* 1984a). Although seed transmission is a very successful strategy for the perpetuation of the endophytes, occasionally seeds from infected maternal parent plants appear to escape infection and on germination develop into endophyte-free plants (Sampson 1935).

Seed transmission of *Acremonium* endophytes is effected by the vegetative proliferation of endophytic mycelium among the cells of the terminal stem meristem of the host plant which gives rise to the inflorescence. The mechanism by which a host embryo becomes infected has been studied in detail on several occasions. There is still some confusion as to the precise sequence of events, as some results appear to contradict others. There is also the possibility that the mechanism and timing of the infection sequence is not the same for all *Acremonium* endophytes.

One of the first detailed studies of seed transmission of endophytes was by

Freeman (1903). In this study, Freeman observed a layer of densely woven hyphae just outside the aleurone layer in the crushed remnants of the nucellus of endophyte-infected darnel seed sections. Freeman (1903) also found an isolated patch of hyphae occurring at the junction of the scutellum and endosperm on the ventral proximal end of the grain, and termed this the 'infection layer'. Freeman (1903) concluded that it was from this layer that infection of the embryo took place. McLennan (1920) also found hyphae to be associated with the aleurone layer and the scutellum, but was unable to demonstrate the existence of an 'infection layer'. However, McLennan (1920) studied the endophyte of perennial ryegrass rather than darnel making a direct comparison invalid. McLennan (1920) also reported that many intracellular hyphae could be seen in the cells of the aleurone layer and scutellum, a claim which was later shown to be incorrect (Neill 1940, Lloyd 1959, Philipson and Christey 1986). McLennan (1920) reported early invasion of the megagametophyte and the presence of the endophyte mainly in the vicinity of the egg apparatus. However, with the advantages of electron microscopy, Philipson and Christy (1986) observed that hyphae were not present in the immature megagametophyte and that invasion of the egg cell occurred later than McLennan (1920) thought. Philipson and Christy (1986) found that hyphae, which are present in the nucellus, gain entry into the mature megagametophyte and then subsequently enter the embryo sac soon after fertilization. During early embryogenesis, hyphae occur on the surface of the embryo and penetrate it at the 'notched' stage. At seed maturity, hyphae occur within the embryo, as well as below the testa, between the cells of the aleurone layer, and between the cells of the scutellum and endosperm. Penetration of the embryo at the 'notched' stage was also observed with *A. coenophialum*. This contradicted the findings of Hinton and Bacon (1985), who failed to observe infection of the embryo even in the early stages of germination and emergence of the coleoptile.

A seed infected with *A. lolii* will, under most circumstances, germinate into an

endophyte-infected seedling. However Neill (1940) found that endophyte viability was lost in seed which had aged 18 months or more. Thus germination of aged endophyte-infected seed resulted in endophyte-free plants. Neill (1941) found that this was also the case with *A. coenophialum*, the viability of which was lost if 12 or more months had elapsed since harvest. Subsequent research has shown that the viability of the endophyte in seed is highly dependant on the conditions under which the seeds are stored. Viability of *A. lolii* and *A. coenophialum* has been shown to remain high when seed is stored at low temperature (-15^{0}C to 5^{0}C), and low relative humidity (Latch and Christensen 1982, Rolston *et al.* 1986, Welty *et al.* 1987), and when the moisture content of the seed is not allowed to exceed 12% (Hare *et al.* 1990). Under such conditions, *A. lolii* viability has been retained in ryegrass seed stored for at least 15 years (Latch 1983). Otherwise, *A. lolii* and *A. coenophialum* viability in seed stored under the usual warehouse conditions of fluctuating temperatures and relative humidity, may be lost within 1-5 years (Latch 1983, Siegel *et al.* 1984b, Rolston *et al.* 1986, Rolston and Latch 1991).

1.2.4 Isolation, maintenance in culture, modification and reinoculation of *A. lolii* and the *Acremonium* endophytes.

Initial attempts to isolate and culture *Acremonium* endophytes were unsuccessful (Freeman 1903, McLennan 1920). Sampson (1937) was the first to isolate and grow the ryegrass endophyte (which presumably was *A. lolii*) but only achieved success using an egg medium. Although Neill (1940) was unable to repeat Sampsons work, he successfully isolated and cultured the ryegrass endophyte by immersing previously sterilized excised leaves or plants in sterile nutritive solution. Neill (1941) also successfully isolated and cultured the endophyte of tall fescue and described an endoconidium stage for the endophyte of perennial ryegrass. Microconidial sporodochia were also observed by Lloyd (1959) who successfully isolated and grew the ryegrass

endophyte in a variety of natural media. Currently, *A. lolii* is commonly isolated from either leaf sheath tissue or seed following the methods of Christensen *et al.* (1991) and Latch and Christensen (1985) respectively. The appearance of *A. lolii* colonies may be variable and is dependant upon the culture medium, the temperature at which the culture is maintained, the age of the colony and the *A. lolii* isolate (Latch *et al.* 1984, Christensen *et al.* 1991). Latch *et al.* (1984) described *A. lolii* on PDA as being tan, hemispherical, convoluted, lacking aerial mycelium and sterile. However, on cornmeal dextrose agar (CMD), conidia were produced but mainly on colonies grown below 15^{0}C. Conidia were ellipsoid and produced singly on long, slender conidiophores. A teleomorphic state has never been observed. Christensen *et al.* (1991) described variants isolated from perennial ryegrass which may deserve the status of a separate species. However, a detailed account of the precise cultural characteristics of *A. lolii* and the other *Acremonium* endophytes is not required in the context of this review and readers are referred to the original publications (Morgan-Jones and Gams 1982, Latch *et al.* 1984, Christensen *et al.* 1991).

Prior to 1985, infection of grasses after inoculation with endophytes was only reported on two occasions. Sampson (1937) described the inoculation of perennial ryegrass seedlings with the 'second' endophyte of ryegrass (probably the *Gliocladium*-like endophyte), and Western and Cavett (1959) infected cut stems of cocksfoot (*Dactylis glomerata* L.) with spores of *E. typhina*. Neill (1941) made many attempts to infect endophyte-free *L. perenne*, *L. italicum* and *L. temulentum* with the endophyte of ryegrass all of which were unsuccessful. The first successful artificial infection of endophyte-free plants with *Acremonium*-type endophytes was achieved by Latch and Christensen (1985). Latch and Christensen (1985) showed that it was possible to infect seedlings of ryegrass, tall fescue and chewings fescue (*Festuca rubra sens. lat.*) with the endophytic fungi originally isolated from each species (*A. lolii*, *A. coenophialum* and *E.*

typhina respectively). They showed that it was also possible to inoculate *A. lolii* and *E. typhina* into tall fescue, and *E. typhina* into perennial ryegrass although this host could not be infected with *A. coenophialum*. Infection could only be achieved by inoculation of seedlings; mature plants could not be artificially infected. The inoculation technique involved placing cultured endophyte mycelium into a slit made at the junction of the mesocotyl and coleoptile of young (9 day old) sterile seedlings. The percentage of seedlings successfully inoculated with the *Acremonium* endophytes varied but was generally low (approximately 10%). Latch and Christensen (1985) also noted that some grass cultivar/endophyte relationships were more easily established than others.

Artificial infection of *A. lolii* into perennial ryegrass was a major breakthrough in endophyte research as endophytes with desirable characteristics could now be inoculated into correspondingly desirable host grasses. However, a limitation of the technique of Latch and Christensen (1985) is that it is not possible to obtain multiple clones of the same plant infected with different endophytes. It is only possible to investigate the individual contributions of host grass and endophyte genotypes in an association if a number of clones of the same plant can be infected with different endophytes. In the case of *A. coenophialum* and tall fescue, this problem was partially overcome when Johnson *et al.* (1986) successfully inoculated endophyte-free calli of tall fescue with *A. coenophialum*. Approximately 17% of the plants regenerated from calli were endophyte-infected. Johnson *et al.* were unable to infect ryegrass calli with *A. lolii*. Kearney *et al.* (1991) then developed a method for infecting tall fescue with *A. coenophialum* via somatic embryos. Like the method of Johnson *et al.* (1986), the method of Kearney *et al.* (1991) involves a callus phase which introduces the possibility of somaclonal variation in the plants. Such variation would reintroduce the possibility of host genotype effects on the association. The longer the callus phase, the greater the likelihood of somaclonal variation. Although Kearney *et al.* (1991) reduced the period of the callus

phase to only one month, and reported no obvious sign of variation within regenerating plants, the possibility of somaclonal variation using callus culture techniques still remains. To overcome this problem, O'Sullivan and Latch (1993) devised a method whereby plantlets derived from single meristems of tall fescue and perennial ryegrass were inoculated. The percentage of ryegrass and tall fescue plantlets successfully infected varied from 0 to 56%. The problem of somaclonal variation is eliminated using this technique as there is no callus phase.

In the case of forage grasses, the primary objective of endophyte research is to maintain insect resistance while eliminating the toxic effects on livestock. This could be achieved by screening a large number of naturally occurring associations, and selecting the desirable few, should they be found. However, naturally occurring endophyte/grass associations with truly desirable characteristics may not exist. This is also a tedious (though not impossible) way of reaching the goal. As a possible short cut to the solution, interest in genetic engineering techniques is increasing. The conditions necessary for producing protoplasts (Leuchtmann and Petrini 1990) and the genetic transformation of endophytic protoplasts from perennial ryegrass (Murray *et al.* 1992, Gurney and Mantle 1993) and tall fescue (Tsai *et al.* 1992) have been described. The endophytes, which were readily transformed, were reintroduced into seedlings of their respective grasses and produced plants which were free of the undesirable toxin (Murray *et al.* 1992). The use of endophytes as vectors to transform grasses opens exciting new possibilities for the introduction of genes conferring a range of agronomic advantages normally associated with endophyte infection.

1.2.5 Ryegrass staggers and other endophyte-related livestock disorders.

Ryegrass staggers has been recognised for many years in New Zealand (Gilruth 1906, Cunningham and Hartley 1959). Although the condition has also been reported in Australia (Aasen *et al.* 1969), Europe (Clegg and Watson 1960, Russell 1975) and the

United States of America (Shaw and Muth 1949), only in New Zealand is ryegrass staggers considered a serious problem. Ryegrass staggers should not be confused with grass staggers and paspalum staggers. Grass staggers may occur in cattle in the spring and is due to a deficiency of magnesium, while paspalum staggers occurs when cattle ingest ergot (*Claviceps paspali*) infected seedheads of *Paspalum dilatatum* (Poir.).

Ryegrass staggers is a condition of temporary tetanic muscle spasm which may occur in sheep, cattle, horses and deer grazing pastures in which perennial ryegrass is dominant (Cunningham and Hartley 1959, Byford 1978). Wapiti deer appear to be particularly susceptible (Mackintosh *et al.* 1982, Brooks and Cahill 1985). In New Zealand ryegrass staggers outbreaks typically occur in the summer-autumn period and are usually associated with the close grazing of swards during, or subsequent to, prolonged warm dry conditions when feed supply is low (Keogh 1973, 1978, Byford 1978, Fletcher *et al.* 1990).

Symptoms of ryegrass staggers usually appear within 7-14 days of ingesting toxic pasture (Mortimer 1978, Fletcher 1982). The clinical symptoms, which appear to differ slightly between species, have been described in detail by Cunningham and Hartley (1959). The symptoms of ryegrass staggers may vary considerably in severity, from slight stiffness of the limbs while walking, to complete tetanic spasm in a prostrate animal. Symptoms usually become visible after a short period of exercise. Sheep affected by ryegrass staggers are often left behind on mustering. Typically the head becomes retracted, the ears droop, and the legs become stiffly extended. The animal walks with fore and hind limbs in unison and, after a total loss of coordination, falls over. After a short period (5-10 seconds) of severe muscular tetanus, the spasm relaxes and the animal walks away apparently unaffected. Calves often mark time vigorously before falling over. Like sheep, symptoms of ryegrass staggers in calves are usually exacerbated by exercise and recovery is rapid. Horses affected by ryegrass staggers

exhibit a reeling drunken gait which in some serious cases may progress to posterior paralysis.

Characteristic of ryegrass staggers is the minimal tissue abnormality, although cerebellar lesions have been noted in affected deer (Mackintosh *et al.* 1982), sheep and cattle (Mason 1968). Focal muscle lesions have also been noted in sheep (Cunningham and Hartley 1959). Ryegrass staggers is not permanent and stock usually recover quickly when rainfall stimulates the production of new grass growth (Cunningham and Hartley 1959, Fletcher 1986). Stock may also be removed from toxic pasture and usually make a full recovery in 1-3 weeks (Mortimer *et al.* 1984).

Mortality from ryegrass staggers is generally low and the condition is more of a nuisance than an economic concern (Cunningham and Hartley 1959). However, during serious outbreaks such as occurs during periods of drought, stock losses can be considerable (Mortimer *et al.* 1982, 1984). Deaths do not usually occur as a direct result of the symptoms of ryegrass staggers; rather mortality is more often a result of indirect effects. An animal in spasm may fall into water and drown or be crushed by other stock during mustering. Animals unable to walk may also die from dehydration or starvation (Hewitt 1983). The economic cost of ryegrass staggers to the individual farmer in ryegrass staggers prone areas may be quite substantial (Everest 1983, Hewitt 1983).

The neuromuscular dysfunction and hypersensitivity, and the near total absence of gross pathological changes, indicated from the outset that ryegrass staggers was probably caused by the action of one or more potent neurotoxic agents, most probably fungal tremorgens (Latch 1972). Not surprisingly therefore, vitamin deficiencies (Cunningham and Hartley 1959) and the plant alkaloid halostachine (Aasen *et al.* 1969) were discounted as possible causes. Aasen *et al.* (1969) were able to induce ryegrass staggers like symptoms when sheep were intravenously injected with perloline, another plant alkaloid. However, symptoms were not induced when perloline was administered

orally. Ergot sclerotia were also eliminated as a possible cause of ryegrass staggers (Gilruth 1906, Cunningham and Hartley 1959, Thornton 1964).

Evidence that ryegrass staggers was caused by the ingestion of tremorgenic mycotoxins began to accumulate in the mid to late 1970's. An investigation of fungi in soil, ryegrass leaves and dead leaf litter producing tremorgenic mycotoxins was undertaken by di Menna *et al.* (1976). These workers found that cultures of tremorgen-producing strains of *Penicillium* produced symptoms similar to ryegrass staggers when fed to sheep. Gallagher *et al.* (1977) also induced ryegrass staggers like symptoms when sheep were administered with the mycotoxins penitrem A, verruculogen, fumitremorgin B and paxilline intravenously. Fungi able to produce tremorgens were found in areas where ryegrass staggers was occurring or had occurred (Gallagher *et al.* 1980). However, known tremorgens were not detected chemically in ryegrass from toxic pastures (Gallagher *et al.* 1977, 1981) and there was no evidence of tremorgen production in the natural habitat (Gallagher *et al.* 1977). Also a field survey by Latch *et al.* (1976) found that fungal populations did not differ significantly between toxic and non toxic pastures. No free living fungus, or group of fungi, could therefore be incriminated as the cause of ryegrass staggers.

The first report investigating the possibility of a connection between the endophyte of ryegrass and ryegrass staggers was by Neill (1941). Chickens, rats, mice and one sheep were fed endophyte-infected ryegrass seed, but no toxic effects were observed. It was also considered by Neill (reported by Cunningham and Hartley 1959) that "a widely distributed fungus such as *A. lolii* could not be responsible for localized outbreaks of ryegrass staggers". Additionally, Neill (1952) concluded that the endophyte of perennial ryegrass had no effect on the palatability of its host. A later study by Cunningham (1958) found that cultures of ryegrass endophyte on rye and wheat grains were not toxic to sparrows and mice. Cunningham (1958) also fed

endophyte-infected ryegrass seed to mice, rats and one sheep. No ryegrass staggers symptoms were observed. Interestingly, Cunningham (1958) also investigated the possibility of whether the endophyte of tall fescue was responsible for the condition known as fescue lameness. Cows were grazed on endophyte-free and endophyte-infected plots of tall fescue, but no lameness occurred in either group. Cunningham (1958) therefore eliminated the tall fescue endophyte as a possible cause of fescue lameness.

Following the work of Neill (1941) and Cunningham (1958) who dismissed the involvement of the ryegrass endophyte in ryegrass staggers, attention in New Zealand focussed on the free living fungi. However, a significant discovery in the United States of America concerning the aetiology of a livestock condition known as fescue toxicosis, brought the *Acremonium*-type endophytes into prominence and provided a clue as to the cause of ryegrass staggers.

In many ways, tall fescue in the United States of America is the equivalent of perennial ryegrass in New Zealand. Tall fescue is a major component of grass pasture systems and the livestock industry in much of the eastern U.S.A. where it is currently grown in over 14 million hectares (Stuedemann and Hoveland 1988). However, livestock feeding on tall fescue dominant pastures may develop fescue toxicosis, a collective name for a group of animal health and performance problems that arise when cattle consume tall fescue. In a landmark discovery, Bacon *et al.* (1977) proposed that *Acremonium coenophialum* was the cause of fescue toxicosis. This was substantiated by Hoveland *et al.* (1980,1983).

Shortly after the link between fescue toxicosis and *A. coenophialum* infection was established, Fletcher and Harvey (1981) independently reported a similar association between ryegrass staggers and *A. lolii* infection in perennial ryegrass. In a replicated cultivar evaluation trial, Fletcher and Harvey (1981) noted that almost all of

the lambs grazing an old perennial ryegrass ecotype developed severe ryegrass staggers, while only a moderate level of ryegrass staggers developed in lambs grazing c.v. Nui perennial ryegrass. No lambs grazing c.v. Ruanui developed ryegrass staggers. After grass tillers from all of the cultivars were examined for endophyte, a correlation was established between the occurrence of ryegrass staggers and the presence of *A. lolii*. However, the results of this trial were complicated by the fact that different ryegrass cultivars were used, introducing the possibility that plant genotype differences were the cause of the ryegrass staggers. Therefore, in a subsequent grazing trial, seed lines of the same cultivar possessing high (more than 90% of seed infected) and low (less than 3% of seed infected) rates of *A. lolii* infection were compared (Mortimer *et al.* 1982, Mortimer and di Menna 1983). 100% of the sheep on the high endophyte plot developed ryegrass staggers, while the incidence in the low endophyte plots was 0%. The results of this trial thus demonstrated conclusively that ryegrass staggers was caused by ingestion of *A. lolii*-infected ryegrass.

Before the involvement of *A. lolii* in ryegrass staggers had even been suggested, Gallagher *et al.* (1981) isolated two new potent neurotoxins, lolitrems A and B, from herbage collected from pastures on which ryegrass staggers had occurred. Studies were undertaken to determine whether these new mycotoxins were indeed the ryegrass staggers causing tremorgens. Mice dosed with the lolitrems showed neurotoxic symptoms similar to ryegrass staggers (Gallagher *et al.* 1981). Also when pellets made from ryegrass seed containing lolitrem B ($C_{42}H_{55}NO_7$) were fed to sheep, several of the animals developed symptoms identical to ryegrass staggers (Gallagher *et al.* 1982a). Sheep fed pellets made from non-toxic seed showed no sign of ryegrass staggers. Following the discovery that *A. lolii* was the probable cause of ryegrass staggers, Gallagher *et al.* (1982a, b) also made the link between the presence of the lolitrems in plant tissue, and the presence of the endophyte. The chemical properties of the known

lolitrems were elucidated (Gallagher *et al.* 1984) and a method of detection using high performance liquid chromatography devised (Gallagher *et al.* 1985).

Analysis of plant material has shown that both *A. lolii* and lolitrem B are concentrated in the basal leaf sheath region of vegetative plant tissues (Musgrave 1984, Gallagher *et al.* 1987, Keogh and Tapper 1993). This distribution of endophyte and tremorgen supports the observation that the risk of developing ryegrass staggers is greater when stock are forced to closely graze ryegrass pastures (Keogh 1973, 1978, Keogh and Clements 1993). The quantity of lolitrem B that a sheep must ingest to develop symptoms of ryegrass staggers has not been accurately defined. However, clinical symptoms of ryegrass staggers have been observed when lolitrem B levels exceeded 2µg/g (Prestidge and Gallagher 1988a, di Menna *et al.* 1992). Results correlating ryegrass staggers occurrence with *A. lolii* content or concentration are conflicting. Mortimer *et al.* (1984) found that hyphal counts of *A. lolii* in the leaf sheath were related to the incidence of ryegrass staggers, while Fletcher (1986) observed that the most severe outbreaks of ryegrass staggers peaked in a trough of endophyte concentration as measured by enzyme-linked immunosorbent assay (ELISA). It is difficult to make a direct comparison between these studies as many of the trial conditions and methods were different. However, the different results highlight the need for further research in this area.

The biosynthetic origin of the lolitrems was unclear for several years. Gallagher *et al.* (1982b) suggested that lolitrem B could be a phytoalexin (a compound produced by the ryegrass in response to being infected by the endophyte). Evidence that the lolitrems were of fungal rather than plant origin appeared when Weedon and Mantle (1987) isolated the related indole-isoprenoid paxilline from cultures of *A. lolii*. These workers also detected paxilline in *A. lolii*-infected seed samples. Recently, lolitrem B has been detected in cultures of *A. lolii* (Penn *et al.* 1993) confirming the fungal origin

of this compound. Miles *et al.* (1992, 1993) have isolated and characterized a number of other indole-diterpenes from *A. lolii*-infected ryegrass, and have proposed the following biosynthetic pathway: Paxilline→ paxitriol→ lolitriol→ lolitrem E→ lolitrem B (→ lolitrem C)→ lolitrem A→ lolitrem D.

With such a large number of related secondary metabolites, it is possible that indole-diterpenes other than lolitrem B could be involved in ryegrass staggers. Miles *et al.* (1992) have tested lolitrem B and a number of their derivatives on mice. Although not closely related to sheep, mice are considered good models and are often used in preference to sheep as only small quantities of toxin are required. Miles *et al.* (1992) found that of the alkaloids tested only two, lolitrem B and paxilline, were tremorgenic when injected intraperitoneally. Although lolitrem B was approximately 5 times more tremorgenic than paxilline, Miles *et al.* (1992) concluded that the potential contribution of paxilline to ryegrass staggers could not be ignored. Lolitriol, -paxitriol and ß-paxitriol were non tremorgenic although toxic effects were observed with all three. Miles *et al.* (1993) have also administered (intravenously) lolitrem B and paxilline to sheep and preliminary results are similar to those obtained for the mice.

Evidence that compounds other than lolitrem B can induce ryegrass staggers has also come from field trials. Fletcher *et al.* (1991, 1993) reported that lambs grazing a lolitrem B free cultivar of endophyte-infected ryegrass still suffered from mild ryegrass staggers. Chemical analyses indicated that paxilline was most strongly correlated with the development of the staggers (Fletcher *et al.* 1993).

Following the discovery of lolitrems A-D, a number of other non indole-diterpene alkaloids were found in *A. lolii*-infected ryegrass plants. These included the pyrollopyrazine alkaloid peramine (Rowan and Gaynor 1986) and 8 ergopeptine alkaloids (Rowan and Shaw 1987, Rowan *et al.* 1990b) of which ergovaline was the most prevalent. None of these alkaloids are thought to be involved in the ryegrass

staggers syndrome, although they may cause other disorders.

Once the association between *A. lolii* and ryegrass staggers was made, subsequent research showed that the presence of the endophyte was also associated with a number of other, more subtle, livestock disorders. Compared to animals grazing endophyte-free ryegrass, live weight gains were reduced in sheep grazing endophyte-infected plots (Fletcher 1983, 1986, Fletcher and Barrell 1984, Fletcher *et al.* 1990, Eerens *et al.* 1992, Fletcher and Sutherland 1993b). Ryegrass staggers itself can affect animal growth rates by impairing the ability of the animal to graze effectively (Fletcher *et al.* 1993, Fletcher and Sutherland 1993b). However, reduced live weight gains have also been observed in the absence of clinical signs of ryegrass staggers (Fletcher 1982, 1983, Fletcher and Barrell 1984, Eerens *et al.* 1992). This situation is somewhat ideal though, as herbage allowances in these experiments were controlled and equal. Fletcher (1986) found that the live weight gain advantage was reversed in summer and autumn when the effects of Argentine stem weevil decreased pasture production in endophyte-free plots.

It is difficult to link live weight changes with one alkaloid. Fletcher and Sutherland (1993b) found that paxilline, lolitrem B, peramine and ergovaline concentrations were all negatively correlated with live weight changes. However, after the dominant endophyte effect was removed, only paxilline was significantly correlated.

Ingestion of *A. lolii* infected ryegrass is also associated with endocrine imbalance in livestock. Plasma testosterone levels in rams (Peterson *et al.* 1978) and bulls (Peterson *et al.* 1984) grazing *A. lolii*-infected perennial ryegrass were reduced compared to stock feeding on *A. lolii*-free ryegrass. Decreases in serum prolactin levels have also been reported in sheep grazing endophyte-infected ryegrass (Fletcher and Barrell 1984). The effects of these hormone imbalances on livestock fertility are not known, although they may be expected to impair reproductive function. There is

evidence that *A. lolii*-infected ryegrass may affect reproduction. Varney *et al.* (1989, 1992) observed a reduction in the reproductive potential of mice fed on a high endophyte diet compared to mice on an endophyte-free diet. However, the situation may be different in grazing mammals. A study by Eerens *et al.* (1992) showed that lambing percentages of ewes were unaffected by the degree of *A. lolii* infection.

Serum prolactin levels which had been suppressed by ingestion of *A. lolii*-infected ryegrass, were restored when the dopamine antagonist metaclopramide was administered (Piper and Fletcher 1990). This suggested that the ergopeptine alkaloids, which are dopamine agonists, were the likely cause of the observed reduction in serum prolactin. Interestingly, Piper and Fletcher (1990) also noted that metaclopramide did not relieve the symptoms of ryegrass staggers, indicating that dopaminergic neurones and the ergopeptines were not involved in the ryegrass staggers syndrome.

Ryegrass infected with *A. lolii* may cause symptoms of heat stress in the stock which graze it. Fletcher (1993) observed that rectal temperatures and respiration rates were generally higher in lambs grazing endophyte-infected cultivars than those grazing an endophyte-free line. Although Fletcher (1993) could not ascribe these effects to one alkaloid, he suggested that the symptoms of heat stress observed in the trial could have been related to the levels of ergovaline and/or paxilline.

Recently, the presence of *A. lolii* in ryegrass has been linked with both fly strike and faecal contamination (Eerens *et al.* 1992, Fletcher and Sutherland 1993a, Pownall *et al.* 1993). Of the alkaloids measured, peramine was most closely correlated with faecal moisture (Fletcher and Sutherland 1993a, Pownall *et al.* 1993).

Although there is no evidence from New Zealand studies, Australian studies have shown that milk production and fat and protein yields in cows grazing pure swards of *A. lolii*-infected perennial ryegrass were lower than in cows grazing *A. lolii*-free ryegrass (Valentine *et al.* 1993). In another Australian study, lambs grazing *A. lolii*-

infected ryegrass had lower survival rates than lambs grazing *A. lolii*-free ryegrass (Foot *et al.* 1988). This effect has not been observed in New Zealand.

A number of strategies for the control of ryegrass staggers and the other *A. lolii* associated disorders have been suggested. However, with the probable exception of ryegrass staggers, it is difficult to define a specific control strategy for the disorders associated with *A. lolii*, as the alkaloids responsible for these disorders, and their dynamics have yet to be conclusively defined. Discussion will therefore be largely restricted to control of ryegrass staggers, about which far more is known. However, many of these control measures will also be effective against the subclinical disorders.

The simplest method of control of animal ill thrift is by control of the endophyte. After the significance of *A. lolii* in the aetiology of ryegrass staggers was established, there was an understandable reaction to remove the endophyte from pastures. Besides storage of seed for prolonged periods, treatment of seed and mature plant tissue with fungicides such as prochloraz, propaconazol and benomyl (Harvey *et al.* 1982, Latch and Christensen 1982), and heat treatment of seed (Nott and Latch 1993), have been used to successfully control the endophyte in small scale studies. However, the treatments were usually harmful, to varying degrees, to the plant, and the fungicides failed to control the endophyte in field trials (Mebalds 1990). The discovery that *A. lolii* also conferred resistance to the ryegrass against Argentine stem weevil attack led to the conclusion that the presence of the endophyte was necessary to ensure the persistence of the ryegrass (Prestidge *et al.* 1985a). Maintaining an intermediate level of endophyte infection is not a viable option as Argentine stem weevil feeding would quickly lead to a predominance of *A. lolii*-infected plants. Some endophyte-free ryegrass plants with resistance/tolerance to Argentine stem weevil have been identified (Easton and Popay 1990), although the extent of this resistance/tolerance has yet to be established.

Grazing management procedures may also be helpful in controlling ryegrass

staggers. Before the significance of *A. lolii* had been established, Keogh (1973, 1978) and Byford (1978) observed that ryegrass staggers was induced in sheep forced to graze the base of ryegrass dominant pastures, particularly during or immediately after prolonged dry periods. These and subsequent observations on factors such as the distribution of alkaloids made when the significance of the endophyte was known, led to the following grazing management recommendations:

1. A rotational grazing management with daily shifts to prevent overgrazing, encourage white clover growth and reduce neurotoxin acquisition (Keogh 1973, 1983, Keogh and Clements 1993).

2. Moving animals with the first clinical signs of ryegrass staggers to non-toxic pastures containing grasses such as cocksfoot and prairie grass, or green feed crops such as brassica and lucerne.

There is evidence that applications of herbicides (Fletcher and Piper 1990), fungicides (Latch *et al.* 1976, Keogh 1978, Fletcher and Piper 1990) and nitrogen (Keogh 1978), may exacerbate the symptoms of ryegrass staggers.

A popular long term strategy for the control of ryegrass staggers is the selection or manipulation of endophytes, which when introduced into suitable hosts, will produce the insect resistance factors but not the mammalian toxins. Selection of endophyte strains with low levels or free of lolitrem B, has already met with some degree of success. Lambs grazing three cultivars inoculated with the non lolitrem B producing 187BB strain suffered no ryegrass staggers, while those grazing plots containing lolitrem B producing associations had serious ryegrass staggers (Fletcher *et al.* 1991). Similar results were obtained with grazing bulls (Cosgrove *et al.* 1993). However, one cultivar (Ruanui) with the 187BB endophyte did cause mild staggers in lambs (Fletcher *et al.* 1991). The staggers were thought to be due to the alkaloid paxilline, a precursor to lolitrem B (Miles *et al.* 1992) which appears to be common to all isolates of

Acremonium in the section *Albo-lanosa* (Penn *et al.* 1993). Penn *et al.* (1993) concluded that to produce a 'safe' endophyte, it may be necessary to block the indole-diterpene pathway before paxilline. Such an endophyte may not occur in natural populations, or if it does, it may be present at such low frequencies that it may not be found. However, the genetic engineering techniques discussed earlier may make this goal attainable.

Breeding ryegrass staggers resistant animals is another possible control option, as there is evidence of heritable variability in susceptibility of sheep to ryegrass staggers (Hewitt 1983, Campbell 1986).

Immunizing stock against the lolitrems and other toxic compounds may also control the adverse effects of *A. lolii* on livestock. To the author's knowledge, no information regarding this aspect has been published.

1.2.6 *A. lolii* mediated resistance of perennial ryegrass to Argentine stem weevil and other invertebrates.

A. lolii confers resistance to perennial ryegrass against a number of insect pasture pests. However, this review will focus primarily on the *A. lolii* mediated resistance of perennial ryegrass against Argentine stem weevil attack for several reasons. Firstly, Argentine stem weevil was the first insect pest to be associated with endophyte-mediated resistance. Secondly, in comparison to any other insect affected by the endophyte, more is known about the relationship between Argentine stem weevil and *A. lolii*. Thirdly, Argentine stem weevil is the most serious pest in New Zealand to be adversely affected by *A. lolii*.

Argentine stem weevil is ranked as the most important pasture insect pest in New Zealand (Prestidge *et al.* 1991). A native of south America, Argentine stem weevil was first observed in New Zealand in 1927 (Marshall 1937). Marshall (1937) incorrectly identified the insect as *Hyperodes griseus* Hustache. The correct identification was later given by Kuschel (1955). Argentine stem weevil was not regarded as a major pasture

pest until the late 1950's and early 60's, when its importance, particularly in ryegrass based pastures was shown in a series of studies in both the South Island (Kelsey 1958) and the North Island (Hoy 1960, May 1961). Most pasture grasses and other gramineae are susceptible to Argentine stem weevil feeding and oviposition (Barker *et al.* 1981).

Argentine stem weevil infests virtually every improved pasture in New Zealand. Pasture damage is most severe during hot, dry summer conditions, as the ability of some cultivars to initiate new tillers and evade the effects of Argentine stem weevil in such an environment is restricted. Adults cause only minor damage to gramineae by foliar feeding (Kelsey 1958). The tiller mining and stem boring habit of the larval stage causes most of the damage to pastures (Kelsey 1958, Pottinger 1961b). Larval Argentine stem weevil mining may eventually destroy the crown causing tiller, or even plant death (Pottinger 1961a, Barker *et al.* 1986). Although up to six eggs per tiller may be laid, only one larva becomes established in each tiller (Pottinger 1961a). One larva may require between 3-8 tillers to complete its development (Pottinger 1961a, b).

Research prior to 1982 indicated that the susceptibility of perennial ryegrass cultivars to Argentine stem weevil was highly variable with many of the results being contradictory (Pottinger 1961a, Kain *et al.* 1977, 1982a, b, Goldson 1982, Gaynor and Hunt 1983). The apparent contradictions were explained when it was revealed that ryegrasses infected with *A. lolii* were resistant to Argentine stem weevil (Mortimer *et al.* 1982, Presidge *et al.* 1982). In a trial designed to investigate ryegrass staggers, Mortimer *et al.* (1982) found that plots sown with low endophyte ryegrass seed (less than 3% of the seed infected with *A. lolii*) contained five times as many Argentine stem weevil adults and suffered far greater pasture damage than plots sown with high endophyte seed (more than 90% of the seed infected with *A. lolii*). Prestidge *et al.* (1982) hypothesised that if *A. lolii* conferred resistance to ryegrass against Argentine stem weevil attack, pastures not protected with insecticide should contain a higher

proportion endophyte-infected plants than insecticide protected pastures. Examining 11 month old ryegrass pastures, Prestidge *et al.* (1982) found that this was indeed the case, explaining the observations of Neill (1941) and Lloyd (1959) that the highest incidence of *A. lolii* infection occurred in established pastures. Prestidge *et al.* (1982) also found that there was a good correlation between endophyte levels and Argentine stem weevil numbers confirming the findings of Mortimer *et al.* (1982).

Barker *et al.* (1983, 1984b), Gaynor *et al.* (1983) and Gaynor and Hunt (1983) showed that in comparison to endophyte-free ryegrass, adult Argentine stem weevil were deterred from feeding and ovipositing on *A. lolii*-infected ryegrass. Barker *et al.* (1984a) also found that larval Argentine stem weevil survival was lower on *A. lolii*-infected ryegrass compared to *A. lolii*-free ryegrass. Barker *et al.* (1984a) suggested that with respect to Argentine stem weevil larvae, the presence of *A. lolii* was associated with antibiosis or feeding deterrency.

Detailed field studies in the Waikato have shown Argentine stem weevil infestations in ryegrass based pastures are primarily determined by the numbers of favourable host plant tillers (carrying capacity) in the sward (Barker *et al.* 1986, 1989a, b). Favourable host plant tillers are defined as *A. lolii*-free ryegrass tillers and suitable volunteer weed grass tillers. Where most perennial ryegrass tillers are infected with *A. lolii* such as occurs in old pastures, and where volunteer weed grasses are scarce, the carrying capacity is low. When such a pasture is resown with *A. lolii*-free seed renewing the favourable resource, adult Argentine stem weevil respond by increased reproductive effort. The resulting increase in the number of larvae and the level of damage then leads to the gradual elimination of endophyte-free plants from the pasture as they succumb to the effects of larval mining. This decrease in the carrying capacity of the pasture leads to a reduction in the Argentine stem weevil population to a stable low level.

The population limit is also influenced by other density dependent factors. Barker *et al.* (1989a, b) showed that fecundity was suppressed by weevil crowding, particularly when favourable host plant resource was limited. Under such conditions, the proportion of weevils that develop flight musculature for dispersal increases at the expense of ovarian development. Additionally, as a result of competitive interactions, fecundity is reduced amongst the reproductive weevils (Barker *et al.* 1989a).

Although lab studies indicated that larval survival was reduced on *A. lolii*-infected ryegrass (Barker *et al.* 1984a, Prestidge and Gallagher 1988b), field studies indicated that population survival (egg to adult) was not influenced by *A. lolii* infection frequency, but was strongly associated with the size of the initial egg population (Prestidge and Van der Zijpp 1985). Also, larvae were not deterred from transferring from *A. lolii*-free to *A. lolii*-infected tillers (Prestidge *et al.* 1986). In fact Prestidge *et al.* (1986) found that Argentine stem weevil larvae transferring from companion grass species such as cocksfoot could inflict considerable damage to *A. lolii*-infected ryegrasses. Prestidge *et al.* (1986) also concluded that the retarded larval growth observed on *A. lolii*-infected plants actually exacerbated damage as larvae would require more *A. lolii*-infected tillers to complete their development, than *A. lolii*-free tillers. The opposite appeared to be the case in a study by Barker *et al.* (1986) who suggested that more tillers were damaged per larva at low endophyte levels. However, irrespective of the numbers of tillers damaged, the survival chances of *A. lolii*-infected ryegrass plants appear to be greater, as stem weevil larvae in *A. lolii*-infected plants did not mine as close to the crown as they did in *A. lolii*-free plants (Prestidge and Gallagher 1988b). Also the unfavourable nature of *A. lolii*-infected tillers may increase searching for more suitable hosts (Prestidge and Gallagher 1985, 1988b), increasing the likelihood of aggressive encounters between larvae (Pottinger 1961a). Increased larval mobility may also increase the risk of larvae succumbing to invertebrate predators and desiccation

(Barker *et al.* 1990). However, concentration of oviposition activity on the favourable endophyte-free host plants effectively increases the number of larvae per unit of infested resource which also increases the frequency of larval combat. Thus *A. lolii*, both alone and in conjunction with density dependent factors, has a considerable influence on the population dynamics of all of the life stages of Argentine stem weevil.

Resistance of *A. lolii*-infected ryegrass to the Argentine stem weevil is due primarily to the reduced feeding and oviposition of the adult stage, although the reduced larval survival may also be important. Several *A. lolii* associated alkaloids have been shown to have activity against Argentine stem weevil. Gaynor *et al.* (1983) were the first to show that an extractable compound in *A. lolii*-infected ryegrass deterred adult Argentine stem weevil from feeding. Although the compound was not identified, Gaynor *et al.* (1983) suggested it had a low molecular weight, and was highly polar and hydrophillic. Thus it was different to the lolitrems. Gaynor *et al.* (1983) were unable to detect the deterrent compound in cultures of *A. lolii*. However, a deterrent compound with the same chemical characteristics was isolated from *A. lolii* cultures by Prestidge *et al.* (1985c), indicating that the compound was of fungal origin rather than a phytoalexin. A bioassay-guided fractionation of extracts from *A. lolii*-infected and *A. lolii*-free ryegrass clones, gave a number of fractions where adult Argentine stem weevil showed less feeding on *A. lolii*-infected extracts (Rowan and Gaynor 1986). A trial performed under more rigorous conditions indicated that only extracts containing the alkaloid peramine ($C_{13}H_{17}N_3O_2$) were significantly deterrent to adult stem weevil (Gaynor and Rowan 1985). Peramine was assigned a structure by Rowan *et al.* (1986). Since peramine and lolitrem B were chemically different, Gaynor and Rowan (1985) suggested that it may be possible to produce a ryegrass resistant to Argentine stem weevil which does not cause ryegrass staggers.

Following the discovery that peramine was the major feeding deterrent against

adult Argentine stem weevil, a number of experiments incorporating peramine and some of the other alkaloids into artificial diets were undertaken to investigate firstly their precise effects on the two feeding stages, and secondly to define the levels at which they were effective. Popay *et al.* (1990) found that in a choice test, peramine deterred adult Argentine stem weevil from feeding at concentrations of less than 1μg/g, although a concentration of 10μg/g was necessary in a no choice test. Dymock *et al.* (1988) and Rowan *et al.* (1990a) found that adult stem weevil were deterred from feeding on diets containing 0.1μg/g. Larval Argentine stem weevil are also sensitive to peramine, although slightly less so than the adult stage. Dymock *et al.* (1988), Popay *et al.* (1990) and Rowan *et al.* (1990a) found that in a choice test, peramine deterred stem weevil larval feeding at 10μg/g, while in a no choice test, fewer larvae fed and pupated on diet containing 2μg/g in comparison with an untreated diet. However, in both studies, the larvae which did feed on the peramine diets developed at the same rate as the larvae on untreated diet indicating that toxicity was not a factor.

Studies incorporating lolitrem B into artificial diets have shown that this compound is neither toxic nor a feeding deterrent to adult stem weevil (Prestidge and Gallagher 1985, Popay *et al.* 1990). However, stem weevil larvae are highly sensitive to lolitrem B. Dymock *et al.* (1989) and Popay *et al.* (1990) found that compared to untreated controls, larvae fed less on diet containing lolitrem B at 2μg/g. Stem weevil larval growth rates were also significantly reduced and there was greater larval mortality on diet containing lolitrem B at 5μg/g (Prestidge and Gallagher 1985, 1988b, Popay *et al.* 1990) and 6μg/g (Dymock *et al.* (1989). Gallagher and Prestidge (1990) also found that the closely related indole-isoprenoid paxilline reduced larval growth rates and survival.

The final group of metabolites which have been shown to have activity against Argentine stem weevil, are the ergopeptine alkaloids. Ergotamine at 0.1μg/g

significantly reduced adult stem weevil feeding, while ergocryptine was effective at 1.0μg/g (Dymock *et al.* 1988). Ergotamine at 10μg/g had no effect on larval development or feeding (Dymock *et al.* 1988). Although studies incorporating alkaloids into artificial diets are useful for investigating the effects of individual alkaloids on insects, their value is limited as they do not account for the possible effects of chemical synergism.

A. lolii-infected perennial ryegrass does not offer absolute resistance to the Argentine stem weevil. Some feeding and oviposition does occur on endophyte-infected plants (Popay and Mainland 1991), and several factors may account for this. Most important perhaps is the amount of endophyte and/or alkaloid in the plant. Prestidge *et al.* (1985a) suggested that there was a minimum density of *A. lolii* mycelium in the ryegrass sheath needed to maintain Argentine stem weevil resistance below which feeding deterrence was reduced. Pilkington (1988) also showed that oviposition level was significantly negatively related with endophyte levels as measured by ELISA. Other factors such as Argentine stem weevil genotype differences (Gaynor *et al.* 1983), host plant genotype differences (Gaynor and Hunt 1983, Prestidge *et al.* 1985a), endophyte genotype differences (Prestidge *et al.* 1985a) and infection by other endophytes (Gaynor *et al.* 1983, Barker 1987) could also elicit varied responses from adult weevils.

The discovery that *A. lolii*-infected ryegrasses are resistant to Argentine stem weevil has revolutionized the control of this pest. Control measures in the past have primarily centred around the use of insecticides (Kelsey 1958, Hoy 1960, May 1961, Pottinger *et al.* 1984). However, in most situations, the costs of applying insecticide for control of pasture pests have been and still are prohibitive (Pottinger 1981, East and Pottinger 1984). Other control strategies have included good pasture management techniques (Batten 1964, Barker *et al.* 1981), selection of resistant perennial ryegrass lines (Goldson 1979, Kain *et al.* 1982a, b) and tolerant pasture grasses (Kelsey 1958,

Goldson 1982). Other potential biological control agents have also been identified (Malone *et al.* 1984, Malone 1987, Barker *et al.* 1989, Goldson *et al.* 1992). An integrated approach incorporating all of the above strategies including *A. lolii* infection of ryegrass, is likely to be the most effective means of controlling Argentine stem weevil (Prestidge *et al.* 1991).

A. lolii infection is known to confer resistance to perennial ryegrass against many other insect species, several of which are present in New Zealand (Table 1.1). Not all of the life stages of some of these insects are equally affected by *A. lolii*. For example, McDonald *et al.* (1993) found that the later instars of *A. infusa*, *T. commodus* and *T. oceanicus* showed no preference for *A. lolii*-free ryegrass. Additionally, sixth instar larvae of *M. convecta* actually preferred *A. lolii*-infected ryegrass over endophyte-free (McDonald *et al.* 1993).

The insects or life stages adversely affected by *A. lolii* in ryegrass, are all foliar feeders. *A. lolii* hyphae and the alkaloids tested to date, are present in far larger quantities in the above ground parts of infected plants compared to the roots (Musgrave 1984, Musgrave and Fletcher 1984, Ball present study). Thus foliar feeding insects are more likely to be affected by the endophyte than soil dwelling root feeding species or insect stages. However, not all foliar feeding insects are adversely affected by the presence of *A. lolii* in perennial ryegrass. Examples of foliar feeding insects which apparently are resistant to the effects of *A. lolii* include the bird cherry-oat aphid (*Rhopalosiphum padi* L.) (Latch *et al.* 1985a), the English grain aphid (*Sitobion avenae* Fab.) (Johnson *et al.* 1985a) and the frit fly (*Oscinella frit*) (Lewis and Clements 1986). There is no evidence to suggest that *A. lolii*-infected ryegrass plants are more resistant to root feeding insects than endophyte-free plants.

Acquired resistance to insect herbivory is a general property of Clavicipitaceous fungal endophyte-infected grasses (Cheplick and Clay 1988, Clay 1989). Argentine

Table 1.1 **Insect species adversely affected by the presence of *A. lolii* in perennial ryegrass.**

Insect Species	Specific Name	Reference
Argentine stem weevil	*Listronotus bonariensis* Kuschel	Prestidge *et al.* (1982)
Black beetle	*Heteronychus arator* F.	Ball & Prestidge (1991)
Pasture mealy bug	*Balanococcus poae* Maskell	Pearson (1988)
Cutworm	*Graphania mutans* Walker	Dymock *et al.* (1988)
Sod webworm	*Crambus* spp.	Funk *et al.* (1983)
Fall armyworm	*Spodoptera frugiperda* Smith	Hardy *et al.* (1985)
Southern armyworm	*S. eridania* Cramer	Ahmad *et al.* (1987)
Common armyworm	*Mythimna convecta* Walker	McDonald *et al.* (1993)
Common cutworm	*Argotis infusa* Boisduval	McDonald *et al.* (1993)
Flour beetle	*Tribolium castaneum* Herbst	Cheplick & Clay (1988)
Bluegrass billbug	*Sphenophorus parvulus* Gyllenhall	Ahmad *et al.* (1986)
Hairy chinch bug	*Blissus leucopterus hirtus* Montandon	Mathias *et al.* (1990)
Locust	*Locusta migratoria* L.	Clements & Lewis (1993)
Black field cricket	*Teleogryllus commodus* Walker	McDonald *et al.* (1993)
Queensland field cricket	*T. oceanicus* Le Guillou	McDonald *et al.* (1993)
House cricket	*Acheta domesticus* L.	Ahmad *et al.* (1985)
Corn leaf aphid	*Rhopalosiphum maidis* Fitch	Johnson *et al.* (1985a)
Yellow sugarcane aphid	*Sipha flava* Forbes	Breen (1993b)
Greenbug	*Schizaphis graminum* Rondani	Siegel *et al.* (1990)

stem weevil resistance is conferred by *A. coenophialum* infection of tall fescue (Barker *et al.* 1983) and *A. starrii* infection of *Bromus anomalus* (Bell and Prestidge 1991). Peramine is produced in both of these associations (Siegel *et al.* 1990). A number of other insects are adversely effected by *A. coenophialum* in tall fescue (see Clay 1989, Latch 1993, West and Gwinn 1993 for reviews). Loline alkaloids (Johnson *et al.* 1985a, Yates *et al.* 1989, Siegel *et al.* 1990, Patterson *et al.* 1991) and ergopeptine alkaloids (Clay and Cheplick 1989) have been implicated as the resistance factors against several of these insects. Unlike *A. lolii* in perennial ryegrass, *A. coenophialum* appears to confer a degree of resistance to tall fescue against some soil dwelling root feeding insects. There is evidence that the Japanese beetle (*Popillia japonica* Newman) and the Southern masked chafer beetle (*Cyclocephala lurida* Arrow) are affected by *A. coenophialum* in tall fescue (Potter *et al.* 1992). Popay *et al.* (1993) also found that weights and survival of third instar grass grub larvae (*Costelytra zealandica* White) were lower on *A. coenophialum*-infected tall fescue compared to endophyte-free tall fescue. This effect is thought to be due to loline alkaloids which are found in endophyte-infected tall fescue roots (Patterson *et al.* 1991).

The effect of endophyte infection on plant parasitic nematodes has been investigated in both perennial ryegrass and tall fescue. Research suggests that *A. lolii*-infected perennial ryegrass is no more resistant against nematodes than *A. lolii*-free ryegrass although not all nematode species have been tested (Yeates and Prestidge 1985, Pedersen *et al.* 1988, Cook *et al.* 1991, Gwinn and Bernard 1993, Prestidge and Ball 1993). This contrasts with the situation in tall fescue where *A. coenophialum*-mediated resistance against both ectoparasitic and endoparasitic nematodes has been recorded (Pedersen *et al.* 1988, West *et al.* 1988, 1990, Kimmons *et al.* 1990, Gwinn and Bernard 1993).

Thus *A. lolii* and many other Clavicipitaceous endophytes confer resistance to

grasses against a wide range of invertebrate pests. The large array of secondary compounds produced in most of these associations is probably largely responsible for the wide ranging effect of endophytes, and indicates their potential usefulness as biocontrol agents.

1.2.7 Suppression of pathogens by *A. lolii* and other *Acremonium* endophytes.

In vitro studies have shown that cultures of *A. lolii* and other *Acremonium* endophytes exhibit antifungal activity. *In vitro* inhibition of fungi was first reported by White and Cole (1985b) who found that cultures of *A. coenophialum* from tall fescue inhibited growth in seven out of the ten fungi tested. White and Cole (1986) also found that culture filtrates of *A. lolii* from perennial ryegrass and an *Acremonium* endophyte from *Festuca versuta* Beal inhibited growth of the fungal pathogen *Rhizoctonia cerealis* van der Hoeven in agar culture. In similar studies, Schmidt (1990) and Siegel and Latch (1991) extended the number of endophytes and grass pathogens examined. As well as *R. cerealis*, *A. lolii* isolates inhibited cultures of *Colletotrichum graminicola* (Ces.) Wilson, *Limonomyces roseipellis* Stalpers *et* Loerakker and *R. zeae* Vorrhees (Siegel and Latch 1991). Isolates of *A. coenophialum*, *A. starrii*, *E. typhina*, *Phialophora*-like sp. and *Gliocladium*-like sp. also showed *In vitro* inhibition of a number of grass pathogens (Siegel and Latch 1991). Additionally, Siegel and Latch (1991) observed that individual isolates of the same species of endophyte, including *A. lolii*, differed in their ability to inhibit the growth of the grass pathogens. This was also noted by Christensen *et al.* (1991) and Christensen and Latch (1991) for *A. lolii* and *A. coenophialum* respectively. Christensen *et al.* (1991) suggested that antibiotic activity was correlated with colony morphology.

Endophyte-mediated inhibition of beneficial and pathogenic fungi and a pathogenic virus has been observed *in vivo*. Chu-Chou *et al.* (1992) found that

populations of potentially beneficial mycorrhizae were lower in *A. coenophialum*-infected tall fescue compared with endophyte-free tall fescue. Gwinn and Gavin (1992) and Blank *et al.* (1993) also showed that loss of tall fescue seedlings to the pathogenic *R. zeae* and *R. solani* Kuhn fungi respectively, were greatest in seed lots with low levels of *A. coenophialum*. Endophytes can reduce the spread of insect transmitted disease indirectly. West *et al.* (1990) showed that in three year old plots of tall fescue, 31% of *A. coenophialum*-infected plants were infected with the barley yellow dwarf virus, while the infection rate in endophyte-free plants was 73%. The bird cherry-oat aphid (*R. padi*) is the main vector of the virus and is deterred from feeding on endophyte-infected tall fescue (Latch *et al.* 1985a) explaining the difference in viral infection rates. No instances of *in vivo* inhibition of fungi, bacteria or viruses by *A. lolii* in perennial ryegrass have been recorded.

1.2.8 Direct influence of *A. lolii* and other *Acremonium* endophytes on host plant performance.

The effect of *A. lolii* on insect resistance and animal performance reflect the beneficial and detrimental aspects respectively of endophyte infection in perennial ryegrass. Another aspect which may affect the agronomic performance of perennial ryegrass (or any other grass which can be infected with an endophyte) is the possible direct influence of *A. lolii* on the plant itself, viz the endophyte may alter the physiology and/or morphology of the host such that dry matter production, competitive ability and tolerance to stress are affected.

Evidence concerning the effect of *A. lolii* on ryegrass yield is conflicting. Neill (1941, 1952) noted that ryegrass yield was not influenced by endophyte infection, while Gaynor and Hunt (1983) and Mortimer and di Menna (1983) found that field plots of *A. lolii*-infected ryegrass produced more biomass than endophyte-free plots, although Argentine stem weevil may have been largely responsible for this effect. Latch *et al.*

(1985b) compared growth of *A. lolii*-infected and free isolines under controlled environment conditions, and found that endophyte-infected clones produced 38% more biomass than endophyte-free clones. Clay (1987) also found that growth of *A. lolii*-infected ryegrass plants was enhanced under controlled conditions, compared to endophyte-free plants. However, the results of Clay (1987) may be confounded by the fact that distinctly different genotypes were used in the comparison. As well as dry matter production, *A. lolii* infection has also been associated with increases in tiller numbers and leaf area (Latch *et al.* 1985b, Clay 1987). However, results from a number of other studies have also indicated that *A. lolii* infection either has no effect (Eerens *et al.* 1993, Hume *et al.* 1993) or actually impairs (Keogh and Lawrence 1987, Marks *et al.* 1991) production of grass matter. The truth concerning the effect of *A. lolii* infection on dry matter production and tillering appears to be a combination of the good, the indifferent and the bad, as suggested by the diverse nature of the observations outlined above. This view is supported by work from Lewis and Clements (1990) who found that one *A. lolii*-infected isoline produced significantly more herbage, another *A. lolii*-infected isoline produced significantly less herbage and the remaining seven *A. lolii*-infected isolines produced the same amount of herbage, as *A. lolii*-free isolines. Also, in another study, biomass production of *A. lolii*-infected and *A. lolii*-free seedlings and adults of perennial ryegrass varied with soil nutrient concentration (Cheplick *et al.* 1989), viz there was a trend for *A. lolii*-infected plants to perform better than *A. lolii*-free plants at higher nutrient levels, but this was reduced at low nutrient levels. Marks and Clay (1990) also found that *A. lolii*-infected ryegrass exhibited greater growth in response to elevated CO_2 levels than *A. lolii*-free plants. Thus, the effect of *A. lolii* infection on perennial ryegrass dry matter yield is probably dependant upon a number of factors, including plant genotype, endophyte genotype and the prevailing environmental conditions.

The effect of endophyte-infection on dry matter production has also been studied in tall fescue. Most of the evidence points to *A. coenophialum* enhancing dry matter production and/or tillering rate (Read and Camp 1986, Belesky *et al.* 1987a, Clay 1987, West *et al.* 1988, Arachevaleta *et al.* 1989, De Battista *et al.* 1990, Hill *et al.* 1990). Although there is some disagreement (Maclean *et al.* 1993), dry matter production and persistence of *A. coenophialum*-infected tall fescue appear to be enhanced still further under conditions of drought stress (Read and Camp 1986, West *et al.* 1988, Arachevaleta *et al.* 1989). A combination of morphological and physiological changes in the host plant appear to enhance the tolerance of *A. coenophialum*-infected tall fescue to drought stress. Morphological changes in endophyte-infected plants include the earlier shedding of old leaves (Arachevaleta *et al.* 1989, Belesky *et al.* 1989a) and the rolling of younger leaves (Arachevaleta *et al.* 1989, Hill *et al.* 1990). Physiological mechanisms of drought tolerance in *A. coenophialum*-infected tall fescue include low stomatal conductances (Belesky *et al.* 1987a) and an enhanced osmoregulatory system (West *et al.* 1989). Enhanced dry matter production and persistence during periods of moisture stress have not been consistently noted in *A. lolii*-infected perennial ryegrass compared to endophyte-free ryegrass (Cheplick *et al.* 1989, Eerens *et al.* 1993, Hume *et al.* 1993). Like *A. lolii*-infected perennial ryegrass, *A. coenophialum*-infected tall fescue dry matter production is only enhanced compared to endophyte-free plants when nutrients are not limited (Arachevaleta *et al.* 1989, Cheplick *et al.* 1989). There is also evidence suggesting that dry matter production of *A. coenophialum*-infected tall fescue varies according to host plant genotype (Belesky *et al.* 1989a, Bates and Joost 1990, Hill *et al.* 1990, Richardson *et al.* 1990).

There is evidence to suggest that due to increased competitiveness, *A. lolii*-infected plants may have an adverse effect on companion plant species. For example, Sutherland and Hogland (1989) and Stevens and Hickey (1990) found that in a summer

dry environment, the white clover content in mixed swards of ryegrass and white clover, was lower when the companion ryegrass was infected with *A. lolii*. Additionally, *A. lolii*-infected ryegrass in comparison to *A. lolii*-free ryegrass significantly lowered white clover seedling survival and stolon growth (Sutherland and Hogland 1989 Stevens and Hickey 1990). However, working in a non-moisture stressed environment, Prestidge *et al.* (1992) found that *A. lolii*-infection did not significantly effect white clover survival, vigour or leaves and stolons per plant when ryegrass tiller densities were similar. It was only when the tiller density of *A. lolii*-free plants decreased due to the effects of Argentine stem weevil that white clover vigour and growth improved. The importance of Argentine stem weevil as a major factor contributing to the release of white clover from competitive pressures from *A. lolii*-free ryegrass plants was also noted by Watson *et al.* (1993).

In the studies by Sutherland and Hogland (1989) and Stevens and Hickey (1990), only 50% of the reduction in white clover was attributed to the extra competitiveness of endophyte-infected ryegrass. Selective grazing of white clover in *A. lolii*-infected plots by sheep was not a factor, suggesting an allelopathic effect of *A. lolii*-infected ryegrass on white clover (Sutherland and Hogland 1989). Using shoot and root extracts of *A. lolii*-infected and *A. lolii*-free ryegrass, endophyte-mediated allelopathic effects on white clover yield were observed by Sutherland and Hoglund (1990). However, both negative and positive endophyte effects on white clover yield were observed depending upon whether ryegrass root or shoot leachates were used, complicating the notion of an allelopathic effect in the field. Also, a study by Prestidge *et al.* (1992) indicated that there were no *A. lolii*-mediated allelopathic effects influencing the perennial ryegrass white clover relationship. Whether allelopathic effects exist in the field or not, excessively competitive grass growth will suppress white clover growth and therefore in time, may limit the nitrogen content and productivity of the sward.

In trials investigating competition between endophyte-infected and free ryegrass, there is evidence that *A. lolii* confers both a competitive advantage (Francis and Baird 1989) and a competitive disadvantage (Marks *et al.* 1991). Marks *et al.* (1991) also found that *A. lolii*-free ryegrass plants performed better than infected plants under interspecific competition although only one cultivar was tested.

The presence of *A. coenophialum* in tall fescue has been shown to enhance host competitive ability (both intra- and inter-specific) relative to endophyte-free plants (Marks *et al.* 1991).

Seed production and germination percentages have also been shown to be influenced by endophyte status. Seed production in both *A. lolii*-infected ryegrass and *A. coenophialum*-infected tall fescue and germination percentages of seeds from such plants, are generally higher compared to seed production and germination percentages in endophyte-free plants and seeds respectively (Clay 1987, Pinkerton *et al.* 1990, Rice *et al.* 1990).

A link between *A. coenophialum* infection and photosynthetic rate has been observed in tall fescue. However, in common with many of the direct endophyte effects on the host plant already discussed, the evidence is conflicting. Belesky *et al.* (1987a) showed that *A. coenophialum*-infected tall fescue had a lower photosynthetic rate than endophyte-free plants, even though dry matter production in infected plants was greater. Work by Richardson *et al.* (1990) partly supported the findings of Belesky *et al.* (1987a) although there were no consistent differences in apparent photosynthesis or gas exchange parameters between *A. coenophialum*-infected and *A. coenophialum*-free tall fescue. Conversely, Richardson *et al.* (1993) found that under water deficit conditions, some genotypes of symbiotic tall fescue plants had greater photosynthesis than endophyte-free plants. However, evidence to date suggests that the impact of endophyte infection on photosynthesis is only minor.

In summary, direct endophyte-mediated effects on host plants have been observed. However, plant genotype differences may influence plant performance parameters to the same or even greater degree than the endophytes do.

1.2.9 General summary.

As research has progressed, the symbiotic association between endophytes and their host plants has come to be regarded more and more as a mutualistic relationship rather than as a parasitic or commensal relationship (Siegel *et al.* 1984a, Clay 1988). Thus there are benefits to both the fungus and the host plant.

Benefits to the fungus:

1. Nutrition: *Acremonium* endophytes subsist on host plant derived nutrients. Being intercellular, endophytes obtain their nutrients from the apoplast.

2. Protection: Being in a relatively constant environment, endophytes are protected to some degree from external influences.

3. Dissemination: *Acremonium* endophytes are seed disseminated, and therefore do not need to expend a large amount of energy in sexual reproduction. Infection rates using the seed dissemination strategy are extremely high, making this a very successful method of propagation.

Benefits to the host grass:

1. Resistance to invertebrate pests: endophyte infection usually confers a degree of resistance to the host grass against attack by a number of insect and sometimes nematode pests.

2. Tolerance to grazing herbivores: Damage to endophyte-infected pasture grasses is reduced as herbivores tend to ingest less grass matter, and do not graze swards as close to the crown.

3. Disease resistance: Endophyte-mediated resistance to pathogens has been observed, although the extent of this effect is still largely unknown.

4. Phytotonic effects: A number of changes in host plant physiology in response to endophyte infection have been observed. These include increased dry matter production and ability to out compete other plants, higher seed production, increased germination percentages and greater tolerance to stress. Phytotonic effects appear to be dependant upon environmental conditions as well as plant and endophyte genotypes.

The best evidence for the grass/endophyte association being mutualistic, comes from observations in the field which show that the level of endophyte infection in an area, increases over time (Neill 1940, Lloyd 1959, Latch and Christensen 1982, Prestidge *et al.* 1982, 1985b, Barker *et al.* 1986, Latch *et al.* 1987, Francis and Baird 1989). Such an increase can only be due to the differential survival of the endophyte-infected plants.

Having reviewed the relevant aspects of *Acremonium* endophyte/host grass associations, the research undertaken in the current study to further investigate this relationship between *A. lolii* and perennial ryegrass will be described.

CHAPTER 2

DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION AND QUANTIFICATION OF *ACREMONIUM LOLII* IN PERENNIAL RYEGRASS.

2.1 INTRODUCTION

2.1.1 Introduction:

In order to answer a number of questions concerning mycelial concentrations of *Acremonium lolii* (ie µg of *A. lolii* mycelium per g (dry weight) of plant tissue) and how these are related to alkaloid concentration, season, host plant tissue type or host plant genetics, an accurate means of determining the endophyte concentration within the perennial ryegrass host is required.

A. lolii is a true endophyte in that it completes its entire life cycle within the perennial ryegrass host with no external sign of infection. Because of this, detection of the fungus is made more difficult. Endophytic fungi can be detected using a wide variety of techniques. Microscopic examination of stained host plant material has undoubtedly been the most popular technique. Using stains such as aniline blue, trypan blue or Rose Bengal, leaf sheaths, seeds and culm piths may be stained to reveal the presence of endophyte (McLennan 1920, Sampson 1933, Neill 1940 and 1941, Lloyd 1959, Bacon *et al.* 1977, Latch and Christensen 1982, Clark *et al.* 1983, Welty *et al.* 1986a, Saha *et al.* 1988, Hignight *et al.* 1993). Endophytes have also been detected using electron microscopy (Hinton and Bacon 1985, Philipson and Christey 1986, Siegel *et al.* 1987), enzyme-linked immunosorbent assay (ELISA) (Johnson *et al.* 1982, Musgrave 1984, Welty *et al.* 1986b, Reddick and Collins 1988, Arese *et al.* 1990), tissue print-immunoblot (Gwinn *et al.* 1991), insect bioassay (Hardy *et al.* 1985, Latch *et al.* 1985a,

Wilson *et al.* 1991), analysis of associated chemicals (Bush *et al.* 1982, Lyons *et al.* 1986, Yates and Powell 1988), *in vitro* assay (Conger and McDaniel 1983, Gwinn *et al.* 1991) and chemical and spectral analysis of chitin content (Roberts *et al.* 1988).

For the simple detection of endophyte, the staining technique is unsurpassed as it is quick, simple, cheap and relatively accurate. However, quantification of the concentration of *A. lolii* is the desired goal of the current study, and of the various means of endophyte detection, only staining, chitin analysis and ELISA (or immunoassays in general) can be used quantitatively (although detection of endophyte-associated alkaloids is quantitative using methods such as high performance liquid chromatography, alkaloid concentrations may be totally unrelated to endophyte concentrations). Quantification of endophyte concentration by measuring the chitin content has been only moderately successful (Roberts *et al.* 1988) and appears not to be a viable option for at least two reasons. Firstly, which ever method is used, it would not be able to differentiate between the chitin of the endophyte in question and that of other fungi. Secondly, there is evidence indicating that there is no direct correlation between the amount of fungal hyphae and the amount of chitin (Mayama *et al.* 1975).

Both the staining and ELISA techniques have been successfully used to quantify *A. lolii* concentrations in perennial ryegrass. Di Menna and Waller (1986) quantified levels of *A. lolii* by counting the number of hyphal strands across the whole breadth of a stained leaf sheath. By dividing the resulting figure by the breadth of the sheath examined, they obtained a value for the number of hyphal strands per mm of leaf sheath. Although they always sampled the outer most sheath associated with a green leaf, and counted the hyphae at a point as close as possible to the point of attachment of the sheath to the tiller to avoid unnecessary variation, their technique has limitations. Firstly, hyphae were only counted in one leaf sheath per tiller; numbers may be quite different in the other sheaths let alone other plant tissues. Secondly, hyphae could only be counted

in the basal sheath region where they could be seen and it would be impossible to count hyphal strands in tissues such as the leaf blade, crown, root or inflorescence as these plant tissues mask the endophyte. Thirdly, counting hyphae is extremely time consuming. Fourthly, variations in the number of hyphae from one tiller to the next on the same plant may be considerable (di Menna pers. comm.). Additionally, hyphal counts are only a semi-quantitative measure of endophyte mycelium, as it is impossible to quote results using physical units of concentration.

In principal, serological techniques such as ELISA, can circumvent all of these problems. Many quantitative ELISA's using polyclonal antisera have been developed against plant viruses (Clark and Adams 1977, McLaughlin *et al.* 1981, Edwards and Cooper 1985). This contrasts with the relatively low number of immunological assays which have been developed for the detection of specific fungi in diseased plants (Halk and De Boer 1985). This may be attributable to the fact that fungal infections of plants are often easily identifiable by eye or by simple light microscopy. More significantly however, it may reflect the difficulties encountered by researchers in raising antisera that are specific enough. Antisera to fungi are notoriously non-specific when tested by immunofluorescence or ELISA (Dewey 1988). They cross react widely with antigens from closely related or even distantly related species, and with host molecules (Johnson *et al.* 1982, Mohan and Ride 1982, Gendloff *et al.* 1983, Kough *et al.* 1983, Musgrave 1984, Aldwell *et al.* 1985, El-Nashaar *et al.* 1986, Gleason *et al.* 1987, Notermans *et al.* 1987, Dewey 1988, Dewey and Braisier 1988, Dewey *et al.* 1989). However, it is often possible to increase the specificity of an assay while lowering background readings. Gerick *et al.* (1987) found that antiserum specificity improved after cross absorption with unrelated fungal species although this did decrease the antibody titre. Methods used to reduce background readings include cross absorbing antisera with kaolin to remove serum lipids which cross-react non-specifically (Mohan 1988), allowing tissue extracts

to remain at 4^{0}C overnight before testing (Musgrave and Fletcher 1986, Mohan 1988, Dewey 1989), the use of tween 20 and antioxidants in extraction buffers (Musgrave and Fletcher 1986), and the development of Protein A sandwich ELISA tests (Reddick and Collins 1988). There are also a number of reports of more specific assays (Gerik *et al.* 1987, Barker and Pitt 1988, Unger and Wolf 1988) and antisera raised against specific fungal molecules have shown that species specificity is possible in polyclonal antisera (Dickerson and Pollard 1982, Svircev *et al.* 1988).

For the purposes of the present study, a high degree of species specificity was not important, as qualitative detection of endophyte was not the aim. The presence of *A. lolii* will already have been determined by staining. The antiserum must however, be specific enough to distinguish between the host grass tissue and its associated microflora, and the endophyte. The only other requirement of the assay is that it should be quantitative.

Quantitative polyclonal based ELISA's have been developed against *A. lolii* in perennial ryegrass (Musgrave 1984, Musgrave and Fletcher 1984) and *A. coenophialum* in tall fescue (Johnson *et al.* 1982, Siegel *et al.* 1984a). Although these antisera were not species specific, they were essentially genus specific and they demonstrated the potential of the ELISA technique as a quantitative tool in endophyte biology.

Although the ELISA developed by Musgrave (1984) was effective in quantifying *A. lolii* in grass samples, many questions regarding various aspects of the relationship between the fungus and its host remain largely unanswered. For example, are alkaloid concentrations in perennial ryegrass simply dictated by endophyte concentrations, or are they independent of this? Are relative endophyte concentrations maintained throughout the year in a particular plant? Is there a seasonal trend in endophyte concentration? How does endophyte concentration vary with plant tissue type and alkaloid content? Only with the aid of a technique such as ELISA is it possible to answer many of these

questions. The aim of chapter 2 therefore was to develop an ELISA which was effective in quantifying *A. lolii* in perennial ryegrass samples. Before discussing how this was achieved, a brief overview of the mammalian immune system is given, after which historical and practical aspects of immunoassays and ELISA's in particular are reviewed.

2.1.2 A brief review of the mammalian immune system:

The following is only a brief review. For a more indepth review of the mammalian immune system, refer to Roitt (1988).

The mammalian immune system is a defence system which protects the individual from certain death by invading infectious agents such as viruses, fungi, bacteria and invertebrate parasites. There are two broad classes of immune response, the humoral antibody response, and the cell mediated response. The humoral antibody response involves the production of antibodies which circulate in the blood stream and bind specifically to foreign antigens which induced their production in the first place. The formation of antibody-antigen complexes enables other factors in the blood to then eliminate the antigen. Cell mediated immune responses involve the mobilization of cells which react directly with the antigen, either killing it, or, as in the case of the humoral antibody response, inducing other cells to destroy the antigen.

The cells responsible for the humoral and cell mediated immune responses are white blood cells called lymphocytes and the two classes of immune response are mediated by two classes of lymphocytes. B cells produce the antibodies used in the humoral response, and T cells are responsible for the cell mediated response. B and T cells are produced in the central lymphoid tissues (liver and bone marrow for B cells and thymus for T cells) and are both derived from pluripotent haemopoietic stem cells. Some of the B and T lymphocytes then migrate to the peripheral lymphoid tissues (lymph nodes, spleen, tonsils and appendix). Most T and B cells continuously recirculate

between the blood and lymph to maximize the probability that an antigen will come into contact with the specific lymphocyte. The enzyme-linked immunosorbent assay uses B cell-produced antibodies rather than T cells to detect a specific antigen. Discussion will therefore focus on B cells and their antibodies.

A significant feature of the immune system is that it can respond to millions of different antigens in a highly specific way. In other words, for virtually every antigen, there is an antibody. This phenomenon used to be explained by the 'instruction hypothesis' which inferred that the shape of an antibody was dictated solely by the shape of the antigen once the two came into contact. This has been replaced by the clonal selection theory which surmises that during development, each lymphocyte is committed to react with a particular antigen before ever having come into contact with it. Once activated by a specific antigen, a B cell will divide into (among other things) many antibody producing cells, each a clone of the original cell and each producing the same antibody; hence the term clonal selection.

Any substance capable of eliciting an immune response may be called an antigen. Most macromolecules, including virtually all proteins and most polysaccharides can serve as antigens. The area of the antigen which combines with the antibody is called the antigenic determinant or epitope while the corresponding area on the antibody is termed the antigen binding site or paratope. Most antigenic substances have an array of different epitopes and will usually stimulate the production of many different antibodies. Even a single epitope will usually activate a number of different B cells. Such responses are termed polyclonal. When only a small number of different B cells are activated, the response is said to be oligoclonal. A monoclonal response occurs when only a single B cell line is activated.

Antibodies themselves do not kill or denature antigens. They become lethal by recruiting the complement proteins, phagocytic cells or killer cells which are also

components of the immune system. Antibodies may however bind to viruses or bacterial toxins preventing them from binding to receptors on their target cells.

After exposure to a particular antigen, a mammal usually develops a lifelong immunity to it. The first contact between a mammal and an antigen results in a primary immune response. This is characterised by a relatively long lag period before an immune response occurs, followed by a short peak, and a rapid decline. Should the mammal come into contact with the same antigen sometime in the future, a secondary immune response is induced. This is characterised by a shorter lag period, a stronger response and a slower decline. The secondary response is also different from the primary in that the principal antibody class produced is IgG as opposed to IgM. IgG antibodies are considerably more useful in ELISA's than IgM antibodies indicating that the immunization and bleeding protocols used to set up an ELISA must be such that the animal is bled during the peak period of the secondary response.

Immunological memory is a result of the production of memory cells. When virgin B cells encounter their specific antigens, some of them multiply to become effector cells which produce the antibodies. Other virgin cells multiply and differentiate into memory cells instead. These do not make a response but can become effector cells during a secondary response. Consequently, memory cells are long lived (months/years) in comparison to effector cells (days/weeks).

Several weak, non-covalent forces, are involved in antibody-antigen interactions. These include hydrophobic interactions, hydrogen bonds, Van der Waals forces and ionic interactions. These forces are only effective if the molecules of the antigen are close enough to allow some of its atoms to fit into complementary areas of the antigen binding site on the antibody. The equilibrium of the equation describing the reaction of an antigen (Ag) with an antibody (Ab) :

$$Ag + Ab \rightleftharpoons AgAb$$

depends upon the concentrations of the antigen and antibody, and the affinity constant, which describes the strength of the interaction between an antigen and its specific antibody. The affinity of an antibody reflects the goodness of fit of an epitope to a single antigen binding site, and is independent of the number of binding sites. The avidity describes the total binding strength of all of the binding sites of an antibody for a multivalent antigen (macromolecules with two or more identical antigenic determinants). The avidity of an antiserum for a particular antigen is an important consideration when developing an ELISA.

Antibodies are mildly glycosylated proteins which collectively are called immunoglobulins (Ig). They are unique proteins in that they can exist in millions of different conformations, each one with a specific and unique paratope. All antibodies produced by one B cell are identical and therefore have the same antigen binding site. A mature effector B cell is called a plasma cell and this may secrete approximately 2000 antibody molecules per second. Antibodies constitute approximately 20% of the total plasma protein by weight.

The simplest antibody molecules are basically Y shaped with two identical antigen binding sites, one at the end of each arm of the Y (Fig. 2.1). Because they are bivalent, antibodies can cross-link antigen molecules into large lattices as long as the antigen molecules each have three or more antigenic determinants. The efficiency of antigen binding and cross-linking is greatly enhanced by the flexible hinge at the base of the arms (Fig. 2.1).

Antibody molecules consist of four polypeptide chains, two identical light (L) chains (25 KDa) and two identical heavy (H) chains (50-70 KDa) (Fig. 2.1). The amino-terminal regions of both L and H chains form the antigen binding sites. Immunoglobulins are broadly classified according to their H chains. Based on this, there are five isotypes, IgA, IgD, IgE, IgG and IgM. Due to the different nature of the tail

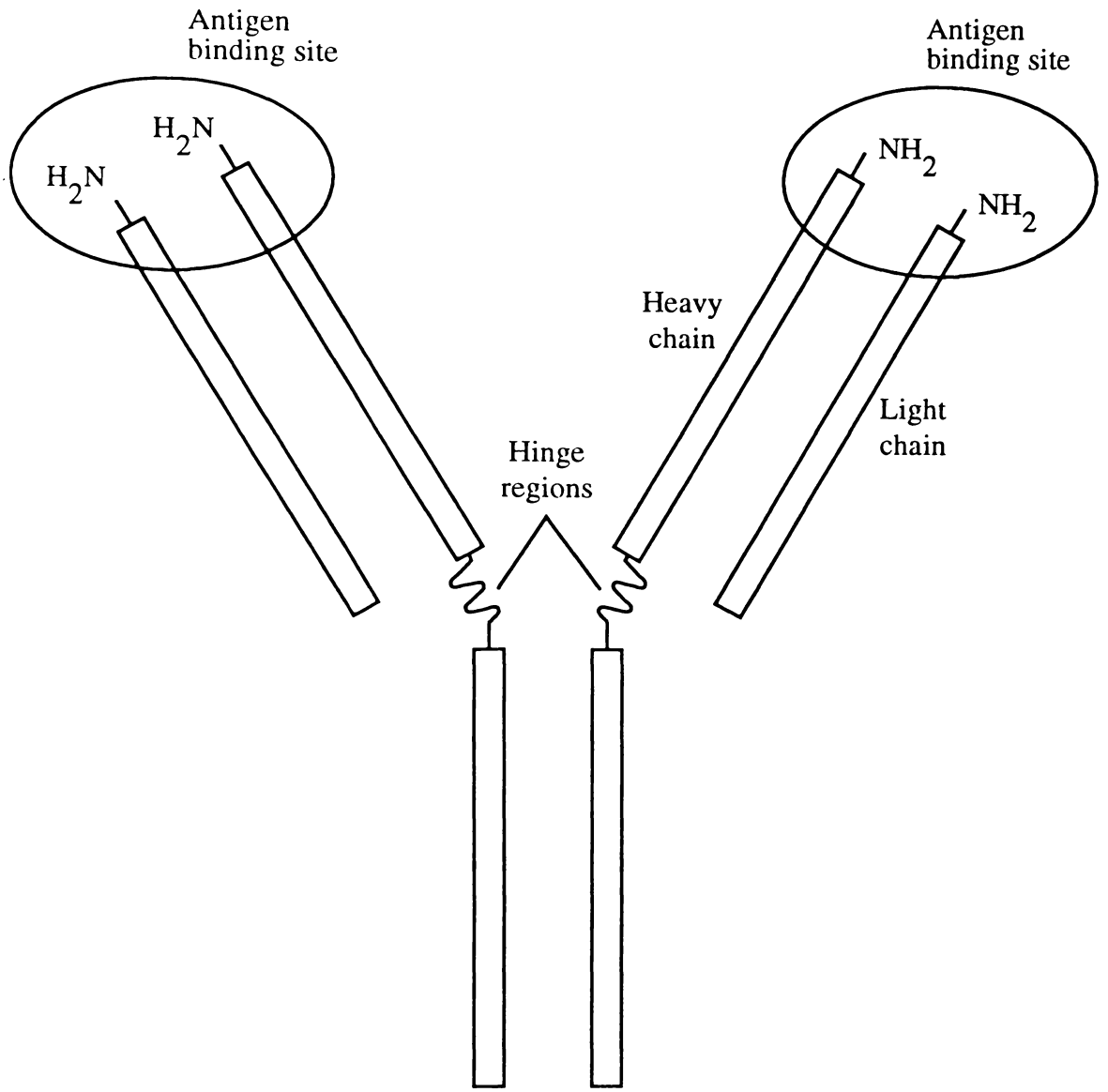


Figure 2.1
Schematic diagram of a typical antibody molecule showing the two heavy chains, the two light chains, the antigen binding sites, and the flexible hinge region.

regions imparted to the antibodies by the different H chains, each immunoglobulin class has characteristic properties of its own. Mammals also have two types of L chain, K and λ, which can associate with any of the H chains. Little needs to be said about the different types of antibody except that IgG constitutes the major class of antibody in the blood and that it is the most common class of antibody used in ELISA's.

The question of how the seemingly infinite number of different antibodies is generated, warrants a little more attention. Both L and H chains have a variable (V) amino acid sequence at their amino-terminal ends and a constant (C) sequence at their carboxyl-terminal ends. Since the amino-terminal ends constitute the antigen binding site, the variability of the amino acid sequence at this point provides the structural diversity of the antigen binding sites. In fact, within the variable domain, only 5-10 amino acids in three so called hypervariable regions in each chain form the antigen binding site. By changing the amino acid sequences in the hypervariable regions only, an enormous number of antibodies with different antigen binding sites can be generated without affecting the overall structure and therefore function of the antibody.

The antibody repertoire of an animal such as a mouse is apparently large enough to ensure that there will be a paratope to fit almost any epitope. In the mouse for example, it is estimated that between 10^6 and 10^9 different antibody molecules are capable of being produced in a single animal. Being proteins, antibodies are coded for by genes; antibody diversity is therefore generated by genetic mechanisms.

Initially, the DNA sequences encoding the V and C regions of an immunoglobulin chain are located on different parts of the genome. During B cell development, the DNA is rearranged (by translocation) so that the V and C sequences are brought together.

For each chain (K light chain, λ light chain and heavy chain) there is a distinct pool of genes (each situated on separate chromosomes) from which a single

immunoglobulin polypeptide chain is made. Each gene pool contains a set of different V genes, and one or more different C genes. In embryonic DNA the V genes are located hundreds of thousands of nucleotides upstream from the C genes. During B cell development, any one of the V genes is translocated so that it lies close to a particular C gene. Only after this has occurred can an immunoglobulin gene be transcribed.

The situation regarding the DNA coding of the V region of the immunoglobulin chain is in fact more complicated than this. In L chains, the V_L region is encoded by two distinct V_L gene segments called V_L and J_L segments. The J_L segment always lies close to the C_L gene sequence and consequently does not need to be translocated. Only the upstream V_L segment is translocated so that it lies beside the J_L segment, and together they make up the V_L sequence. In mice, there are several different J gene segments in each immunoglobulin gene pool. In the λ chain gene pool, there is one J gene segment associated with each C gene, while in both the K and H gene pools there are four. During B cell development, any of the V gene segments can be joined to any of the J gene segments in the K and H gene pools. This joining of various V and J gene segments to create a V region gene is an important factor in generating antibody diversity. Additional variation in the exact site of the V-J join generates more antibody diversity.

In H chains, the V_H region is coded for by three gene segments, a V_H segment, a J_H segment and a D_H segment. In mice there are at least 10 different D_H gene segments. In creating a functional V_H gene, any D_H segment may be joined to any V_H segment which in turn may be joined to any J_H segment, increasing possible antibody diversity still further.

Studies indicate that a mouse inherits several hundred V_K gene segments, a similar number of V_H gene segments, and two V_λ gene segments. Thus by joining the

different V, D and J gene segments in various combinations, a mouse could make at least 10000 different V_H regions and 1000 different V_L regions. By various combinations of the different V_L and V_H chains, 10^7 different antigen binding sites should be possible. Additionally, somatic mutations occurring in and around the V region genes probably increase the number of different antibodies by a factor of at least 10-100.

Now that the origin, structure and properties of antibodies are known, the principles behind the enzyme-linked immunosorbent assay can be discussed.

2.1.3 Development of the enzyme-linked immunosorbent assay: historical and practical perspectives:

All immunoassays are dependant upon the power of discrimination which an antibody has for its homologous antigen. Immunoassays which employ antibody or antigen bound labels are also dependant upon the detectability of the label.

Although it was not known at the time, development of competitive binding assays using radio labelled ligands (Berson *et al.* 1956, Yalow and Berson 1959, Yalow 1978), heralded the first stage in the development of the enzyme-linked immunosorbent assay (ELISA). The disadvantages associated with the use of radio nuclides as immunochemical labels (short half life of the isotopes used limiting reagent shelf life, potential health hazards from the use and disposal of radioactive reagents, the need for expensive detection equipment and the denaturing effect of radioactive disintegrations) probably accelerated the search for suitable alternative labels.

Before the radioimmunoassay (RIA) was developed, antibodies tagged with fluorescent dyes were being used, with considerable success, for the localization of antigens in tissue sections (Coons 1956). However, immunofluorescence techniques also had their drawbacks. Samples tended to fade with time and naturally occurring

autofluorescence could obscure the specific fluorescence (Nakane and Pierce 1967). There was also the trouble and expense of employing dark field ultraviolet microscopy. In circumventing these problems for immunohistochemical applications, Nakane and Pierce (1966) conjugated the antibodies to enzymes rather than fluorescent dyes. Addition of the correct substrate resulted in the formation of a coloured product which permanently stained preparations. These could then be viewed under the light microscope.

At approximately the same time, Wide and Porath (1966) found that antibodies which were bound to an insoluble polymer (cellulose) retained their antigen binding capacity and could be used in a competitive form of RIA. This made separating bound and unbound radioactively labelled antigen components much easier. Catt and Tregear (1967) went one step further by using tubes as a solid phase, to which antibodies were adsorbed and in which the entire assay took place. Miles and Hales (1968) then described the use of isotope labelled antibodies in a non-competitive RIA, and suggested that enzymes or coenzymes could replace the isotopic label.

By replacing radioactive labels with enzymes, Engvall and Perlmann (1971, 1972) and Van Weeman and Schuurs (1971, 1972) were the first to use antigen-enzyme and antibody-enzyme conjugates in an immunoassay. The enzyme labels were easily detectable by addition of a non chromogenic substrate which the enzyme converted into a highly chromogenic product. The concentration of the product as measured by spectrophotometry was found to be proportional to the amount of enzyme, and therefore antigen, bound to the solid phase (Scharpe *et al.* 1976). Like Wide and Porath (1966), Engvall and Perlmann (1971), (who coined the term enzyme-linked immunosorbent assay, 'ELISA' for solid phase enzyme immunoassays) and Van Weeman and Schuurs (1971, 1972) used cellulose as the solid phase. Subsequently, Engvall *et al.* (1971) and Engvall and Perlmann (1972) adapted the method of Catt and Tregear (1967) for the

ELISA and used polystyrene tubes as both the solid phase and the reaction vessel.

Although this method was very successful, it was wasteful of valuable reagents and did not lend itself to large scale use. These problems were overcome when Voller *et al.* (1974) introduced the now popular microplate method of ELISA. Using the microplate method, Voller *et al.* (1976b) developed the 'double antibody sandwich' ELISA which is a variant of the 'indirect' assay used by Engvall and Perlmann (1972). In the indirect ELISA, antigen adsorbed onto the solid phase is used to capture sample antibody which is then detected using an enzyme conjugated anti-IgG antibody (Fig. 2.2.2). The double antibody sandwich ELISA differs in that antibody, rather than antigen, is adsorbed onto the solid phase. Following incubation with the sample, antigens captured by the immobilized antibody are then detected using enzyme conjugated antibodies (Fig. 2.2.3).

Voller *et al.* (1976a) described the first ever successful application of the sandwich assay for the detection of infectious agents (two plant viruses). Clark and Adams (1977) described some of the characteristics of this method, and many of their procedures are currently used in assays.

ELISA has many advantages over other serodiagnostic techniques. It is highly sensitive and quantitatively precise and a large number of samples can be analyzed in a short space of time. It is also amenable to automation and kit development. Additionally, reagents are inexpensive, nonhazardous and have a long shelf life. Finally, the equipment necessary to perform a basic ELISA is simple. Although it was a contentious area, it is now generally accepted that ELISA and RIA techniques have similar sensitivities (Engvall and Perlmann 1971 and 1972, Maggio 1980, Nakamura 1992).

2.1.4 A review of solid-phase enzyme-immunoassays:

The term solid phase enzyme-immunoassay stems from the fact that either the antigen in question, or the antibody used to detect the antigen, is immobilized onto a

solid phase for the duration of the assay. The solid phase enzyme-immunoassay is also known as the heterogeneous enzyme-immunoassay or the enzyme-linked immunosorbent assay (ELISA).

The most commonly used solid phases are plastics such as polystyrene and pvc. Solid phases should have a high capacity for binding immunoreactants and they should be able to bind a wide variety of immunoreactants. They should also cause minimal denaturation of the immobilized molecules and they should enable the immobilized antibodies to bind in such a way that the antigen binding sites are orientated towards the solution rather than the solid phase. Plastic solid phases do not have all of these attributes. Plastic surfaces have a limited capacity of adsorption (Kemeny and Challacombe 1988) and, because immobilized molecules are physically adsorbed (probably as a result of hydrophobic interactions between the nonpolar proteins and nonpolar plastic matrix (Standefer 1985)) rather than covalently bound to the plate, loss of protein by desorption during the assay will occur. Adsorbed antibodies also undergo a degree of denaturation when adsorbed to plastic with a consequent loss of antigen binding activity. However, the ease with which bound antigen-antibody complexes can be separated from free components, far outweigh these drawbacks (Clark and Engvall 1980).

Another important consideration in enzyme-immunoassays is the enzyme itself. Enzymes have considerable catalytic power. One molecule of enzyme may transform 10^3-10^4 molecules of substrate to product every second (Guesdon 1988). Thus by repeating the catalytic event again and again, enzymes in immunoassays act like amplifiers and are easily detected.

Enzymes used in immunoassays should have a high turnover rate, a pH optimum which is compatible with good ligand-antibody binding, an easily detectable product, long term stability, a high retention of activity after coupling, be cheap and

easily available. The enzyme used should also not be present in the sample itself as this would lead to catalytic breakdown of the substrate and could result in false positive readings.

An ideal enzyme-antibody conjugation should yield 100% conjugate. The presence of any unconjugated antibody will lead to a reduction in assay sensitivity as labelled antibodies will be unable to bind to sites already occupied by unlabelled antibodies. While the conjugate should be stable, this must be achieved without a reduction in enzyme or antibody activity. None of the methods currently available satisfy these requirements completely. Covalent linkage of an enzyme to an antibody can result in the partial blocking of the antibody paratope, or the active site of the enzyme, by steric hinderance (Avrameas *et al.* 1978). Thus enzyme-antibody conjugates usually have less activity than their separate parent molecules.

Until recently, radioisotopic labels were the most widely used type of label. Enzyme labels are now the most popular, and of these, horseradish peroxidase, alkaline phosphatase and ß-D-galactosidase are the most commonly used (Gould and Marks 1988, Gosling 1990). The enzyme labels used in enzyme immunoassays and procedures for their conjugation to macromolecules are reviewed by Tijssen (1985), Gould and Marks (1988) and Ishikawa *et al.* (1988). However, a brief review of the use of alkaline phosphatase will now be given as this was the enzyme used in the current study.

Alkaline phosphatase was chosen for several reasons. Firstly, it has proven its worth in the many successful enzyme immunoassays in which it has been used (Clark and Adams 1977, Musgrave 1984). Secondly, alkaline phosphatase is found primarily in animals and microorganisms. It is therefore absent from tissues of higher plants, eliminating possible endogenous enzyme effects in the perennial ryegrass samples (Tijssen 1985). Thirdly, alkaline phosphatase conjugates are easily prepared and are very stable (Tijssen 1985).

The glutaraldehyde method, pioneered by Avrameas (1969), is the most suitable method for cross-linking alkaline phosphatase to antibodies. Glutaraldehyde induced cross-links are irreversible and are predominantly a result of the modification of lysine residues (Avrameas and Ternynck 1969). However, this method results in a heterogeneous mixture of products (Avrameas 1969), many of which are high molecular weight aggregates (Kabakoff 1980). Excessive self-linking of components may reduce the sensitivity of the assay (Clark and Engvall 1980). This lack of control during the conjugation phase is one of the few drawbacks of enzyme labels. The substrate most often used with alkaline phosphatase is the relatively non-chromatic para-nitrophenyl phosphate which is hydrolysed to the highly chromatic para-nitrophenol (Clark and Engvall 1980, Tijssen 1985). Product (and therefore sample) concentration may then be determined by measuring the extinction at 405nm using a spectrophotometer.

The performance of an enzyme immunoassay may be defined using the following criteria: accuracy, specificity, precision and sensitivity (Feldkamp 1992). The accuracy is the property of an assay to give the 'correct' answer without interference from factors such as cross reacting molecules, non specific conditions (lipemia), or colour-producing molecules. Specificity is largely a function of the antibodies present in an antiserum and it describes the ability of an antiserum to discriminate between homologous antigens and all other antigens. Precision is the property of an assay to give the same result when repeated. Feldkamp (1992) describes sensitivity as the least amount of analyte that can be consistently detected, which also takes into account the slope of the standard curve near zero. However, Tijssen (1985) makes a strong distinction between the sensitivity (the slope) and the detectability (the least amount of analyte which can be detected) of an assay and this distinction will be observed in this thesis.

Enzyme immunoassays are broadly classified as being heterogeneous or

homogeneous. In the context of the current research, the homogeneous assay (reviewed by Tijssen 1985) is irrelevant and will therefore not be discussed. Unlike homogeneous assays, heterogeneous assays require the use of a solid phase for the immobilization of the participating antibodies or antigens. For this reason heterogeneous assays are also termed solid-phase enzyme immunoassays or enzyme-linked immunosorbent assays (ELISA). Heterogeneous assays are further classified as being competitive or non-competitive. Although competitive assays (reviewed by Tijssen 1985) are generally simpler to develop than non-competitive assays, they are potentially less sensitive and are more likely to be affected by the presence of sample interferants (Gould and Marks 1988). In non-competitive assays, which are also known as immuno-enzymometric assays, the antigen or antibody to be measured is allowed to react with an excess of immune reactant. These assays may be classified according to which immunoreactant is immobilized and/or the valency of the antigen in question. Three broad classes of non-competitive ELISA are recognised, direct ELISA with antigen immobilized on solid phase, indirect ELISA with antigen immobilized on solid phase, and the double antibody sandwich ELISA (DAS ELISA) with antibody immobilized on solid phase. In order to increase sensitivity or specificity, many variations on these central themes have been developed (Flegg and Clark 1979, Shamsuddin and Harris 1983, Edwards and Cooper 1985, Torrance 1987, Ben-Ze'ev *et al.* 1988, Reddick and Collins 1988).

Direct ELISA with antigen immobilized on solid phase:

The principles of this method are illustrated in Figure 2.2.1. The antigen is immobilized on the solid phase and is reacted directly with the enzyme labelled antibody. The enzyme is then detected by reaction with a colourless substrate which is converted into a highly chromatic product. The colour reaction can be assessed visually or more accurately by using a spectrophotometer. This assay is primarily used for qualitative rather than quantitative purposes (Tijssen 1985). Antigens may be univalent or

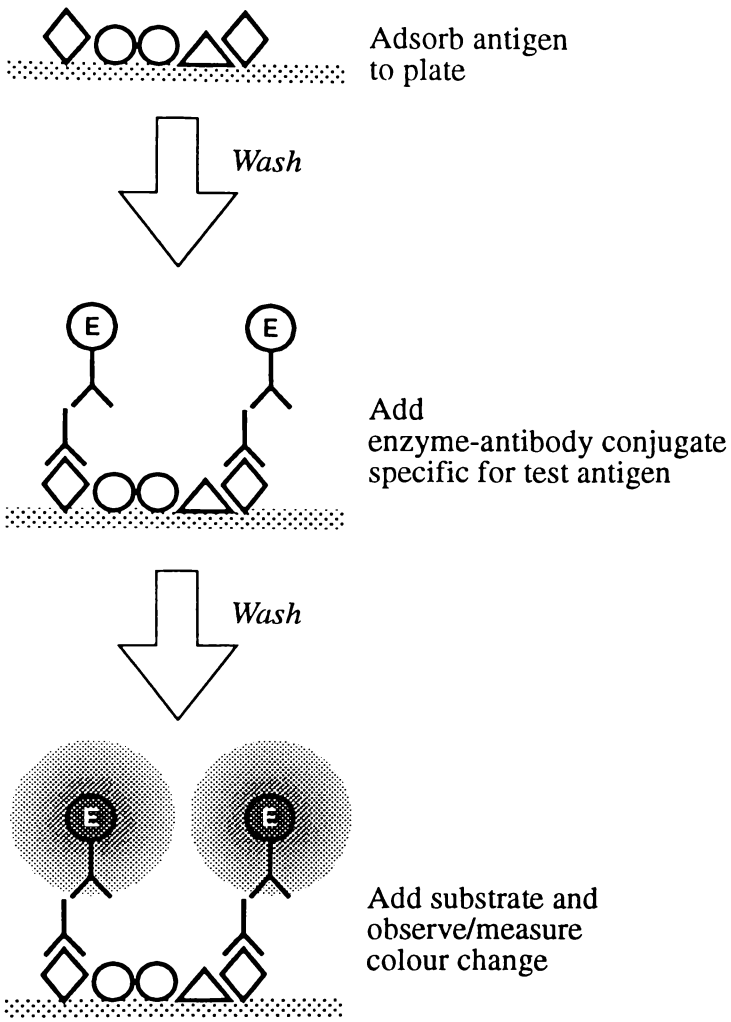


Figure 2.2.1
Schematic diagram of a direct ELISA for measuring antigen

polyvalent.

Indirect ELISA with antigen immobilized on solid phase:

This assay was first described by Voller *et al.* (1974) and is widely used for the determination of antibody activity. The immobilized antigen captures the test antibody, which itself is used to capture enzyme-labelled anti-IgG antibodies. Enzyme label is then revealed as described for the direct assay (Fig. 2.2.2). The limit of detection of this assay is generally superior to the direct method (Crook and Payne 1980, Tijssen 1985). Once again, antigens may be univalent or polyvalent.

Double antibody sandwich (DAS) ELISA with antibody immobilized on solid phase:

This method is widely used for the quantification of antigens in test samples and was first described by Voller *et al.* (1976a and b). Immobilized antibody is used to capture test antigens. After incubation with enzyme-labelled antibody specific to the antigen, enzyme activity is revealed by the addition of substrate (Fig. 2.2.3). The only prerequisite of the sandwich method is that the antigen must be bivalent or polyvalent. Using DAS ELISA, antibody binding must occur at two sites (epitopes) on the antigen; univalent antigens would therefore be undetectable. Of the three basic ELISA methods, the DAS ELISA has been shown to have the greatest specificity (Crook and Payne 1980, Murphy and D'Arcy 1991).

In any type of ELISA, the conditions under which each incubation step is performed, are often critical (Kemeny and Challacombe 1988). Optimum incubation times, incubation temperatures and reagent concentrations may differ considerably between different assays. Optimum conditions should therefore be determined separately for each assay (Clark and Adams 1977, Feldkamp 1992). The washing steps between each incubation step are also critical. Insufficient washing may lead to contamination in the next incubation step, this is particularly important after the conjugate incubation as

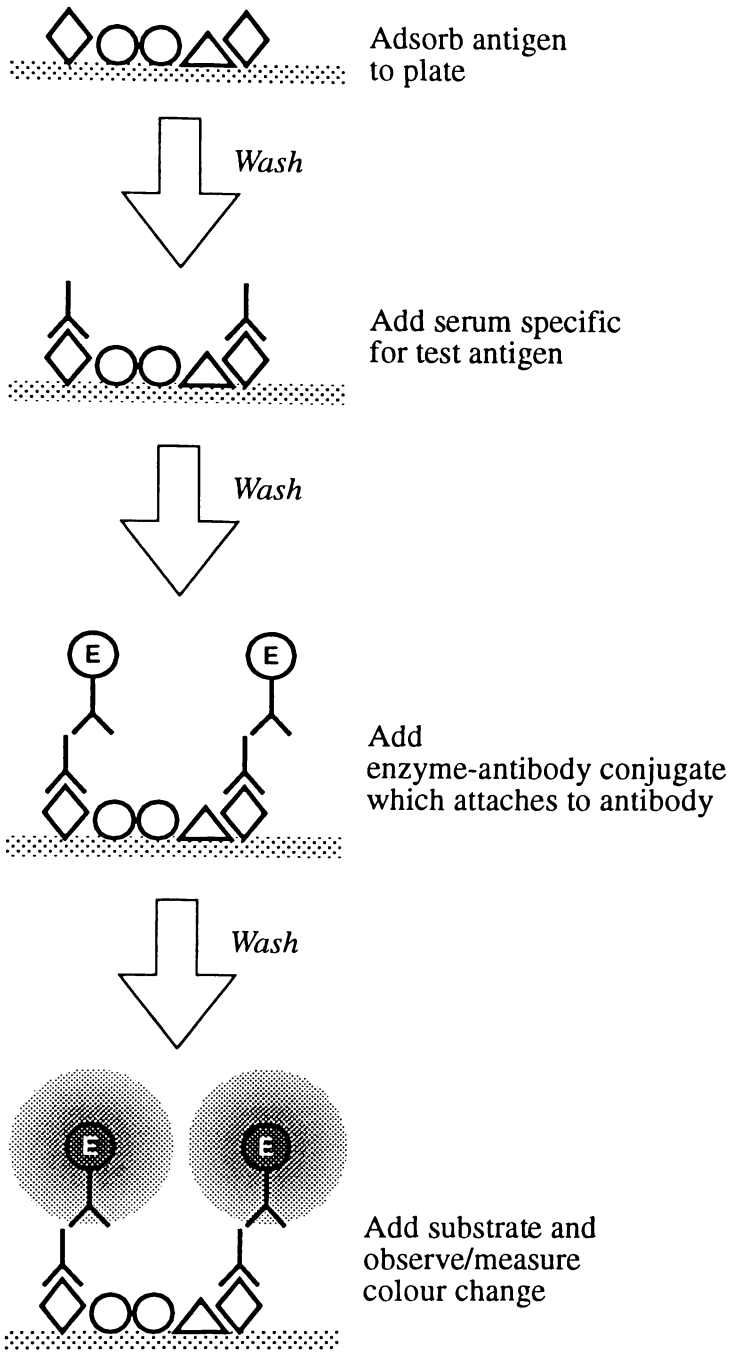


Figure 2.2.2
Schematic diagram of an indirect ELISA for measuring antibody

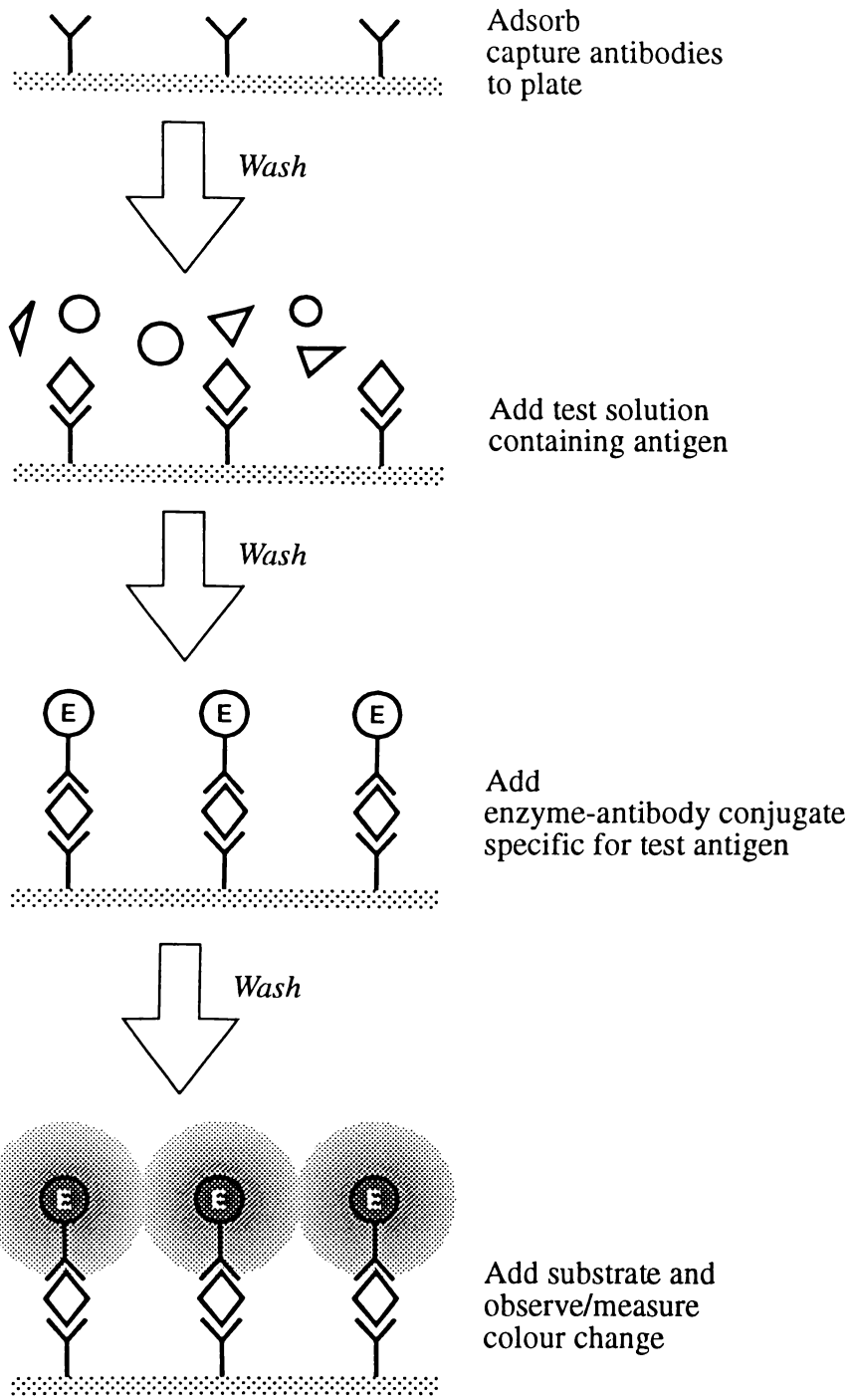


Figure 2.2.3
Schematic diagram of a double antibody sandwich ELISA for measuring antigen

the presence of unbound enzyme conjugate may lead to false positive readings. Inadequate washing after the antigen or antibody coating step may also considerably affect the accuracy and sensitivity of an assay, as weakly bound immunoreactants (ie antigen or antibody that has bound to another protein rather than to the solid phase) carried over to the next step in the ELISA, are likely to be desorbed and lost in the subsequent stages of the assay. Weakly immobilized immunoreactants should therefore be removed as early as possible by extensive washing after the coating step (Tijssen 1985). However, over-vigorous washing should also be avoided as this could result in the desorption of properly immobilized immunoreactants so decreasing the signal.

Having reviewed relevant aspects of enzyme immunoassays, the implementation of an enzyme-linked immunosorbent assay for the detection and quantification of *A. lolii* will now be described.

2.2 PRODUCTION OF POLYCLONAL ANTISERA TO *ACREMONIUM LOLII*.

2.2.1 Antigen preparation:

Both mycelial (particulate) and culture fluid (soluble) antigens were used in the immunization. Preparation of the two antigens will be dealt with separately. All *A. lolii* cultures were obtained from Dr M. E. di Menna, AgResearch, Ruakura Agricultural Research centre, Hamilton.

Mycelial antigen preparation:

A similar methodology to that described by Johnson *et al.* (1982) and Musgrave (1984), was used in this study. Perennial ryegrass seed (cv. Ellett) was surface sterilized and germinated in potato glucose aureomycin agar. Germinated seedlings were then placed into 250ml flasks containing a glucose (2%), peptone (1%), and yeast extract (0.5%) broth (GPYE). *A. lolii* mycelium, which had grown out of the seedlings into the broth, was then transferred to fresh 250ml flasks of GPYE broth. The flasks were incubated at 20^0C with continuous shaking (50-100 oscillations per minute) until cultures were thick with mycelium.

Endophyte cultures were vacuum filtered using a Buchner funnel. *A. lolii* mycelium was collected on a glass fibre wad (Whatman GF/B) while the broth (used to produce the soluble antigen) was collected in a glass vacuum flask. The mycelium was scraped off the glass fibre wad with a spatula, weighed, and resuspended in phosphate buffered saline (PBS) at a concentration of 50mg/ml. The suspended mycelium was then homogenised in a Waring blendor for 10 seconds at high speed, after which it was stored at 4^0C ready for injection.

Culture fluid antigen preparation:

The presence of specific antigens in the soluble antigen fraction of *A. lolii* was confirmed by Musgrave *et al.* (1986). These workers concluded that the soluble antigen

fraction could be used as the immunogen when raising antibodies against *A. lolii*. The ability of the soluble antigen fraction of *A. lolii* to evoke an immune response was therefore tested. This is the first report where antibodies against *A. lolii* have been raised using the soluble antigen fraction of the cultured endophyte. A similar procedure to that described by Musgrave *et al.* (1986) was used to prepare the soluble antigen fraction of *A. lolii*. Measured volumes of broth supernatant were placed into several 250ml Beckman centrifuge tubes after which 2.5 volumes of absolute ethanol were added dropwise. After being stored at -20^{0}C for 16 hours, the precipitate was collected by centrifugation at 10,400g for 20 minutes at 4^{0}C in a Sorvall superspeed RC2-B centrifuge. The supernatant was discarded and the tubes were left to drain for one hour on paper tissues. The pellets were resuspended in 2ml of half-strength PBS, and dialysed (molecular weight cutoff point 10000Da) against two changes of two litres of half-strength PBS at 4^{0}C for 24 hours. The dialysate was then transferred to sterile 10ml Eppendorf centrifuge tubes and stored at a concentration of 1mg/ml at -20^{0}C ready for injection.

2.2.2 Production of antiserum:

Mature New Zealand white rabbits were used to produce antibodies against *A. lolii* as they are a recommended choice (Hurn and Chantler 1980) and have been successfully used in similar projects (Johnson *et al.* 1982, Musgrave 1984). Several rabbits, rather than a single animal were used, as individual variation in response is often great (Hurn and Chantler 1980, Halk and De Boer 1985, Goding 1986).

Two immunizations were performed on two different sets of animals. The first series of injections on rabbits 1-5, commenced on 11th May 1990 and the second on rabbits A-D, were started on 31st January 1991. The injection protocols for the two immunizations were identical. On both occasions, soluble and particulate *A. lolii*

antigens were administered, although throughout the immunization, animals received either soluble or particulate material and not both. Rabbits 1, 4, 5, A and D were injected with particulate antigen, the antibodies from which are referred to as IgG '1p', IgG '4p', IgG '5p', IgG 'Ap' and IgG 'Dp', while animals 2, 3, B and C received soluble antigen, the antibodies from which are referred to as IgG '2s', IgG '3s', IgG 'Bs' and IgG 'Cs'.

Freunds adjuvant (complete for the initial injection and incomplete for the boosters) was used on all rabbits. Adjuvants such as Freunds, are immunopotentiators, enhancing the immune response of an animal to an antigen. In the case of Freunds adjuvant, this is achieved by prolonging the persistence of the immunogen in the tissues resulting in a continuous stimulation of the immune system (Edelman 1980, Tijssen 1985). Freunds adjuvant is composed largely of mineral oils which yield stable water-in-oil emulsions with the immunogen slowing its release. Freunds complete adjuvant also contains heat killed *Mycobacterium tuberculosis* in the oil phase which helps mobilize macrophages. Development of mineral oil adjuvants (Freund and McDermott 1942) resulted in considerably enhanced antibody responses. Freunds adjuvants (complete in particular) however are toxic to mammals and severe side effects or even death may result from their use (Broderson 1989). Consequently, there has been considerable research into alternative adjuvants, without much success. Although they have been in use for some time (Freund and McDermott 1942), Freunds adjuvants are considerably more effective than the majority of new adjuvants in potentiating immune responses (Edelman 1980, Erhard *et al.* 1991, Johnston *et al.* 1991, Smith *et al.* 1992). Freunds adjuvants were therefore chosen for the immunization but injected animals were closely monitored for any possible ill effects.

Thirty minutes before the animals received their first injection, 2ml of homogenised *A. lolii* mycelium or 2ml of soluble *A. lolii* antigen were emulsified with

1.25ml Freunds complete adjuvant (FCA) (Sigma F-5881) in sterile 10ml Eppendorf centrifuge tubes. The FCA was gently shaken before the 1.25ml aliquots were dispensed to ensure even distribution of the bacterial pellet in the oil phase. Emulsification of antigen with adjuvant was achieved using a 5ml syringe fitted with a 20 gauge needle.

Inocula were administered subcutaneously. A fold of skin on the right hind leg was sterilized using ethanol. Injections were performed using 5ml syringes fitted with B-D 20G1 precisionglide needles. The needle was inserted, bevel up, into the skin at an angle until the tip felt free and the contents of the syringe slowly injected. After injection, the needle track was pinched to prevent loss of the inoculated material. The injection site was then gently massaged and sterilized with ethanol.

The first booster injection was given 14 days after the primary inoculation. The inocula were identical to those the animals received in the first injection except that Freunds incomplete adjuvant (FIA) (Sigma F-5506) was used. Also, rabbits were immunized on the left leg. The second booster was administered after another 14 days. This was given on the right leg using identical inoculum to that used in the first booster.

Rabbits 1-5 were bled 12 and 31 days after the final booster while rabbits A-D were bled 7 days after the final booster. Ethanol was applied to one ear to sterilize the area. The area around the marginal ear vein was then shaved with a scalpel blade. Using cotton wool, xylol was applied to the distal parts of the shaved region which was then gently rubbed to promote blood flow. A small nick was made across the marginal ear vein with a sterile scalpel blade and blood was collected in a 50ml Beckman centrifuge tube. Blood flow was maintained by gently massaging the ear. After a sufficient amount of blood had been collected (10-40ml), blood flow was stopped using cotton wool and applying a small amount of pressure to the cut. Ethanol was then rubbed over the ear to prevent infection. Blood was left to clot at room temperature for two hours. Blood

samples were then incubated overnight at 4^{0}C after which they were clarified by low speed centrifugation (1000g for 10 minutes at 4^{0}C) using a Beckman model J2-21M induction drive centrifuge. Sera were stored at -20^{0}C ready for purification.

2.2.3 Immunoglobulin purification:

1ml of each antiserum was diluted with 9ml of sterile distilled water in a 50ml Beckman centrifuge tube, after which 10ml of saturated ammonium sulphate was added dropwise. Tubes were incubated at room temperature for one hour and were then centrifuged at 4340g for 10 minutes at 4^{0}C in a Beckman J2-21M centrifuge. The supernatant was poured off and the pellet resuspended in 15ml of 50% saturated ammonium sulphate (not added dropwise). After a 10 minute incubation at room temperature, samples were spun at 4340g for 10 minutes at 4^{0}C. The pellet was resuspended in 2ml of PBS and dialysed (molecular weight cutoff point 10000Da) against two changes of two litres of half-strength PBS overnight at 4^{0}C. The protein precipitates were transferred to 10ml Eppendorf centrifuge tubes and stored at 4^{0}C ready for ion exchange purification.

Immunoglobulin, in particular IgG, was further purified by ion exchange chromatography with diethylaminoethyl (DEAE) cellulose. This technique relies on the fact that at low ionic strength and neutral pH, IgG carries a neutral or slight positive charge and will therefore not be adsorbed to the ion exchanger, unlike all other serum proteins (Hurn and Chantler 1980, Goding 1986). Before use, the DEAE-cellulose was precycled to charge the matrix. 50g of DEAE-cellulose (Sigma D-3764) was added to 2l of 0.5M hydrochloric acid in a 5l glass beaker and stirred. This was left to stand, with occasional stirring, at room temperature. After 30 minutes, the DEAE-cellulose was filtered using a Buchner funnel and a Whatman GF/B glass fibre wad. Distilled water was then washed through the DEAE-cellulose until the effluent registered a pH of

approximately 4.0. The DEAE-cellulose was then added to 2l of 0.5M sodium hydroxide, stirred, and left to stand as before. After filtration using a Buchner funnel, the ion exchanger was washed through with several litres of distilled water until the pH of the effluent was approximately 7.0.

The charged DEAE-cellulose was packed into a glass column which was incorporated into the apparatus shown in Figure 2.3. The column (305ml) was equilibrated with at least five bed volumes of half-strength PBS, and tested with 1ml of a 1mg/ml bovine serum albumin (BSA) solution, before any samples were added. As much of the assembly as possible was maintained at 4^0C throughout the purification process and a flow rate of approximately 4ml/minute was used. The serum protein samples were passed through the column and the effluent monitored at 280nm. The immunoglobulin peak, the first peak to elute, was collected into a sterile 10ml Eppendorf centrifuge tube. A typical example of the absorbance profile during a purification is shown in Figure 2.4. The immunoglobulin concentration was determined by measuring the absorbance of the purified serum at 280nm (E_{280nm}= 1.4) using a Shimadzu U.V.-visible recording spectrophotometer. The purified serum was adjusted with half-strength PBS to 1mg/ml, and stored at -20^0C.

2.2.4 Enzyme conjugated antibody preparation:

Enzyme-antibody conjugates were prepared by adding 0.5mg of purified IgG to 1mg of alkaline phosphatase (Sigma type VII, specific activity 1200 units/mg) in a sterile 10ml Eppendorf centrifuge tube. The mixture was dialysed (molecular weight cutoff point 10000Da) against two changes of 2l of half-strength PBS overnight at 4^0C. The dialysate was then transferred to a fresh 10ml Eppendorf centrifuge tube and glutaraldehyde (BDH EM grade 36080) was added to a final concentration of 0.06%. The mixture was incubated at room temperature for 4 hours, after which the

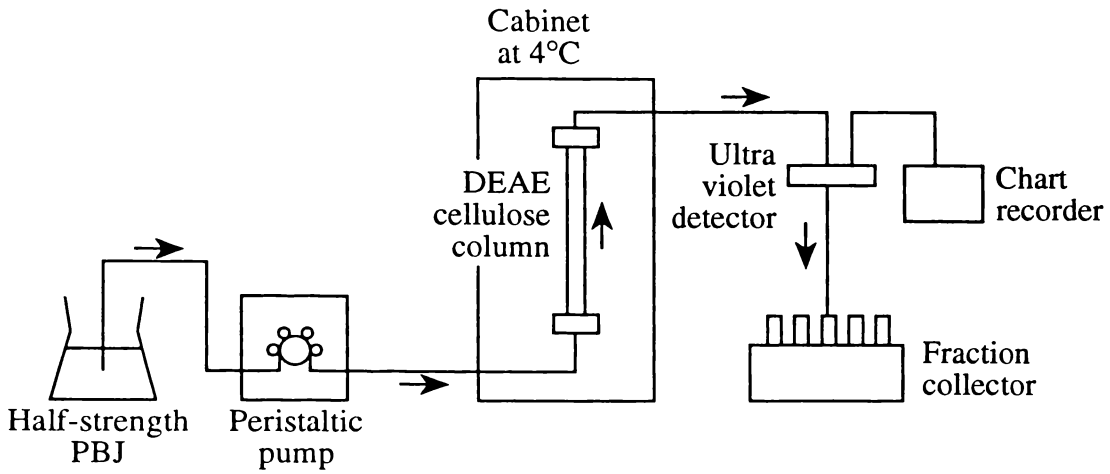


Figure 2.3
Schematic diagram of the ion-exchange chromatography apparatus used to purify rabbit serum

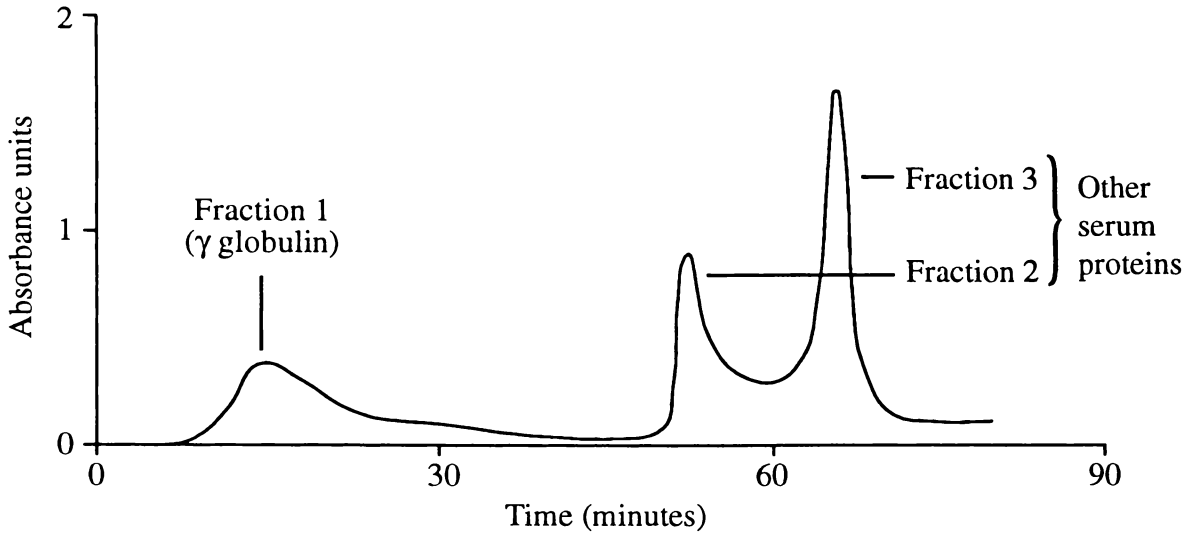


Figure 2.4
Typical example of the absorbance profile obtained during ion exchange purification of rabbit serum

glutaraldehyde was removed by dialysis against half-strength PBS as described earlier. After dialysis, one tenth of the conjugate volume of a 50mg/ml BSA solution was added to the conjugate which was stored at 4^0C.

2.3 DEVELOPMENT OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY TO *ACREMONIUM LOLII*.

Each of the enzyme-linked immunosorbent assays discussed in this section, were performed using one of four general protocols. Different protocols were used depending upon whether the antibodies used were purified or still present in raw serum, and on the stage of development of the assay. Protocols 1, 3 and 4 were of the double antibody sandwich type based on the method of Clark and Adams (1977). Protocol 2 was an indirect assay, based on the method of Voller *et al.* (1976b). Protocol methodologies were as follows:

Protocol 1:

i. Coat plate with purified antibody in coating buffer (200µl/well for 24hrs at 4^{0}C).

ii. Wash three times in PBS/Tween 20 (0.05%).

iii. Block with 220µl ovalbumin (0.1% w/v) in coating buffer (24hrs at 4^{0}C).

iv. Repeat step two.

v. Sample incubation (grass extracted in sample buffer overnight at 4^{0}C at 20mg/ml w/v) for 24hrs at 4^{0}C (200µl/well).

vi. Repeat step 2.

vii. Incubate with conjugate in conjugate buffer (200µl/well for 12hrs at 4^{0}C).

viii. Repeat step 2.

ix. Incubate with substrate in substrate buffer (0.5mg/ml p-nitrophenyl phosphate (pNPP) Sigma 104-0) 30-90 mins at room temperature (200µl/well). Measure extinction at 405nm using a Bio Rad model 3550 microplate reader.

Protocol 2:

i. Sample incubation (200µl/well, overnight at 4^{0}C).

ii. Wash two times with tap water.

iii. Block with 220µl BSA (0.5% w/v) in PBS/Tween 20 (0.05%) for 2hrs at 27^{0}C.

iv. Repeat step two.

v. Raw serum (diluted 1:500 in conjugate buffer) incubation for 2hrs at 27^{0}C (200µl/well).

vi. Repeat step two.

vii. Incubation with goat anti-rabbit alkaline phosphatase labelled antibody (Sigma A 8025) at a 1:1000 dilution in conjugate buffer for 2hrs at 27^{0}C (200µl/well).

viii. Repeat step two.

ix. Substrate incubation (1.0mg/ml pNPP) for 30-90 mins at room temperature (200µl/well). Measure absorbance at 405nm as in step ix of protocol 1.

Protocol 3:

i. Antibody coating step (200µl/well for 6-8hrs at 27^{0}C).

ii. Wash two times with tap water.

iii. Block with 220µl BSA (0.5% w/v) in PBS/Tween 20 (0.05%) for 1hr at 27^{0}C.

iv. Repeat step two.

v. Sample incubation (grass extracted overnight at 4^{0}C at 20mg/ml w/v) overnight at 4^{0}C (200µl/well).

vi. Repeat step two.

vii. Conjugate incubation (200µl/well for 7hrs at 27^{0}C).

viii. Wash two times with tap water, once with PBS/Tween 20 (0.05%), and twice more with tap water.

ix. Substrate incubation (1.0mg/ml pNPP) for 90 mins at room temperature (200µl/well). Measure absorbance at 405nm as in step ix of protocol 1.

Protocol 4:

i. Antibody coating step (200µl/well for 6-8hrs at 27^{0}C).

ii. Wash three times with PBS.

iii. Block with 220µl BSA (0.7% w/v) in PBS/Tween 20 (0.05%) for 1-2hrs at 27^{0}C.

iv. Repeat step two.

v. Sample incubation (grass extracted for 6hrs at room temperature at 20mg/ml w/v) overnight at 4^{0}C (200µl/well).

vi. Wash twice with PBS/Tween 20 (0.05%) and once with PBS.

vii. Conjugate incubation (200µl/well for 7hrs at 27^{0}C).

viii. Repeat step six.

ix. Substrate incubation (1.0mg/ml pNPP) for 90 mins at room temperature (200µl/well). Measure absorbance at 405nm as in step ix of protocol 1.

Flat bottomed polystyrene Nunc Immuno microtitre plates (maxisorp F96 certified) were used throughout. During each incubation step, plates were covered with a strip of parafilm and a plastic lid and were sealed inside a plastic bag. The compositions of the buffers used are shown in appendix one. In all cases, a minimum of two replicates (wells) were used per sample.

2.3.1 Effect of rabbit and time of bleeding on antibody production following immunization with *A. lolii* antigens.

Introduction:

The effectiveness of different polyclonal antisera to a homologous antigen or group of antigens, can be quite variable (Hurn and Chantler 1980, Goding 1986). This may be dependant upon several factors, including:

-the extent of the humoral immune response in a particular animal (ie: the number of different B cells mobilized, the number of effector cell clones of each different B cell produced, and the rate of antibody synthesis of all of these cells)

-the affinity of the different antibodies produced by the effector cells

-the avidity of the different antisera

-the immunization protocol (ie; how often and where the animal was injected, how much was injected, and whether and what types of adjuvant were used)

-the quality and purity of the antigen used to inject the animal.

To increase the overall efficacy of any ELISA, the sensitivity, specificity, detectability and accuracy of the assay should be maximized. Apart from modifying various aspects of the actual assay (Clark and Adams 1977), this can only be accomplished by injecting several animals and using the 'best' antiserum. Purified antibodies from each of the different antisera produced from the immunization programmes, or the raw antisera themselves, were therefore screened against samples of pure antigen to determine their activity against *A. lolii* in an ELISA.

Methods:

Antibodies or raw antiserum from all 14 bleedings were screened against a 10-fold dilution series of *A. lolii* fungus. A 10-fold dilution series was made by successively diluting a 10mg/ml solution of washed, filtered and homogenised endophyte (see section 2.2.1 for preparation of mycelium) in sample buffer. For rabbits

1-5, a purified gamma globulin concentration of 1.0μg/ml for both capture and conjugated antibody was used with protocol 1, while a 1:500 dilution of raw antiserum and a 1:1000 dilution of alkaline phosphatase labelled goat antirabbit conjugate (Sigma A8025) was used with protocol 2 for rabbits A-D.

Results:

Antibodies and antisera from all rabbits showed some degree of anti-*A. lolii* activity (Figs. 2.5, 2.6 and 2.7). Antibodies from the first bleed of rabbits 1-5 all appeared to detect *A. lolii* down to the lowest concentration of 0.01μg/ml (Fig. 2.5). Apart from antibodies from animal number 2 which showed a similar activity, there were considerable reductions in absorbance values with antibodies from the second bleeding of rabbits 1-5 (Fig. 2.6). The limits of detection also appeared to be inferior with antibodies from this bleeding. However results from the first bleed showed that the highest extinction values were obtained using IgG '4p'. IgG '5p' also showed appreciable activity against *A. lolii* , while IgG '2s' and IgG '3s' were of intermediate activity. IgG '1p' showed the least degree of anti-*A. lolii* activity. In animals A-D, the antiserum with the highest activity to *A. lolii* was IgG 'Cs' (Fig. 2.7). This was followed by IgG 'Ap', IgG 'Dp' and IgG 'Bs' in order of decreasing reactivity. Using antisera from animals A-D, *A. lolii* was detected down to a concentration of 1.0μg/ml, although this is statistically unsubstantiated.

Discussion:

The observation that a sloped line (standard curve) corresponding to the antigen dilution series was achieved with all purified antibodies and antisera (Figs. 2.5, 2.6 and 2.7), indicates that immune responses against *A. lolii* antigens had taken place in each animal. Had no immune response taken place, absorbances would have been extremely low and they would not have increased with increasing antigen concentrations. The extent of the immune response in the different rabbits was highly variable. This was not

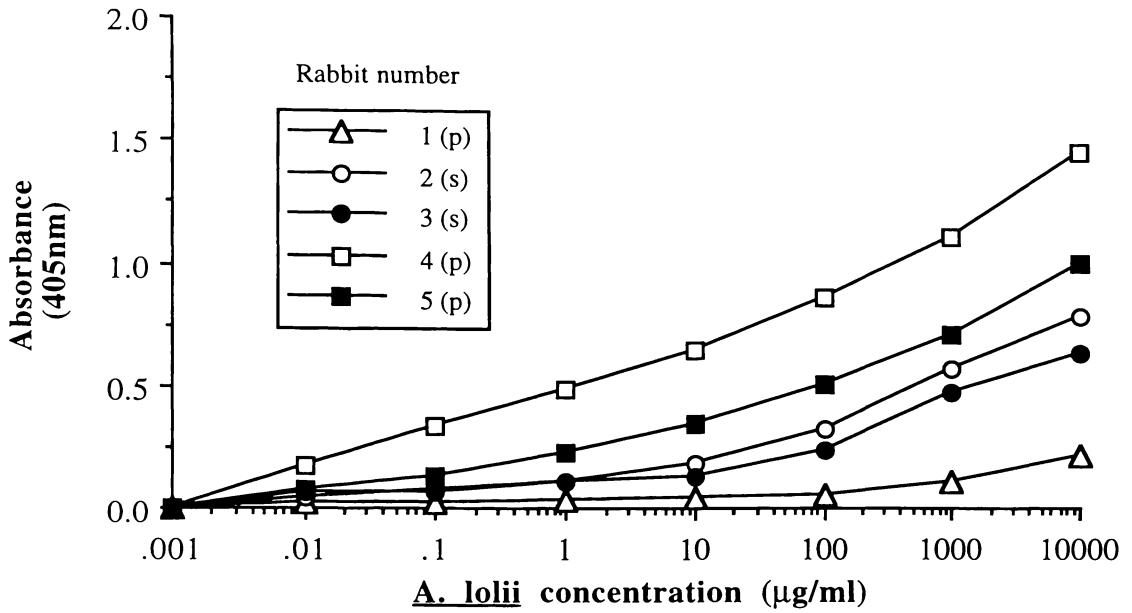


Figure 2.5 Absorbance curves comparing detection of A. lolii using purified antibodies (at 1.0µg/ml) from rabbits 1-5 (12 day bleeding) in a double antibody sandwich ELISA (protocol 1). (P=rabbits injected with particulate antigen, S=rabbits injected with soluble antigen).

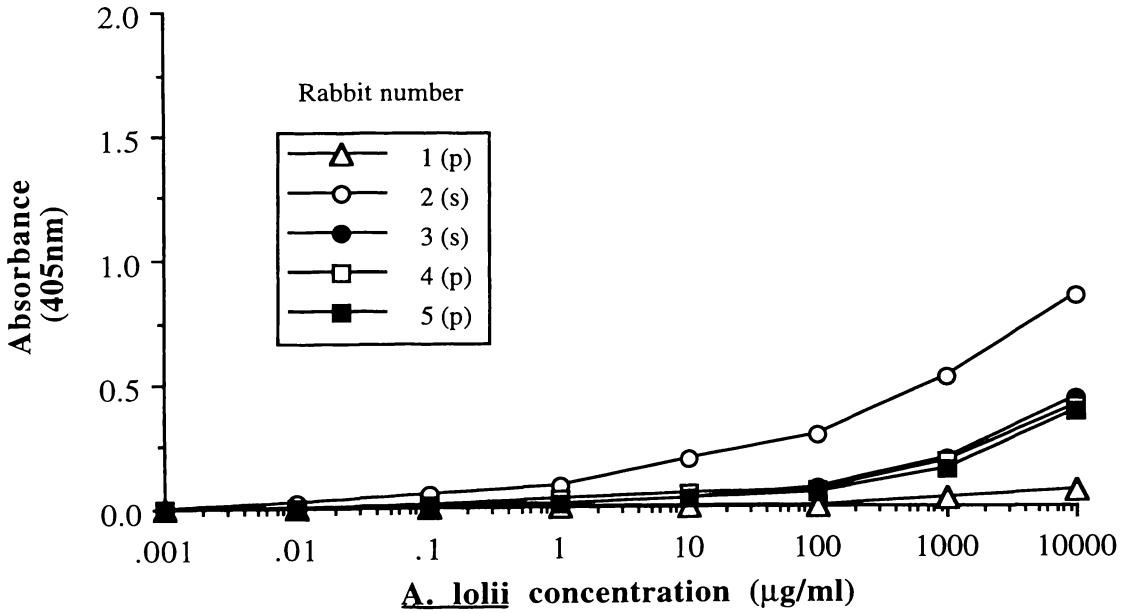


Figure 2.6 Absorbance curves comparing detection of A. lolii using purified antibodies (at 1.0µg/ml) from rabbits 1-5 (26 day bleeding) in a double antibody sandwich ELISA (protocol 1). (P=rabbits injected with particulate antigen, S=rabbits injected with soluble antigen).

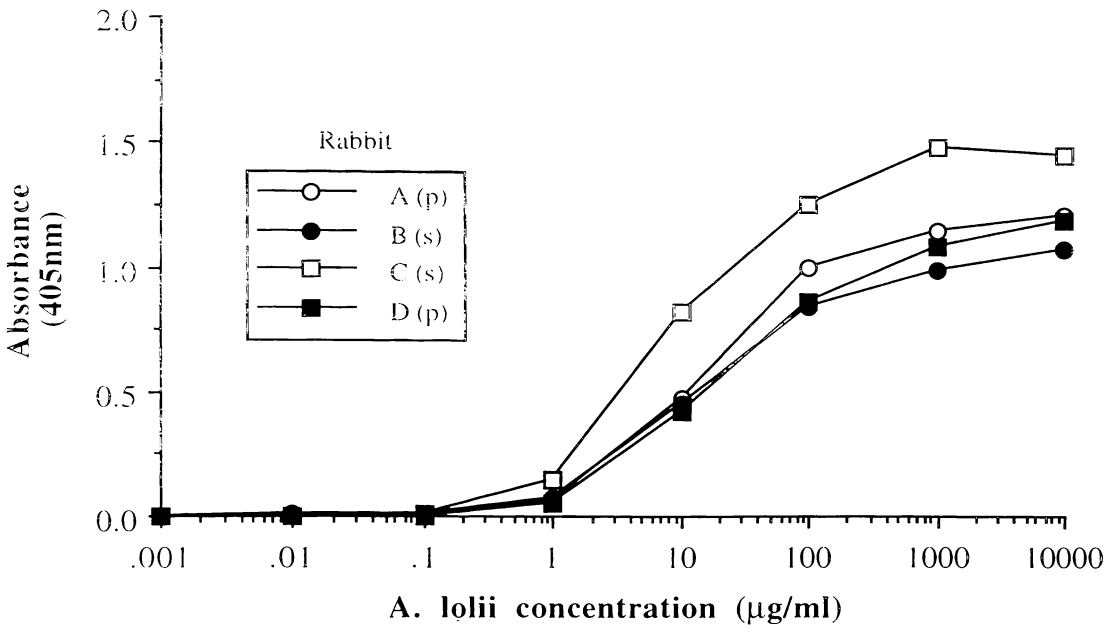


Figure 2.7 Absorbance curves comparing detection of A. lolii using raw antisera (1:500 dilution) from rabbits A-D in an indirect ELISA (protocol 2) with antigen bound to the solid phase. Goat antirabbit conjugate was diluted 1:1000. (P=rabbits injected with particulate antigen, S=rabbits injected with soluble antigen).

unexpected as, in comparison with sheep and goats, immune responses in rabbits are known to be notoriously variable (Goding 1986).

Results from figures 2.5 and 2.6 show that the anti-*A. lolii* activities of antibodies from rabbits 1-5 were generally far greater at the first bleeding, taken 12 days after the final booster, than they were at the second, taken 26 days after the final booster. Although bleedings are often taken 5-7 days after the final booster, antibody titres usually remain high for several weeks (Goding 1986). It is evident that the titre for specific anti-*A. lolii* antibodies had declined considerably by the time the second bleeding was taken, as corresponding absorbances were lower than for the first bleed. The limit of detection of the assay also appeared to be inferior using antibodies from the second bleed. Serum from rabbits A-D, which were bled 7 days after the final booster, showed a similar level of anti-*A. lolii* activity to the antibodies taken at the first bleed from animals 1-5 (Figs. 2.7 and 2.5 respectively).

Although the results from figures 2.5 and 2.7 suggest that the limits of detection using antibodies from rabbits 1-5 may be greater than those using antisera from animals A-D, a direct comparison between the two sets of results is difficult. This is partly due to the different nature of the two ELISA protocols used (O'Sullivan *et al.* 1979). Comparing direct, indirect and double antibody sandwich methods for detection of virus particles, Crook and Payne (1980) found that the most sensitive assay was the indirect one, the least sensitive was the direct, while the sandwich ELISA showed intermediate sensitivity. However, a similar study of the foot and mouth disease virus using the same three assay techniques, showed similar levels of virus detection (Crowther and Abu-el-Zein 1979). The results from figures 2.5 and 2.7 indicate that in the present study, the sandwich assay had the highest limit of detection. The higher limit of detection in the sandwich assay in the current study may however be a function of antibody purity, as antibodies from rabbit 4 had been purified prior to the ELISA whereas only raw serum

from animal C was used. At its optimum, detectability of antigens with raw antiserum is approximately four times less than with purified gamma globulin (Tijssen 1985). Differences may also arise according to the gamma globulin concentration used in the two assays; the gamma globulin concentration in the raw serum was not determined and a simple 1:500 dilution was used, while the gamma globulin concentration used in the sandwich assay was 1μg/ml.

Direct comparisons with results from other endophyte assays regarding the limit of detection are not possible for a number of reasons (eg the endophyte standards and assay protocols used are different). However, a quick check is useful to ensure that detection limits achieved in this study were not unusual. Visual estimation of the data suggests that the limit of detection using antibodies from the first bleed of rabbits 1-5 was approximately 0.01μg *A. lolii* mycelium/ml using the double antibody sandwich method (a more rigorous test in section 2.3.5 which was analyzed statistically indicates that this is a fair estimate). Using a sandwich assay, Musgrave (1984) detected *A. lolii* mycelia down to a concentration of 0.1μg/ml and after subsequent improvements, obtained a detection end point of 0.03μg/ml (Musgrave *et al.* 1986). Working on *Acremonium coenophialum*, Johnson *et al.* (1982), and Arese *et al.* (1990), achieved a detection end point of 0.1 μg/ml, also using sandwich assays. Also working on *A. coenophialum*, Reddick and Collins (1988) obtained a limit of detection of only 10μg/ml using a standard sandwich assay. However, by using a modified protein-A sandwich ELISA, the limit of detection of the assay was enhanced to 0.04μg/ml.

The anti-*A. lolii* activities in the antibodies and antisera from all 9 rabbits indicate that the soluble and particulate antigens used in the immunization were similarly immunogenic (Figs. 2.5 and 2.7). Musgrave *et al.* (1986) also found that the immunogenic reactivity of soluble antigens was similar to that found with mycelia.

To maximize the efficiency of the final assay (and to greatly simplify matters),

the 'best' performing antibodies from each of the two groups of rabbits were selected for further examination. These were IgG '4p' and IgG 'Cs'. Two ml of raw antisera from rabbits 4 and C were therefore purified (as described in section 2.2.3) for further testing.

2.3.2 Effects of capture and conjugate antibody concentrations on detection of *Acremonium lolii*.

Introduction:

The concentrations at which the coating and enzyme conjugated antibodies are used in an assay are critical (Butler *et al.* 1986). Correct antibody concentrations will maximize detectability, sensitivity, specificity and accuracy of an assay. Choosing the 'right' antibody concentrations is more often than not, a compromise, as the above factors which determine the success of an assay are usually highly interrelated (Tijssen 1985). For example, lower specificity is often associated with a higher background as well as a higher detectability (Edwards and Cooper 1985). In general, factors influencing optimal antibody concentrations include antibody avidity, immunogenicity of the antigen(s), the concentration of antigen in the samples, the ELISA protocol used, and finally the presence of interfering compounds in sample extractions.

Optimum antibody coating concentrations may vary widely between different assays. Optimal gamma globulin concentrations are commonly between 1.0-10.0µg/ml (Voller *et al.* 1976b, Clark and Adams 1977, Engvall 1980). Use of an excessively high antibody coating concentration often leads to a reduction in specificity and/or sensitivity (Clark and Adams 1977, Koenig 1978, McLaughlin *et al.* 1981, Butler *et al.* 1986, Hermann 1986). This 'saturation point' effect is thought to be due to steric hinderance of the adsorbed antibody caused either by the formation of multiple antibody layers or by an overcrowding effect on the plate surface (Tijssen 1985, Hermann 1986). Desorption of antibody from the solid phase during the assay will also increase, as

secondary protein-protein interactions are not stable (Tijssen 1985, Hermann 1986). This would also decrease the sensitivity of the assay, as antigens will be free to bind with antibodies in solution reducing antigen uptake by the coat (Engvall *et al.* 1971). The coating antibody concentration must however be high enough to prevent a decrease in the reaction level (McLaughlin *et al.* 1981, Devey and Steward 1988). The optimal coating concentration for each assay should therefore be determined (Nakamura and Kasahara 1992).

Like capture antibody concentrations, enzyme-antibody conjugates are commonly used at between 1.0-10.0µg/ml (Lister and Rochow 1979, Nachmias *et al.* 1979, Crook and Payne 1980, Musgrave 1984). It is perhaps more useful to quote conjugate concentrations in terms of dilution rather than gamma globulin concentrations, as conjugation of antibodies with enzymes inevitably results in a reduction in the affinity of the antibody (Koenig 1978, Crook and Payne 1980). It is thought that the conjugation of the antibody with the enzyme causes steric hinderance or a conformational change at the antibody binding site, so reducing its affinity (Koenig 1978, Crook and Payne 1980). Reduced affinity may however lead to greater specificity.

To determine the 'best' conjugate concentration to use in an assay, one must again compromise between the required limit of detection and the required specificity. Generally, an increase in conjugate concentration will lead to increased levels of detection and higher backgrounds (Lister and Rochow 1979, Musgrave 1984).

Purified samples of IgG '4p' and IgG 'Cs' at several concentrations were screened against a dilution series of pure antigen to determine suitable working gamma globulin concentrations for the assay.

Methods:

IgG '4p' was screened at three coating concentrations (1, 2 and 3µg/ml) using a

conjugate concentration of 1.0μg/ml, and three conjugate concentrations (0.2, 0.5 and 1.0μg/ml) using a coating concentration of 1.0μg/ml, against a ten-fold *A. lolii* dilution series. Protocol 1 was used for the ELISA. IgG 'Cs' was screened against a ten-fold dilution series of the *A. lolii* culture broth instead of the mycelium itself. The highest concentration used was a ten-fold dilution of the original broth. Three antibody coating and conjugate dilutions were tested (1:333, 1:266 and 1:222) for IgG 'Cs' using protocol 3.

Results:

With both sets of antibodies, absorbances rose as *A. lolii* mycelium or antigen concentrations increased (Figs. 2.8, 2.9 and 2.10). This occurred at all antibody concentrations tested. Increasing the coating concentration of IgG '4p' from 1 to 2μg/ml resulted in a corresponding increase in absorbance at all *A. lolii* concentrations except at 0.01μg/ml (Fig. 2.8). Detection (statistically unsubstantiated) at the lowest antigen concentration of 0.01μg/ml was greatest with a coating concentration of 1.0μg/ml. Using a coating concentration of 3.0μg/ml, the limit of detection of the assay was reduced to 0.1μg/ml. Absorbances on the steep part of the slope at a coating concentration of 3μg/ml were no higher than at 2μg/ml.

Extinction values at all antigen concentrations were directly proportional to the conjugate concentration (Fig. 2.9). The apparent limit of *A. lolii* detection using conjugate at 0.25μg/ml was 0.1μg/ml, while a detection limit of 0.01μg/ml using a conjugate concentration of 0.5 or 1.0μg/ml is indicated by the data.

Absorbances using IgG 'Cs' were also directly proportional to coating and conjugate gamma globulin concentrations (Fig. 2.10). *A. lolii* antigens were detected in broth diluted 100,000 times. Assay limit of detection appeared to be marginally improved with increasing gamma globulin concentration.

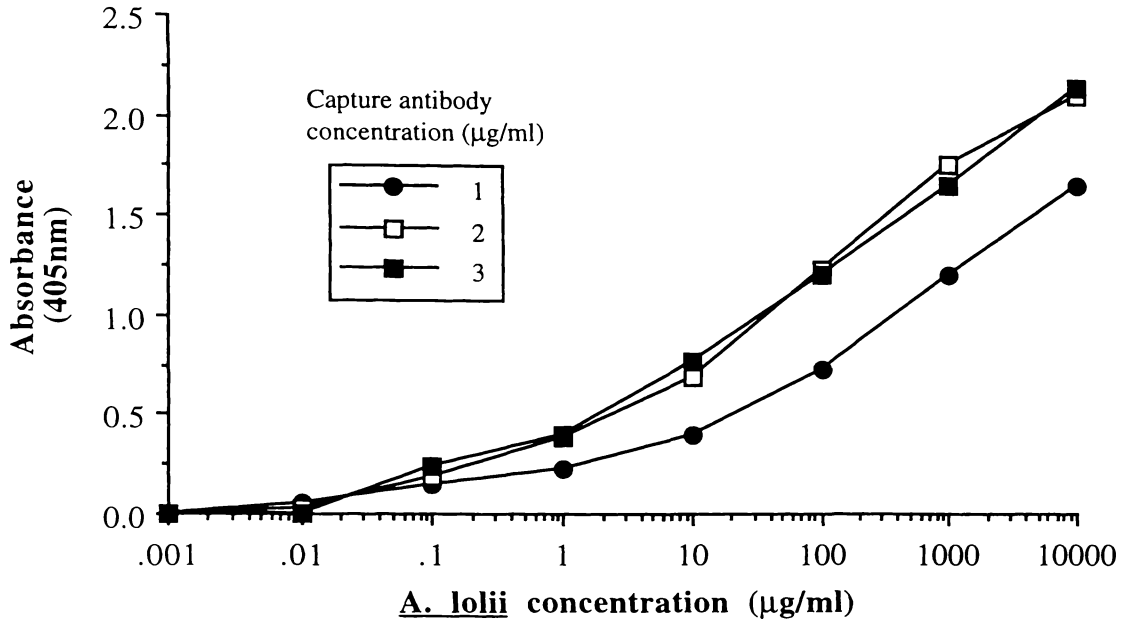


Figure 2.8 Absorbance curves comparing detection of A. lolii using IgG '4p' at coating concentrations of 1, 2 and 3 µg/ml in a double antibody sandwich form of ELISA (protocol 1). Conjugate concentration used was 1.0µg/ml.

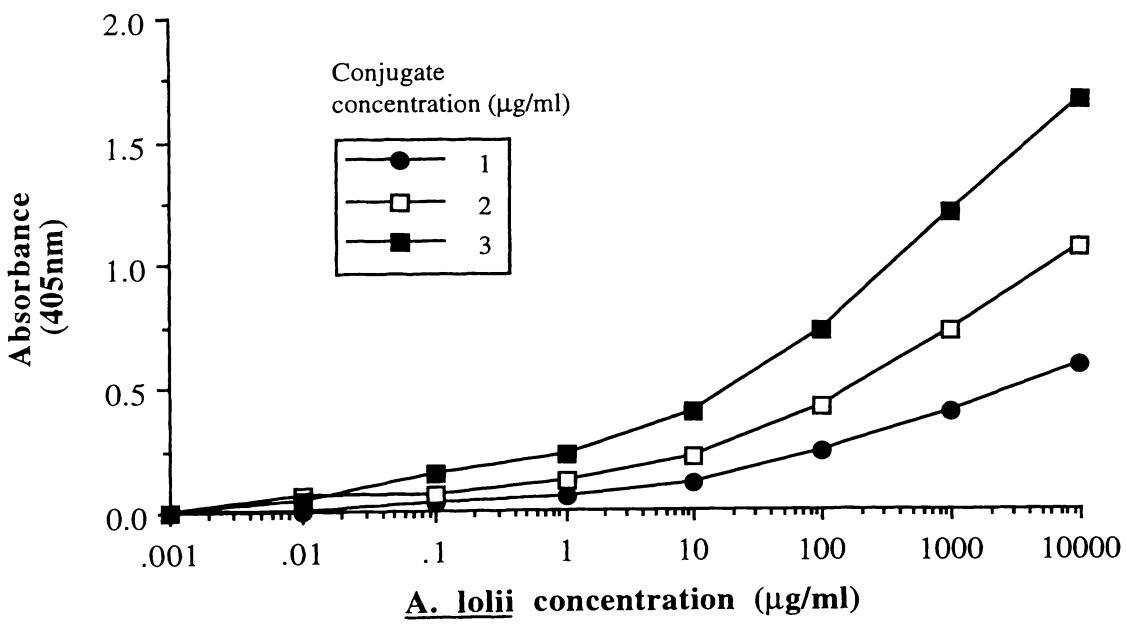


Figure 2.9 Absorbance curves comparing detection of A. lolii using IgG '4p' at conjugate concentrations of 0.25, 0.5 and 1.0 µg/ml in a double antibody sandwich ELISA (protocol 1). Coating concentration used was 1.0µg/ml.

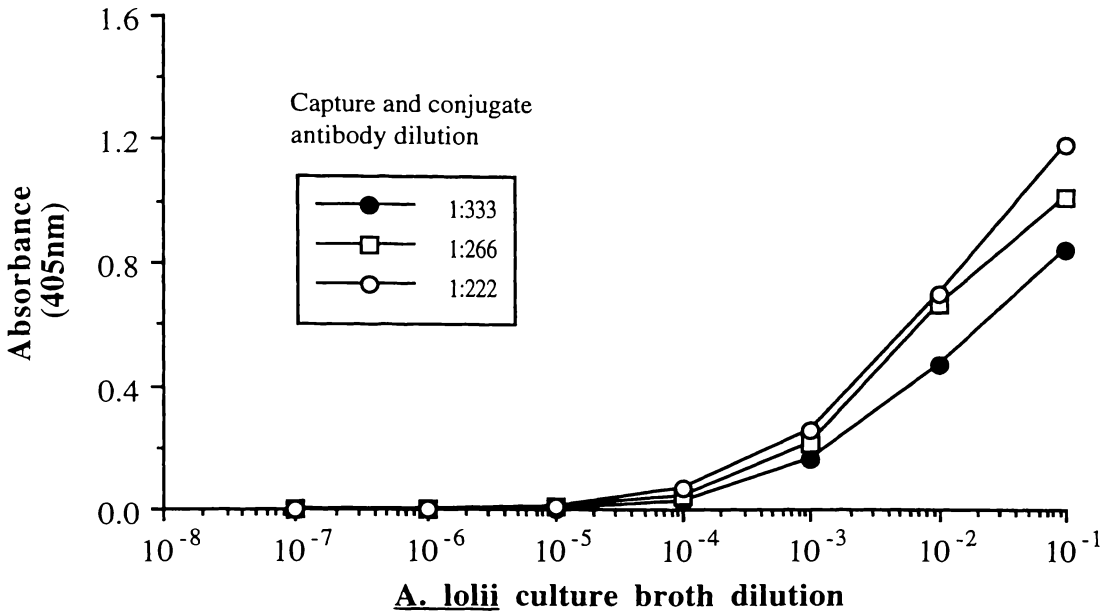


Figure 2.10 Absorbance curves comparing detection of A. lolii using IgG 'Cs' at three capture and conjugate antibody dilutions in a double antibody sandwich ELISA (protocol 3).

Discussion:

The fact that absorbances generally increased as a function of gamma globulin concentration, adds strength to the assumption that IgG '4p' and IgG 'Cs' contain effective anti-*A. lolii* antibodies, as an increase in the number of active binding sites would be expected to increase antigen binding frequency to a point.

The lack of any significant increase in absorbance using IgG '4p' at 3µg/ml rather than 2µg/ml (Fig. 2.8), indicates that the 'saturation point' of the assay was probably reached. This is the point at which the capture antibody concentration is well in excess of the antigen concentration of the sample. Although the sensitivity of the assay was not affected, it led to an apparent reduction in the limit of detection which was expected for reasons outlined in the introduction (Clark and Adams 1977, Koenig 1978, McLaughlin *et al.* 1981, Hermann 1986).

At the concentrations tested, a rise in the conjugate concentration also resulted in an increase in absorbance (Fig. 2.9). This was similar to what was seen when coating antibody concentrations were increased before the point of saturation was reached. However, an increase in conjugate concentration also appeared to improve the limit of detection of the assay which is in marked contrast to what was observed when antibody coating concentrations were increased. Improvements in antigen detectability in other assays with higher conjugate concentrations were also recorded by Clark and Adams (1977), Lister and Rochow (1979) and Musgrave (1984).

Taking the results of Figures 2.8 and 2.9 together, of the concentrations tested the limit of detection and sensitivity of the assay were apparently greatest at coating and conjugate antibody concentrations of 1.0µg/ml. From this point on, IgG '4p' was therefore used at 1µg/ml for both the coating and conjugate steps.

Results from Figures 2.10 and 2.7 show that IgG 'Cs' which was produced using *A. lolii* antigens released into culture broth, was effective against both particulate

and soluble *A. lolii* antigens indicating their similar nature. Musgrave *et al.* (1986) also found that the immunogenic reactivity of the purified antigens was similar to that found with the mycelium.

As expected, increases in the coating and conjugate gamma globulin concentrations of IgG 'Cs' led to higher extinction values (Fig. 2.10). There was little hint of the assay having reached the saturation point at the highest dilution of 1:222 as absorbances were generally well above those at the 1:266 dilution. Additionally, although the limit of detection of the assay was no greater, detection at low antigen concentrations was probably enhanced at the 1:222 dilution. Subsequent ELISA's using IgG 'Cs' were therefore performed at the 1:222 dilution which corresponds to a gamma globulin concentration of 1.4μg/ml.

2.3.3 Detection of *A. lolii* in perennial ryegrass samples: effect of sample preparation and blocking.

Introduction:

The principal reason for developing the ELISA against *A. lolii* was to enable accurate determination of endophyte levels in perennial ryegrass samples. A method for extracting the endophyte from ryegrass samples, without denaturing the antigen(s) was therefore required. Johnson *et al.* (1982), Musgrave (1984), Reddick and Collins (1988) and Arese *et al.* (1990) achieved this by extracting their respective endophytes from fresh tissue. Johnson *et al.* (1982) and Musgrave (1984) also found that this was possible using oven-dried grass. The present ELISA was designed to be quantitative or semi-quantitative, and assessing the amount of fungal mycelium in terms of plant fresh weight is less accurate than assessment using dry weights. It was therefore important that plant material was dried.

Musgrave *et al.* (1986) identified the major antigen of *A. lolii* as a polysaccharide moiety of a high molecular weight protein lipopolysaccharide complex. This antigen was

relatively heat stable as their ELISA functioned perfectly well using grass material which had been oven dried at 80^{0}C (Musgrave and Fletcher 1986). Oven drying at 60^{0}C did not affect detection of *A. coenophialum* in tall fescue samples, indicating that the major antigens of that endophyte are also relatively heat stable (Johnson *et al.* 1983, Siegel *et al.* 1984a). Immunodominant fungal antigens in general appear to be quite heat stable as Notermans and Heuvelman (1985) showed that exposure of water soluble mould antigens to a temperature of 100^{0}C for 10 minutes did not result in their denaturation.

Detection of *A. lolii* in perennial ryegrass samples was therefore attempted using grass material oven-dried at 60^{0}C. The effect of freeze-drying the grass samples instead of oven-drying was also investigated.

Methods:

ELISA 1: This assay was carried out to determine the effects of oven-drying and extraction temperature on detection of *A. lolii* in grass samples. Material from the basal region (The first 8cm above ground level) of vegetative tillers of three different *A. lolii*-infected grasses and three *A. lolii*-free grasses, was harvested in January 1991 from plants grown outdoors. The samples were oven-dried at 60^{0}C for 40 hours, ground to a fine powder in an Ika model MFC culatti micro hammer mill, and stored at -20^{0}C until analysis.

Two 100mg portions of each grass were extracted in 5ml of sample buffer in separate 25ml glass scintillation vials (Kimble). One of the replicates of each grass was incubated at 4^{0}C for 24 hours, while the other replicate was incubated at 25^{0}C for the same length of time. Samples were gently oscillated during the incubation. The ELISA was carried out using IgG '4p' in protocol 1 minus the blocking step (step 3).

ELISA 2: The effect of two blockers at several concentrations on endophyte detection using IgG '4p' was investigated in this ELISA. Material from the basal 8cm

zone of one *A. lolii*-infected and one *A. lolii*-free plant was harvested in February 1991, dried at 60°C for 40 hours, ground and stored at -20°C until use. Each grass was extracted in sample buffer (20mg grass/ml buffer) for 20 hours at 4°C. The effects of blocking the plate with BSA and ovalbumin at 0.1%, 0.5%, 1.0% and 1.5% were examined using protocol 1.

ELISA 3: The effect of blocking the plate with different concentrations of BSA was also investigated using IgG 'Cs'. Plants from two *A. lolii*-infected and two *A. lolii*-free lines of cv. Ellett perennial ryegrass were harvested as before, dried at 60°C for 40 hours, ground and stored at -20°C. Grasses were extracted as described in ELISA 2. The ELISA was performed using protocol 3 at BSA concentrations of 0%, 0.125%, 0.25%, 0.5% and 1.0%.

ELISA 4: A comparison between oven-drying and freeze-drying grass material was made in this ELISA. As described in ELISA 3, material from the basal 8cm region of plants from two *A. lolii*-infected and two *A. lolii*-free lines of cv. Ellett ryegrass was harvested. Each sample was then divided into two approximately equal subsamples. One subsample of each grass was then oven-dried at 60°C for 40 hours, while the other subsample was freeze-dried. All of the material was then ground and stored as before. All of the material was then extracted as described for ELISA 1 and analyzed for *A. lolii* content using IgG 'Cs' with protocol 3.

Results:

ELISA 1: Results in Table 2.1 show that absorances in both *A. lolii*-infected and *A. lolii*-free grasses were considerably higher in samples extracted at 25°C. There were no major differences in absorbances between *A. lolii*-infected and *A. lolii*-free grasses at the two extraction temperatures.

ELISA 2: In both *A. lolii*-infected and *A. lolii*-free samples, increasing the

Table 2.1 **Effect of extraction temperature on absorbance in three endophyte-infected (E+) and three endophyte-free (E-) perennial ryegrass samples. Grass samples were oven-dried at 60°C for 40 hours stored at -20°C and extracted (20 mg grass/ml buffer) for 24 hours at 4°C or 25°C. A double antibody sandwich ELISA (protocol 1) was used with IgG '4p' (1.0 µg/ml).**

	E+(1)	E+(2)	E+(3)	E-(1)	E-(2)	E-(3)
Extraction temperature						
4°C	0.024	0.035	0.053	0.021	0.021	0.049
25°C	0.228	0.265	0.171	0.335	0.104	0.116

concentration of ovalbumin blocker from 0.5% to 1.0% lead to a decrease in absorption (Table 2.2). Ovalbumin at 1.0 and 1.5% reduced the absorbance of the endophyte-free sample to background levels. This pattern was not repeated to the same extent when BSA was used (Table 2.2), although absorbances in the *A. lolii*-infected and *A. lolii*-free sample did appear to decrease at the highest BSA concentration of 1.5%. Extinction values were consistently higher in the *A. lolii*-infected sample.

ELISA 3: Using IgG 'Cs', an increase in BSA concentration resulted in corresponding increases in extinction values in all four grass lines tested (Fig. 2.11). With BSA concentrations of zero and 1.25%, it was impossible to differentiate between *A. lolii*-infected and *A. lolii*-free grasses on the basis of absorbance differences. Small, but consistent, differences between *A. lolii*-infected and *A. lolii*-free grasses were apparent at BSA concentrations of 0.25, 0.5 and 1.0%, with *A. lolii*-infected material registering slightly higher absorbances.

ELISA 4: With endophyte-free material, absorbances were low and were not influenced by the drying method (Fig. 2.12). However, absorbances in freeze-dried *A. lolii*-infected grasses were over five times higher than in their oven-dried counterparts (Fig. 2.12). Using the freeze-drying technique, absorbances in *A. lolii*-infected samples were higher than in *A. lolii*-free samples by a factor of 8.8. Using oven-dried samples, This factor decreased to 1.6.

Discussion:

From Tables 2.1 and 2.2, and from Figure 2.11, it is evident that absorbance differences between *A. lolii*-infected and *A. lolii*-free plants which had been oven-dried prior to extraction, are not significant. As Table 2.1 shows, considerable overlap was possible which is totally unacceptable for a quantitative or qualitative assay.

In comparison with extraction at 4^0C, extinction values of samples extracted at 25^0C were high (Table 2.1). At this point, the assay was obviously not detecting *A. lolii*

Table 2.2 **Effect of different blockers, and their concentration, on absorbance in endophyte-infected and endophyte-free perennial ryegrass. Grass samples were oven-dried at 60°C for 40 hours and extracted (20 mg grass/ml buffer) for 20 hours at 4°C. A double antibody sandwich ELISA (protocol 1) was used with IgG '4p' (1.0µg/ml).**

	Ovalbumin concentration (% w/v)			
	0.1	0.5	1.0	1.5
Endophyte-infected	0.099	0.098	0.047	0.058
Endophyte-free	0.042	0.040	0.000	0.002

	Bovine serum albumin concentration (% w/v)			
	0.1	0.5	1.0	1.5
Endophyte-infected	0.098	0.103	0.112	0.088
Endophyte-free	0.037	0.049	0.046	0.028

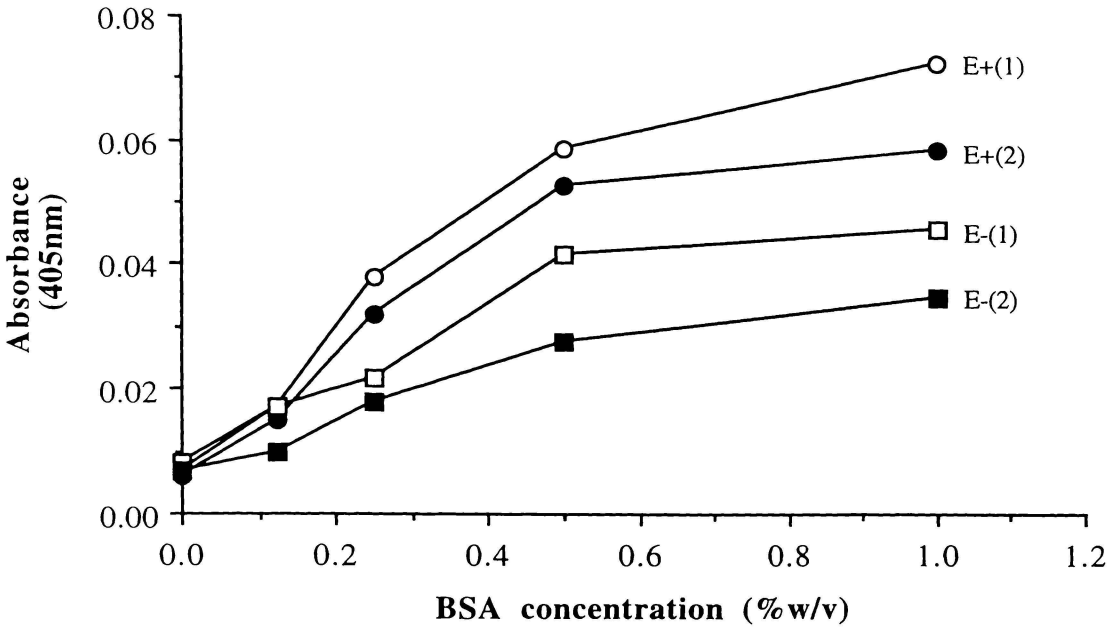


Figure 2.11 Effect of BSA blocking concentration on absorbance in endophyte-infected (E+) and free (E-) ryegrass samples oven-dried at 60°C for 40 hrs. A double antibody sandwich ELISA was used (protocol 3) with IgG 'Cs' at 1.4μg/ml.

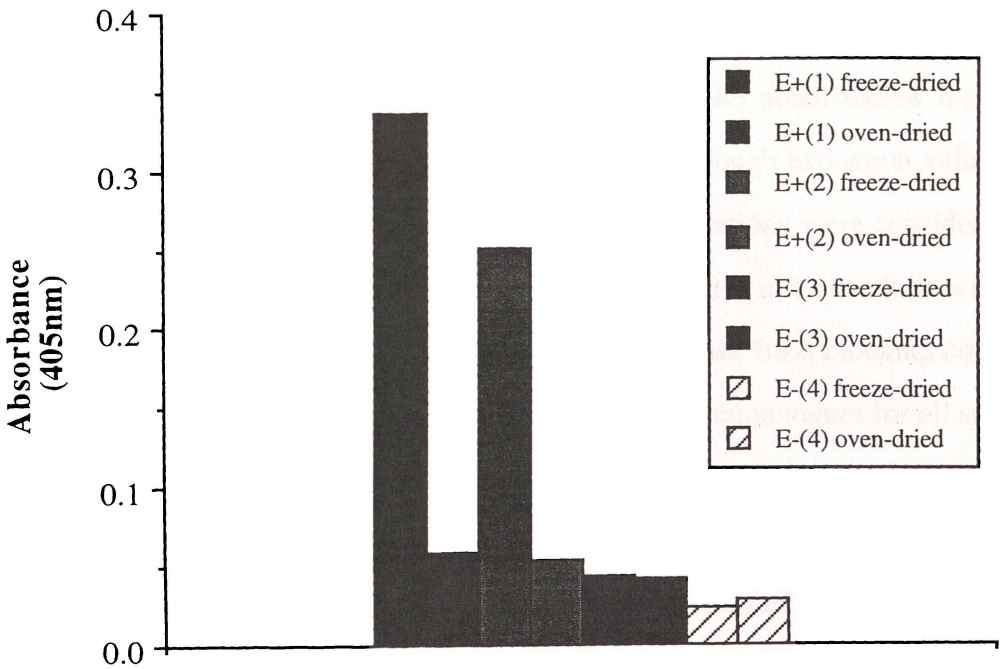


Figure 2.12 Effect of oven-drying (60°C for 40hrs) and freeze-drying on absorbance in two A. lolii-infected (E+(1) and E+(2)) and two A. lolii-free (E-(3) and E-(4)) ryegrasses. Samples were extracted for 24 hrs at 4°C. A double antibody sandwich ELISA was used (protocol 3) with IgG 'Cs' (1.4μg/ml).

antigens, or if it was, they were a minor component, as absorbances obtained with *A. lolii* -free samples were as high as those seen with infected grasses. These results indicate that the assay was therefore almost certainly detecting ryegrass derived antigens, due to shared reactivity (due to the presence of multispecific antibodies which could detect antigens derived from both the endophyte and the grass even if they were very different) and/or due to cross reactivity (due to the presence of common or similar epitopes on the ryegrass and endophyte). In the plant-endophyte context, cross reactivity may be an important factor, as in the majority of studies, serological relationships between plants and their respective fungal parasites have been found (Purkayastha and Ghosal 1985). However, which ever mechanism was operating in the present study, increasing the extraction temperature to 25^{0}C may have resulted in a greater release of reactive antigen, which in turn may have led to the higher extinction values recorded.

At high BSA and ovalbumin concentrations, absorbances in both *A. lolii*-infected and endophyte-free samples decreased when IgG '4p' was used (Table 2.2). Using ovalbumin at 1.0 and 1.5% with IgG '4p', the absorbances recorded from endophyte-free samples fell to background levels. Although extinction values observed in *A. lolii*-infected samples at these blocking concentrations were considerably higher than the background, they were still too low to be effective in a quantitative assay.

In contrast to the situation with IgG '4p', higher BSA blocking concentrations using IgG 'Cs' resulted in correspondingly higher extinction values for all samples (Fig. 2.11). The distinction between *A. lolii*-infected and endophyte-free grass also became clearer at higher blocking concentrations. This was also seen with IgG '4p', and indicates that increasing the protein blocking concentration probably reduced non specific binding of ryegrass antigens. However, in comparison to earlier endophyte studies (Johnson *et al.* 1982 and 1983, Musgrave and Fletcher 1986, Arese *et al.* 1990) the absorbance values for *A. lolii*-infected material were still extremely low, indicating

that the assay was not detecting *A. lolii* antigens effectively enough for it to be of much use.

When samples were freeze-dried rather than oven-dried prior to extraction, absorbances in *A. lolii*-infected samples increased markedly (Fig. 2.12). Corresponding increases were not observed in freeze-dried endophyte-free samples. Thus, to make the distinction between *A. lolii*-infected and endophyte-free perennial ryegrass with a high degree of certainty in this assay, freeze-dried, rather than oven-dried, grass material was required. Detection of plant fungal pathogens by ELISA using freeze-dried plant material has previously been described (Casper and Mendgen 1979, Gleason *et al.* 1987, Barker and Pitt 1988). The results presented in Figure 2.12 also suggest that the assay may have detected different levels of *A. lolii* in the two *A. lolii*-infected samples, as the absorbances are quite different. However, the results also suggest that there is a small grass effect.

Although the results in Figure 2.12 apply only to IgG 'Cs', later results using IgG '4p' showed a similar effect. These results are somewhat surprising as previous enzyme-linked immunosorbent assays against endophytes have worked well using oven-dried grass. Johnson *et al.* (1983) dried grass samples at 60^{0}C, while Musgrave and Fletcher (1986) used a temperature of 80^{0}C. This apparently had no negative effect on endophyte detection and indicates that the major endophyte antigens against which these assays were directed (a polysaccharide moiety of a high molecular weight protein lipopolysaccharide complex in the case of the latter (Musgrave *et al.* 1986)) were relatively heat stable. The loss in signal using oven-dried *A. lolii*-infected grass material in the current study, indicates that the target antigens may have been denatured by heating. Thus the majority of antibodies in IgG 'Cs' (and IgG '4p') appear to be directed against heat sensitive *A. lolii* epitopes. However, it is also possible that other

factors, such as the combination of the 60^{0}C drying temperature and -20^{0}C storage temperature may have been responsible for the reduced signal in oven-dried grass samples.

2.3.4 Detection of *A. lolii* in perennial ryegrass samples: Improvement of detection.

Introduction:

Although it has been established in this study that it is necessary to freeze-dry perennial ryegrass samples to detect *A. lolii* antigens, other factors may be important in influencing the extent of this detection. In the present context, it is important to maximize the sensitivity, detectability, reproducibility and specificity of the assay. An investigation of the factors likely to influence detection of the *A. lolii* antigens is therefore important and may be achieved by selectively manipulating some of the conditions of the ELISA. The effects of Tween 20 concentration in the sample buffer, temperature and duration of sample extraction, sample buffer volume and BSA blocking concentration on detection of *A. lolii* in perennial ryegrass samples were therefore investigated. The effect of using different combinations of IgG '4p' and IgG 'Cs' as conjugate and coating antibodies on detection of *A. lolii* was also tested.

Methods:

ELISA 5: The effect of BSA blocking concentration on detection of *A. lolii* in freeze-dried material, was investigated using IgG 'Cs'. Material from the basal 8cm zone of plants from two *A. lolii*-infected and two *A. lolii*-free lines of cv. Ellett perennial ryegrass was harvested, freeze-dried, ground and stored at -20^{0}C. Grass material from each line was weighed into four separate 25ml scintillation vials and extracted in sample buffer (20mg grass/ml) for 20 hours at 4^{0}C. BSA concentrations of zero, 0.125, 0.25, 0.5 and 1.0% were tested using protocol 3.

ELISA 6: The interactions of the two antibodies were tested in this assay. The basal portions of two *A. lolii*-infected perennial ryegrass plants and one endophyte-free plant, were harvested separately as described in ELISA 5. The material was then freeze-dried and ground as before. Each sample (20mg/ml) was extracted for 20 hours at 4^{0}C, and analyzed for *A. lolii* content using protocol 3. On the plate, each sample was analyzed using the four possible combinations of IgG '4p' and IgG 'Cs' as the coating antibody and the conjugate.

ELISA 7: The effect of sample buffer composition (Tween 20 content), extraction temperature and duration of extraction were tested in this assay. Basal material from one *A. lolii*-infected and one *A. lolii*-free grass was harvested and freeze-dried as before. Each sample (20mg/ml) was extracted over two time periods (6 and 20 hours), at five Tween 20 concentrations (zero, 0.05, 0.2, 0.5 and 1.0%) and at two temperatures (4 and 25^{0}C). IgG 'Cs' was used as the coating antibody and IgG '4p' as the conjugate. Protocol 3 was employed using a BSA blocking concentration of 0.7%.

ELISA 8: The effect of the sample buffer volume on detection of *A. lolii* antigens was tested in this assay. Three *A. lolii*-infected and three endophyte-free plants were harvested and freeze-died as before. Six 100mg samples of each plant were extracted separately (for six hours at room temperature) in six different volumes of sample buffer (3, 4, 5, 7, 10 and 15ml) and assayed for endophyte content using protocol 4. IgG 'Cs' was used as the coating antibody and IgG '4p' as the conjugate.

Results:

ELISA 5: Absorbances in both the *A. lolii*-infected and *A. lolii*-free samples increased as BSA blocking concentrations increased (Fig. 2.13). Absorbances were several times higher in the *A. lolii*-infected samples than in the endophyte-free samples at all BSA concentrations. There was no increase in the intra sample error as BSA concentrations rose. The slope of the relationship between blocking concentration and

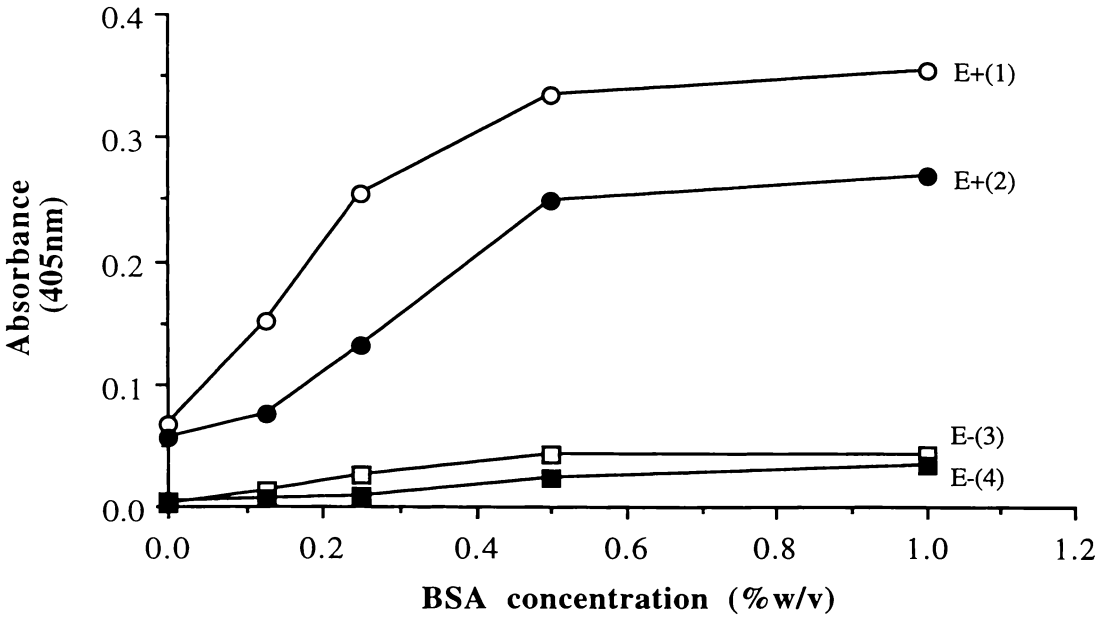


Figure 2.13 Effect of BSA blocking concentration on absorbance in two A. lolii-infected (E+(1) and E+(2)) and two A. lolii-free (E-(3) and E-(4)) ryegrass plants. Grass samples were freeze-dried and extracted (20mg/ml) for 20 hrs at 4°C. A double antibody sandwich ELISA was used (protocol 3) with IgG 'Cs' (1.4μg/ml).

absorbance was particularly steep between BSA concentrations of zero and 0.5%. Absorbances increased only slightly between BSA concentrations of 0.5 and 1.0%. Absorbances between the two *A. lolii*-infected grasses were consistently different in the same direction. This was also the case with the two endophyte-free grasses.

ELISA 6: Results for the *A. lolii*-infected material using the four possible combinations of antibody, show that absorbances were highest when IgG '4p' was used as both the coating antibody and the conjugate, and lowest when conjugate made from IgG 'Cs' was used in a plate coated with IgG '4p' (Fig. 2.14). Absorbances using IgG 'Cs' as the coating antibody and either IgG 'Cs' or IgG '4p' as the conjugate, were intermediate. Absorbances in endophyte-free grass were also considerably higher when IgG '4p' was used as both coating antibody and conjugate (Fig. 2.14). By far the lowest absorbances were achieved using IgG 'Cs' as the coating antibody and IgG '4p' as the conjugate. The mean ELISA index (absorbance of *A. lolii*-infected grass divided by absorbance of endophyte-free grass) using this combination of antibodies was 57.0 and was over three times higher than the ELISA indices achieved using any of the other antibody combinations (Fig. 2.15).

ELISA 7: Irrespective of extraction conditions, absorbances with *A. lolii*-infected material were consistently many times higher than with non-infected material (Fig. 2.16). Raising the extraction temperature from 4 to 25^{0}C resulted in higher absorbances in *A. lolii*-infected grass (Table 2.3). This effect was independent of the duration of the sample extraction, and the Tween 20 concentration. At 4^{0}C and at all but the highest Tween 20 concentrations, absorbances were slightly higher with the longer extraction (Fig. 2.16, Table 2.3). The complete reverse was true for the extractions carried out at 25^{0}C, where shorter extractions tended to yield higher absorbances (Fig. 2.16, Table 2.3). With respect to Tween 20 concentration, absorbances appeared to

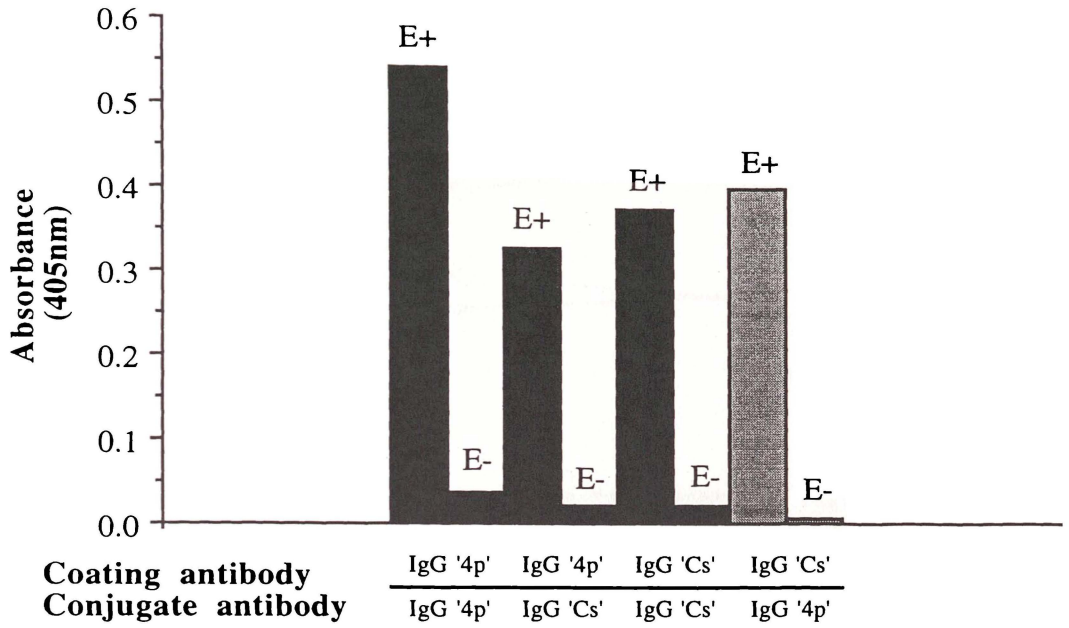


Figure 2.14 Effect of conjugate and capture antibody combinations on absorbance in endophyte-infected (E+) and endophyte-free (E-) ryegrass. Grass samples were freeze-dried and extracted (20mg/ml) for 20 hrs at 4°C. A double antibody sandwich ELISA was used (protocol 3). IgG 'Cs' (1.4μg/ml) was used as the capture antibody and IgG '4p' (1.0μg/ml) was used as the conjugate.

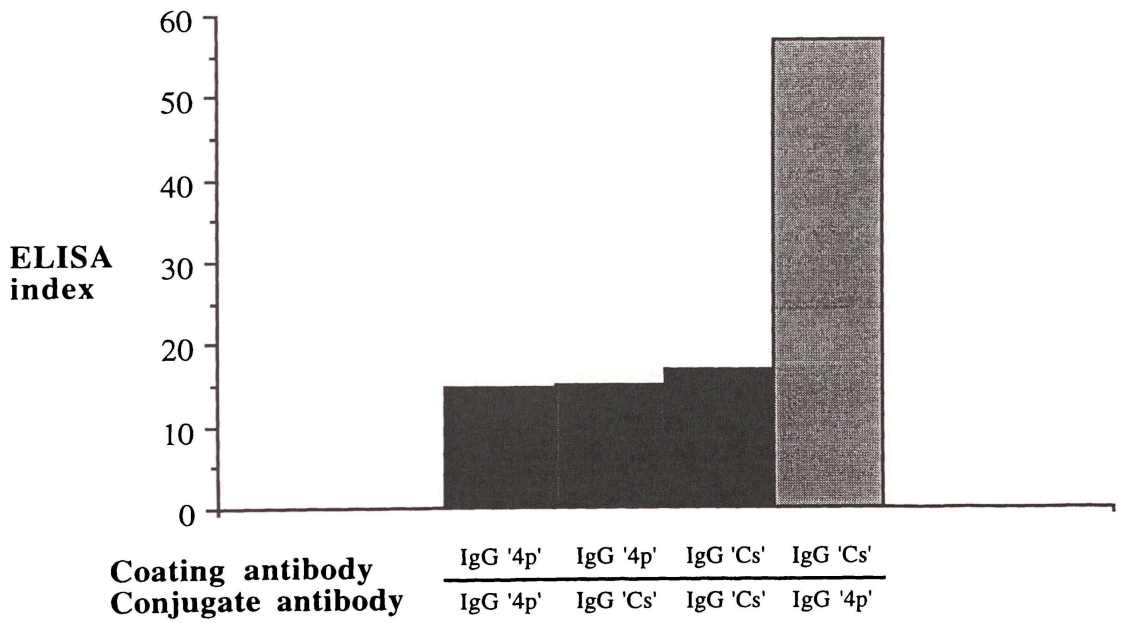


Figure 2.15 Effect of conjugate and capture antibody combination on ELISA index. ELISA conditions were as desribed in Figure 2.14.

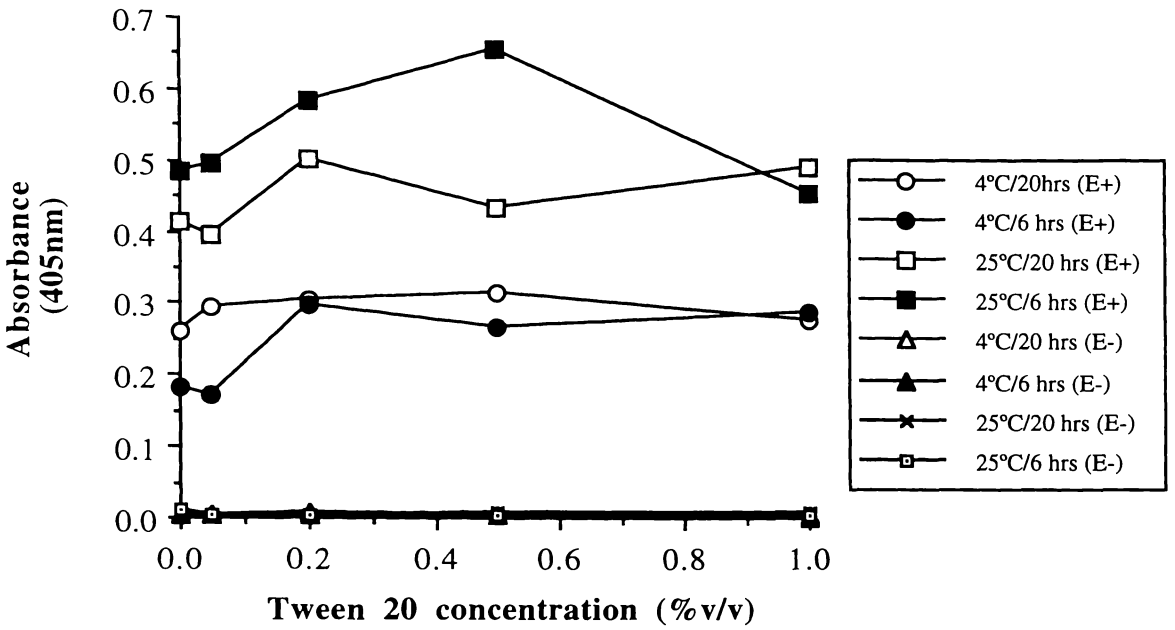


Figure 2.16 Effect of sample buffer Tween 20 concentration, extraction temperature and length of extraction on absorbance in endophyte-infected (E+) and endophyte-free (E-) ryegrass. Grass samples were freeze-dried and extracted at 20mg/ml. A double antibody sandwich ELISA was used (protocol 3) with IgG 'Cs' (1.4µg/ml) as the capture antibody and IgG '4p' (1.0µg/ml) as the conjugate.

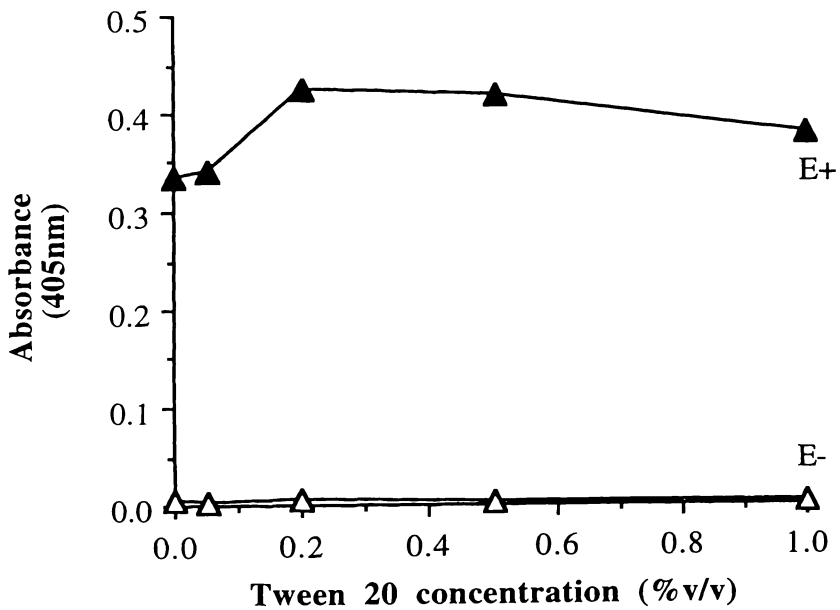


Figure 2.17 Effect of Tween 20 concentration on absorbance in endophyte-infected (E+) and endophyte-free (E-) ryegrass. Grass sample preparation and extraction, and the ELISA conditions used were as described in Figure 2.16.

Table 2.3 **Effects of extraction temperature and duration on absorbance in endophyte-infected and endophyte-free perennial ryegrass samples. The data are adapted from results presented in Figure 2.16. Each datum point is a mean of the five Tween 20 concentrations used. Grass sample preparation and extraction, and the ELISA conditions used were as described for Figure 2.16.**

Extraction conditions	*A.lolii*-infected	*A.lolii*-free
4°C/20 hours	0.291	0.002
4°C/6 hours	0.242	0.004
25°C/20 hours	0.448	0.005
25°C/6 hours	0.536	0.005

peak, or at least tended to be high at concentrations of between 0.2 and 0.5%, irrespective of temperature or duration of extraction (Fig. 2.16). This effect can be seen more clearly in Figure 2.17 where mean values, incorporating the different extraction parameters, have been calculated.

Absorbances with endophyte-free samples were extremely low making it impossible to identify any trends (Figs. 2.16 and 2.17 and Table 2.3).

ELISA 8: With *A. lolii*-infected samples, as the buffer volume increased, absorbances also increased (Fig. 2.18). This trend was completely reversed when the samples consisted of endophyte-free material.

Discussion:

The purpose of blocking a plate (incubating the wells in a solution of protein prior to adding the sample) is to reduce or eliminate non-specific binding of sample material or conjugate onto the solid phase (Clark and Adams 1977). This is achieved by the molecules of protein binding to sites on the plate which are not occupied by immobilized antibody. This then prevents non-specific adsorption in subsequent steps.

It appeared that increasing the concentration of blocker increased the specific reaction, as absorbances in *A. lolii*-infected samples rose considerably (Fig. 2.13). However, the higher absorbances recorded in the endophyte-free samples indicated that non-specific reactions also increased. The higher absorbances as a result of increased blocking in the infected samples, may have been a result of a decrease in the non-specific binding of host grass proteins which would have otherwise sterically interfered with the binding of target antigens onto the immobilized antibody. Most of the non-specific binding was eliminated at a BSA concentration of 0.5%, as indicated by the lack of any appreciable increase in absorbance between BSA concentrations of 0.5 and 1.0% (Fig. 2.13). The absorbance increases recorded in the endophyte-free samples at the higher BSA concentrations may have been due to the BSA promoting some non-specific

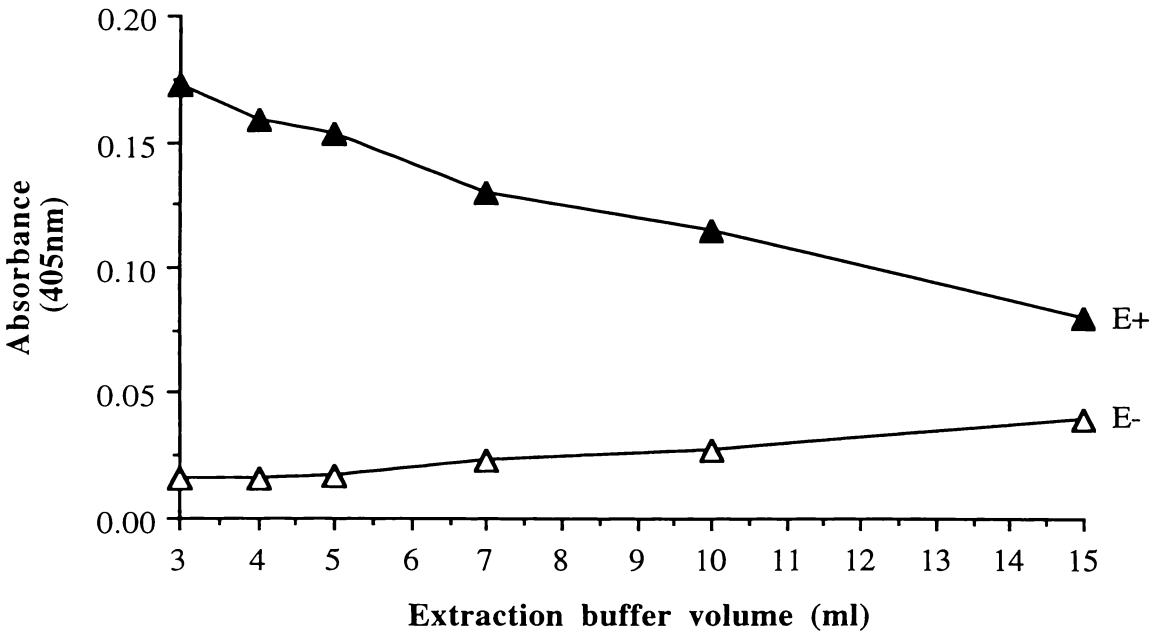


Figure 2.18 Effect of extraction buffer volume on absorbance in A. lolii-infected (E+) and endophyte-free (E-) ryegrass. Grass samples were freeze-dried and extracted for 6 hrs at room temperature. A double antibody sandwich ELISA was used (protocol 4) with IgG 'Cs' (1.4μg/ml) as the capture antibody and IgG '4p' (1.0μg/ml) as the conjugate.

interactions.

Absorbance increases with increasing blocking concentrations in *A. lolii*-infected samples were also noted by Musgrave and Fletcher (1986). However, in their assay, a concurrent increase was not recorded in endophyte-free tissue. This may have been because they used ovalbumin as the blocker, instead of BSA which was used in the current study. Alternatively, the different results may be due to intrinsic differences in the properties of the two groups of antibodies. Whatever the reason, with the exception of the assay developed in this study, the ELISA described by Musgrave and Fletcher (1986) (which was an improved version of an earlier assay (Musgrave 1984) where a blocking step was not included) is the only other anti-endophyte assay to have used a blocking step. Anti-endophyte assays developed by Johnson *et al.* (1982), Reddick and Collins (1988) and Arese *et al.* (1990) have not used an additional blocking step (these studies do not report whether the effects of plate blocking were investigated). The absence of a blocking step appears to be more the rule than the exception in the majority of anti-fungal assays using the double antibody sandwich method (Nachmias *et al.* 1979, Notermans and Heuvelman 1985, El-Nashaar *et al.* 1986). The blocking step is therefore not a crucial element in most assays. Although it was not crucial for the differentiation between *A. lolii*-infected and endophyte-free grass in the current assay, blocking with BSA considerably reduced non-specific binding. Even though this increased absorbances in non-infected as well as infected material, the signal was still very low in endophyte-free grass. A BSA concentration of 0.7% was therefore chosen for future assays, as this was the lowest BSA concentration at which non-specific binding was almost completely eliminated (Fig. 2.13).

Widely differing absorbances in both *A. lolii*-infected and endophyte-free samples were recorded using the four different combinations of antibody (Fig. 2.14). In comparison with IgG 'Cs', absorbances in *A. lolii*-infected samples were substantially

higher when IgG '4p' was used as both capture antibody and conjugate, even though a slightly lower antibody concentration was used (1.0μg/ml as opposed to 1.4μg/ml for IgG 'Cs'). This suggests that IgG '4p' has a higher avidity for *A. lolii* antigens than IgG 'Cs'. A high affinity or avidity is often associated with increased levels of non-specific binding (Tijssen 1985). The substantially higher absorbance recorded in non-infected material suggests that this was the case with IgG '4p'.

The lowest absorbance in endophyte-free material was achieved using IgG 'Cs' as the capture antibody and IgG '4p' as the conjugate (Fig. 2.14). Because of this, and because absorbances in *A. lolii*-infected samples were relatively high, the ELISA index using this antibody combination was considerably higher than that achieved using the other antibody combinations (Fig. 2.15).

In comparison with results obtained by Musgrave (1984) and Musgrave and Fletcher (1986), the ELISA index of 57.0 obtained in the present study, was high. The highest ELISA indices achieved by Musgrave and Fletcher (1986) for plant material, were in the region of 10 - 15. However, it is impossible to compare ELISA indices directly, as these are highly dependant upon the absorbance registered by the reference non-infected grass as well as by the concentration of endophyte in the sample. As the results from Figure 2.13 suggest, absorbances from different endophyte-free samples may be highly variable, indicating a possible grass effect. Even a small amount of variation was likely to have a considerable effect on the ELISA index, as absorbances from endophyte-free samples were so low. Therefore, in the context of the present study, the ELISA index is being used as a measure of the ability of the serum to distinguish between *A. lolii*-infected and *A. lolii*-free samples, rather than as an absolute measure of the concentration of *A. lolii* in the sample material. The higher the ELISA index, the better the distinction between *A. lolii*-infected and endophyte-free samples. As ELISA indices were highest when IgG 'Cs' was used as the capture antibody and

IgG '4p' as the conjugate, this combination and configuration of antibodies was used in all future assays.

Results from Figure 2.16 and Table 2.3 show that extraction temperature had a considerable effect on detection of *A. lolii* antigens in grass material. Absorbances using a 25^{0}C extraction for 20 hours and 6 hours, were, respectively, 1.5 and 2.2 times higher than those recorded at an extraction temperature of 4^{0}C. The fact that absorbances in endophyte-free samples remained virtually unchanged at the higher temperature, suggests that *A. lolii* antigen extraction efficiency, rather than increased non-specific binding, was responsible for the absorbance increase. Determination of the precise factors responsible for the more efficient extraction of *A. lolii* antigens at the higher temperature is beyond the scope of this project but may be explainable in simple thermodynamic terms.

In their investigation into the effects of extraction temperature, Musgrave and Fletcher (1986) concluded that detection of the protein lipopolysaccharide antigen of *A. lolii* was best achieved at 5^{0}C. In complete contrast to the results of the present study, they also found that the antigenic reactivity of the sample decreased following solubilization if the temperature of the sample was allowed to rise above 5^{0}C. These differences are contrary to what one would expect, as oven-drying at 80^{0}C did not appear to reduce the reactivity of *A. lolii*-infected samples (Musgrave and Fletcher 1986), while drying grass material at 60^{0}C in the current study led to a complete elimination of the signal.

In addition to a temperature effect in *A. lolii*-infected material, the time period over which the extraction was performed also appeared to influence absorbance. At a temperature of 4^{0}C, a longer extraction resulted in a higher absorbance at all but the highest Tween 20 concentration (Fig. 2.16, Table 2.3). Completely the reverse was true

using an extraction temperature of 25°C. Once again, determining the precise causes of these effects is beyond the scope of this project. However, the patterns may illustrate that a fine balance exists between efficiency of antigen extraction and antigen breakdown (by enzyme activity in the sample for example). At both temperatures, a longer extraction time may have resulted in the solubilization of more of the *A. lolii* antigens present in the sample. However, at the higher temperature, the rate of antigen denaturation may have been faster than that at the lower temperature. Thus, at 4°C, the advantage of a longer extraction time may not have been negated by the effects of antigen denaturation as was possibly the case at 25°C.

Although the effect was not as pronounced, Tween 20 concentration also appeared to influence absorbance in *A. lolii*-infected material over and above the effects of temperature and duration of extraction. The lowest absorbances were generally recorded with no Tween 20, or at the lowest Tween 20 concentration of 0.05% (Figs. 2.16 and 2.17). With the exception of the six hour extraction at 25°C using a Tween 20 concentration of 1.0%, detection of *A. lolii* antigens improved at Tween 20 concentrations of 0.2, 0.5 and 1.0%. Although assay differences make direct comparisons with other studies difficult, it is interesting to note that a similar effect was observed by Musgrave and Fletcher (1986) who compared Tween 20 concentrations of 0.1, 0.2 and 0.4% and found that absorbance of oven-dried *A. lolii*-infected samples was directly proportional to Tween 20 concentration.

The inclusion of Tween 20 in the sample buffer has a two fold effect. Firstly, it reduces non-specific binding by preventing the formation of new hydrophobic interactions between sample proteins and the solid phase (Clark and Adams 1977, Clark and Engvall 1980). Secondly, as Tween 20 is a wetting agent, its presence would lead to greater infiltration of buffer into the sample material, possibly aiding the antigen

extraction process. Results from Figure 2.17 show that absorbances were higher when *A. lolii*-infected material was extracted in the presence of Tween 20, indicating a more complete antigen extraction. Absorbances in endophyte-free material appeared to be independent of Tween 20 content (Fig. 2.17). This was not the case in the work carried out by Musgrave and Fletcher (1986), who found that absorbances in non-infected samples were directly proportional to Tween 20 content, indicating increased non-specific binding. Once again however, comparing the results obtained in the current study with those of Musgrave and Fletcher (1986) is difficult considering the many differences between the two assays.

The results from ELISA 7 indicate that the assay could operate adequately under all of the conditions tested. However, a 6 hour sample extraction at 25^{0}C with a Tween 20 concentration of 0.5% was decided upon. The extraction temperature of 25^{0}C was favoured as absorbances at this temperature were considerably higher than those at 4^{0}C (Fig. 2.16 and Table 2.3) indicating a more complete extraction of the antigen. The shorter extraction period (6 hours) at 25^{0}C was favoured over the longer one (20 hours) for two reasons. Firstly, absorbances of *A. lolii*-infected samples were generally higher in the 6 hour extraction than in the 20 hour extraction. Secondly, operational convenience was maximized as the time required to complete an assay was much shorter. A Tween 20 concentration of 0.5% was chosen, as the grass extraction at 25^{0}C over 6 hours appeared to function best at this concentration (Fig. 2.16).

As extraction buffer volume decreased, mean absorbances in *A. lolii*-infected samples increased (Fig. 2.18). This would be expected assuming that the amount of antigen extracted at each dilution is constant. Detection of *A. lolii* antigens was possible down to the lowest dilution of 15ml sample buffer per 100mg grass. This effect was also recorded in a completely different assay by Nachmias *et al.* (1979) with *Phoma*

tracheiphila infection in lemon trees. However, in contrast with the current study, these workers also recorded an absorbance increase in healthy material as extraction buffer volumes decreased. The absorbance decrease recorded in non-infected material with decreasing sample buffer volume in the present study, was advantageous to the assay (in terms of the ability of the assay to distinguish between infected and non-infected grass), but mystifying. Unless endophyte concentrations were particularly low and a greater limit of detection was necessary, a tissue/buffer ratio of 20mg/ml was used.

2.3.5 Assay reproducibility.

Introduction:

To be useful, an ELISA must yield highly reproducible results which are also accurate. In the case of *A. lolii* infection in perennial ryegrass, it is difficult to assess the accuracy of the assay as endophyte contained in grass may present quite a different antigen profile to the same amount of endophyte culture with which the sample is being compared. As this is also a common problem in biological systems in general, it has even been previously suggested that ELISA results be expressed in arbitrary units rather than in absolute terms (Voller 1980). However, it is possible to determine assay reproducibility. This can be done by comparing results from two (or more) ELISA's using identical samples in each assay. This aspect was therefore tested, both in *A. lolii*-infected and endophyte-free perennial ryegrass samples. Inter-well (intra-plate) variation in absorbance was also tested, using both *A. lolii*-infected ryegrass samples and a pure *A. lolii* dilution series. As the number of replicates in the dilution series was sufficient, the limit of detection of the assay was ascertained.

Methods:

ELISA 9: Material from the basal 8cm zone of seven different A. *lolii*-infected perennial ryegrass plants was harvested in February 1992, freeze-dried and ground to a fine powder. These samples were analyzed for *A. lolii* content using the ELISA on two

separate occasions. Protocol 4 was used on both occasions with all four antibody combinations. Linear regression analysis was used to determine whether absorbances on the two occasions were related and whether the relative level of detection of each grass sample did not differ between IgG 'Cs' and IgG '4p'.

ELISA 10: This was identical to ELISA 9, except that eight endophyte-free grasses were used.

ELISA 11: Regrowth plant material down to a height of 8cm of seven different *A. lolii*-infected perennial ryegrass plants was harvested in January 1992, freeze-dried and finely ground. As in ELISA 9, these samples were analyzed for *A. lolii* content using the ELISA on two separate occasions (28/4/92 and 3/5/92). Protocol 4 was used on both occasions with IgG 'Cs' as the capture antibody and IgG '4p' as the conjugate. The sample absorbances obtained in the two assays were converted to a percentage by dividing them by the absorbance of a 3µg/ml *A. lolii* standard included on each plate, which was automatically assigned a value of 100%. The results of this ELISA were used to determine whether it would be possible to calculate *A. lolii* concentrations of grass samples by simply including the 3µg/ml *A. lolii* standard on each plate, converting the sample absorbances to a percentage of the standard, and converting the percentages to concentrations using a single *A. lolii* dilution curve. The dilution curve used is described in ELISA 12.

ELISA 12: A liquid culture of *A. lolii* (cv. Ellett) was maintained and filtered according to the description in section 2.2.1. The mycelium was then freeze-dried and ground to a fine powder by mortar and pestle. The mycelium was then extracted in sample buffer (10mg/ml) for 6 hours at room temperature. A three-fold dilution series (from 625µg/ml to 0.011µg/ml) was made from the supernatant which was then subjected to the ELISA test using protocol 4 with IgG 'Cs' as the capture antibody and IgG '4p' as the conjugate. Six replicate wells were used at each antigen dilution. The

percent coefficient of variation (CV%) which simply expresses the standard deviation (SD) as a proportion of the mean absorbance (x) at each antigen concentration was calculated using CV% = (SD/x) x 100 to yield a precision profile (Tijssen 1985).

ELISA 13: Regrowth plant material from three different *A. lolii*-infected ryegrass plants was harvested in February 1992, freeze-dried and ground to a fine powder. 100mg of each sample was extracted as described in section 2.3.3 and analyzed for *A. lolii* content using the ELISA with the same combination of antibodies as that used in ELISA 12. Ten randomly assigned replicate wells were used for each sample. The mean absorbance, SD and CV% were then calculated for each sample.

Results:

ELISA 9: For *A. lolii*-infected samples, absorbances in the first ELISA were positively correlated with absorbances in the second, with all four antibody combinations (Figs. 2.19a-d and Table 2.4). This relationship was significant at the 0.1% level for all antibody combinations with the exception of the combination using IgG 'Cs' as both capture and conjugate antibody, which was significant at the 1.0% level.

ELISA 10: For endophyte-free samples, absorbances between the two ELISA's were not significantly correlated when IgG '4p' was used as the capture antibody and IgG 'Cs' as the conjugate (Fig. 2.20c and Table 2.4). Absorbances were significantly correlated when all other antibody combinations were used (Figs. 2.20a, b and d and Table 2.4). The level of significance was lower when IgG 'Cs' was used as both capture and conjugate antibody ($P< 0.05$) (Fig. 2.20b), compared to when IgG '4p' was used as both capture and conjugate antibody ($P< 0.001$) (Fig. 2.20a) or when IgG 'Cs' was used as capture antibody and IgG '4p' as the conjugate ($P< 0.001$) (Fig. 2.20d).

In comparison with *A. lolii*-infected grasses, $\%r^2$ values in endophyte-free

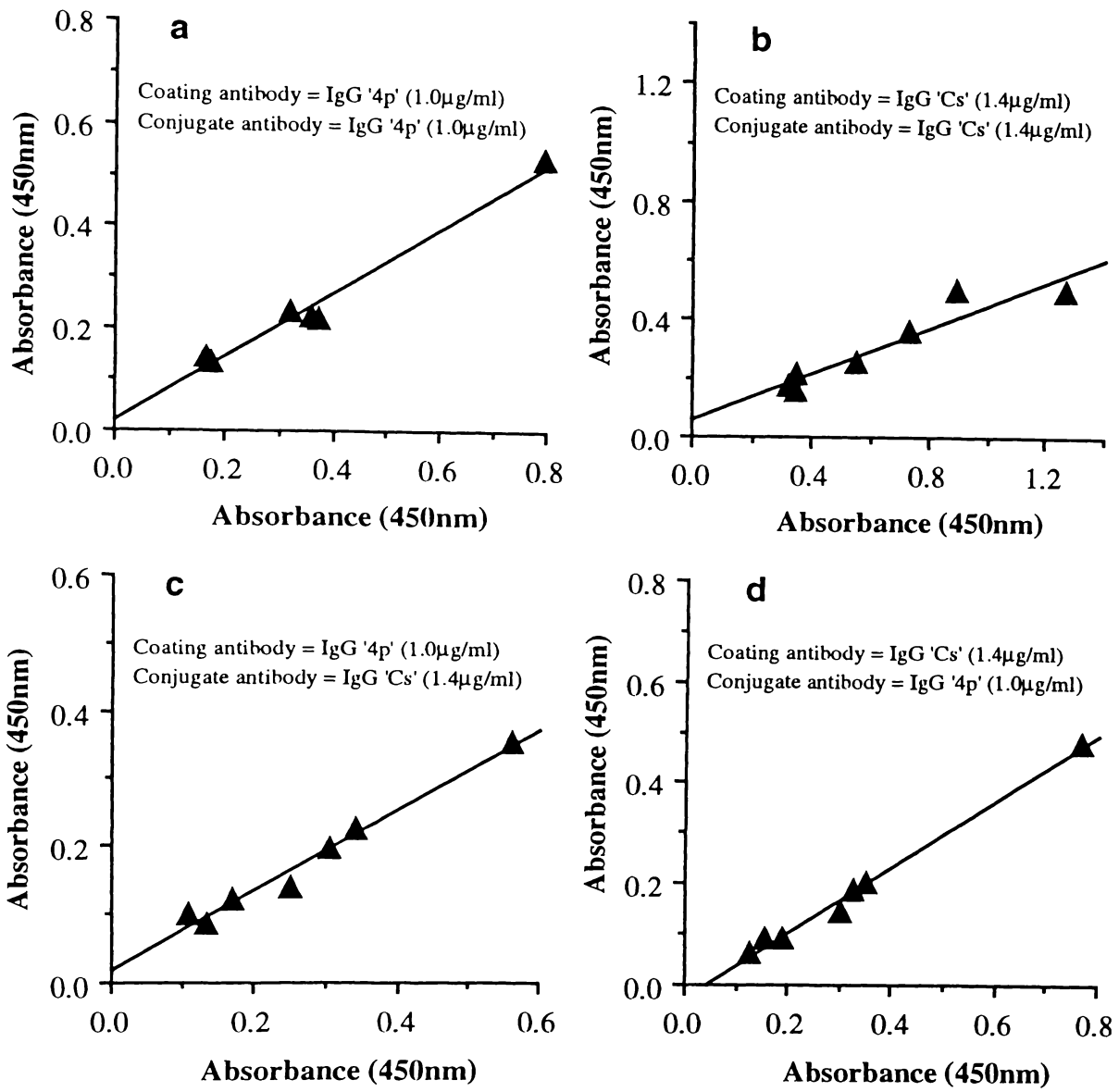


Figure 2.19a-2.19d
Linear regression analysis showing relationships between absorbances obtained from two separate ELISA's performed on seven A. *lolii*-infected ryegrass samples using different combinations of coating and conjugate antibodies. Grass samples were freeze-dried and extracted (20µg/ml) for 6 hrs at room temperature. Double antibody sandwich ELISA's were used (protocol 4).

Table 2.4 **Indicator of assay reproducibility using coefficient of determination values ($\%r^2$) obtained by comparing absorbances of seven endophyte-infected and eight endophyte-free plants assayed on two separate occasions. Grass sample preparation and extraction, and the ELISA conditions used were as described in Figure 2.19.**

	conjugate antibody	4p	Cs	Cs	4p
	capture antibody	4p	Cs	4p	Cs
A.lolii infected		97.6***	87.4***	97.5***	98.9***
A.lolii-free		82.6***	50.4*	30.2	83.5***

* P<0.05
** P<0.01
*** P<0.001

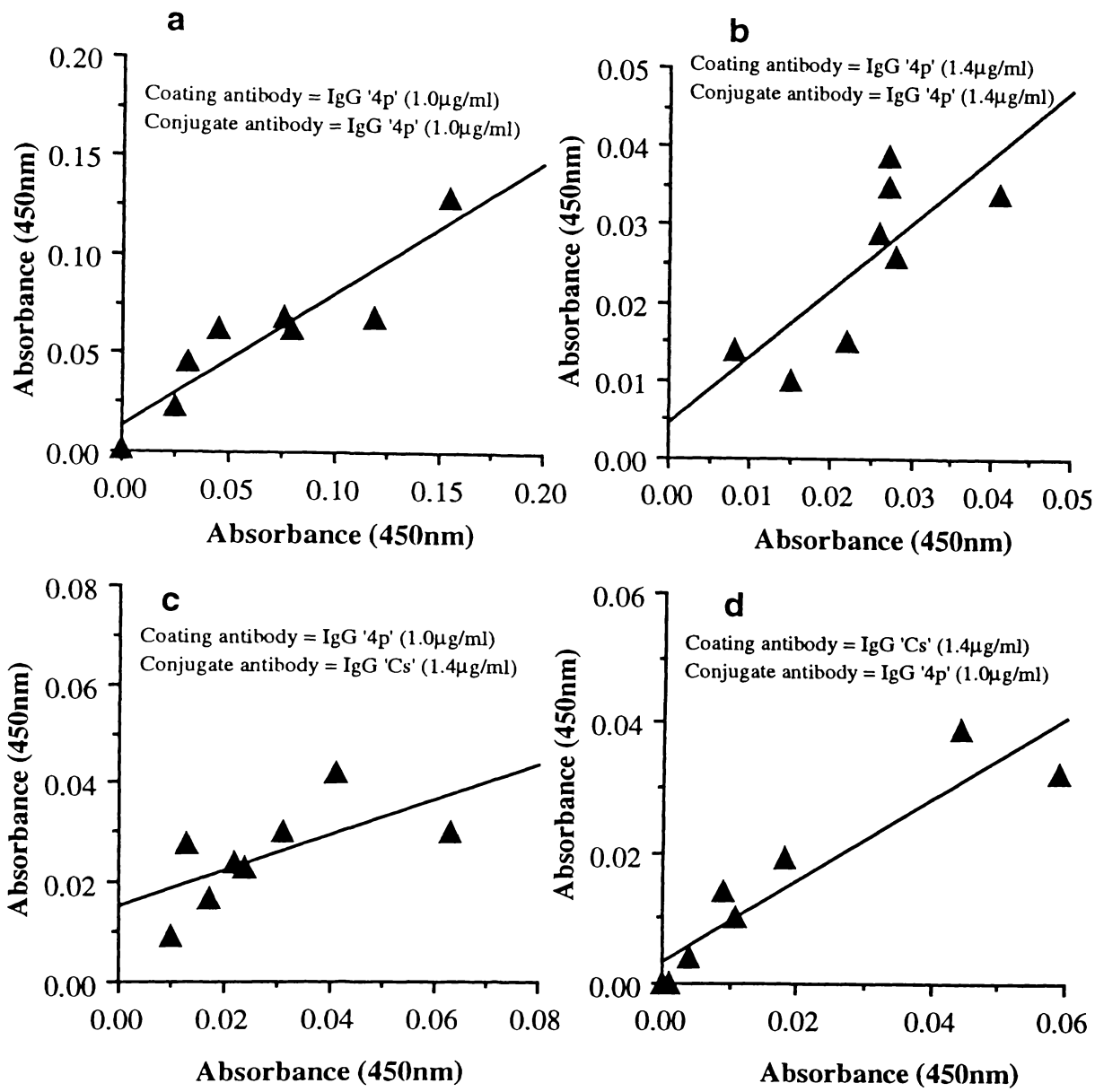


Figure 2.20a-2.20d
Linear regression analysis showing relationships between absorbances obtained from two separate ELISA's performed on eight A. *lolii*-free ryegrass samples using different combinations of coating and conjugate antibodies. Grass sample preparation and extraction, and the ELISA conditions used, were as described in Figure 2.19.

grasses were lower for all antibody combinations (Table 2.4).

Absorbances were highest when IgG '4p' was used as both capture and conjugate antibody (Fig. 2.20a). Absorbances in one particular endophyte-free sample were very high (0.155 and 0.130).

ELISA 11: *A. lolii* concentrations of the seven *A. lolii*-infected ryegrass plants analyzed in the two separate assays, were similar (Fig. 2.21). Concentrations were slightly higher in six out of the seven plants analyzed on 28/4/92.

ELISA 12: Mean absorbances yielded a typical sigmoidal curve for the *A. lolii* dilution series (Fig. 2.22). From the highest antigen concentration of 625µg/ml to the third lowest of 0.095µg/ml, CV% were below 6%. At the two lowest antigen concentrations of 0.032 and 0.011µg/ml, CV% values increased to 13.4 and 40.1% respectively. Absorbances at the lowest antigen concentration of 0.011µg *A. lolii* mycelium/ml were above the mean plus three standard deviations of the absorbance of the negative wells.

ELISA 13: CV% for the three replicated endophyte-infected ryegrass samples ranged between 6.99 and 8.82% (Table 2.5).

Discussion:

Results from Figures 2.19a-d illustrate clearly why, when comparing results from different plates, *A. lolii* concentration cannot be quoted as a simple absorbance. Absorbances for identical *A. lolii*-infected samples varied considerably between the two ELISA's. This may have been caused by a slight change in the conditions in any of the 9 steps of the ELISA. This inter-plate variation may be avoided by including all samples which are to be compared, on the same plate. Comparing sample absorbances against a pure *A. lolii* standard or dilution series included on each plate, should also nullify this inter-plate effect.

Even with the inter-plate effect, the results from Figures 2.19a-d and Table 2.4

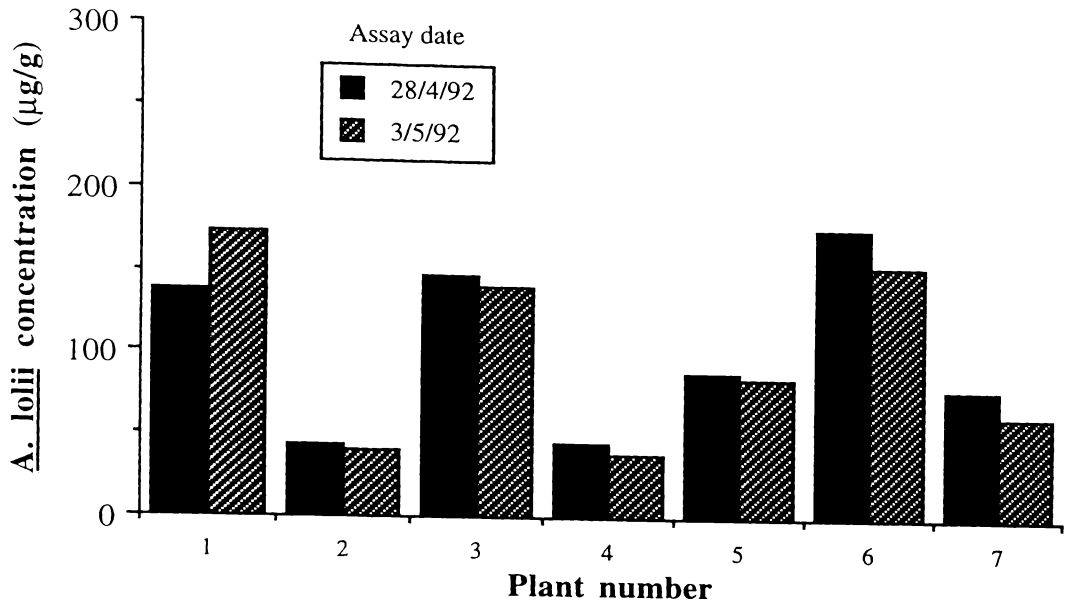


Figure 2.21 A. lolii concentrations of seven endophyte-infected ryegrass plants determined in two separate ELISA's (on 28/4/92 and 3/5/92) using a 3µg/ml A. lolii standard included on each plate. Endophyte concentrations were then calculated using a dilution curve similar to the one shown in Figure 2.22. Double antibody sandwich assays (protocol 4) were used with IgG 'Cs' (1.4µg/ml) as the capture antibody and IgG '4p' (1.0µg/ml) as the conjugate.

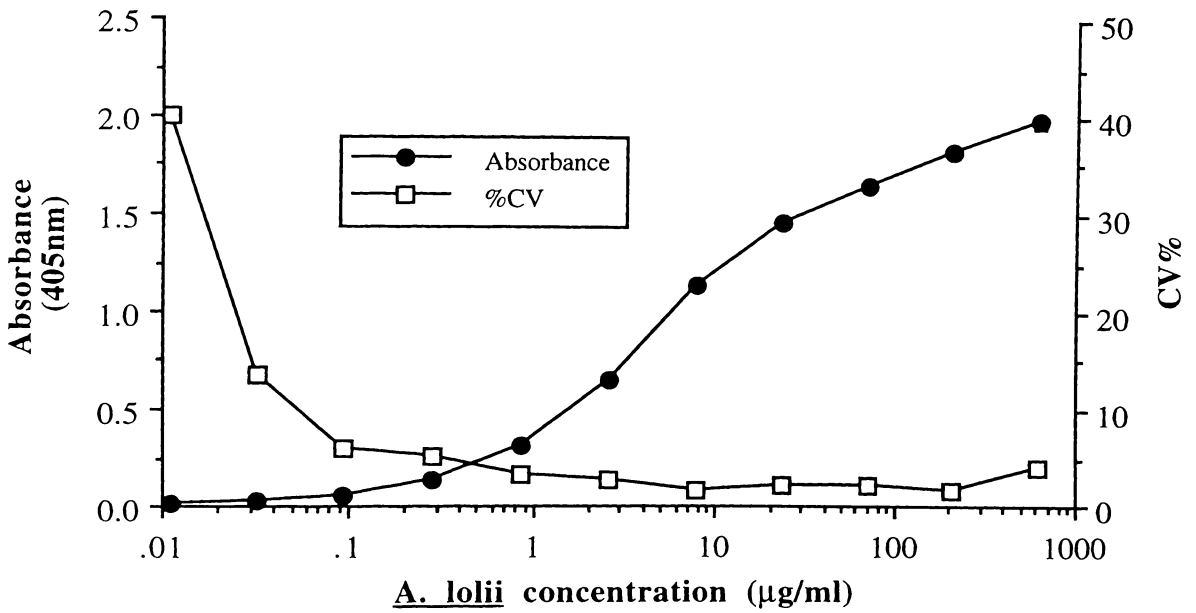


Figure 2.22 Absorbance curve and precision profile (CV%) for detection of A. lolii at a range of concentrations, determined using a double antibody sandwich ELISA (protocol 4) with IgG 'Cs' (1.4µg/ml) as the capture antibody and IgG '4p' (1.0µg/ml) as the conjugate. Freeze-dried A. lolii mycelium was extracted at 10mg/ml for 3 hrs at room temperaure. Bars represent SEM.

Table 2.5 **Results from a double antibody sandwich ELISA (protocol 4) investigating the extent of replicate well variation in three endophyte-infected ryegrass samples. Ten replicate wells were used for each sample and results were used to calculate mean absorbances ($\bar{x}A_{405}$), as well as standard deviations (SD) and percent coefficient of variations (CV%) for each grass. Grass samples were freeze-dried and extracted (20 mg/ml) for 6 hours at room temperature. IgG 'Cs' (1.4µg/ml) was used as the capture antibody and IgG '4p' (1.0µg/ml) was used as the conjugate.**

Plant number	$\bar{x}A_{405}$	SD	CV%
7424 T	0.471	0.0329	6.99
6617 T	0.478	0.0400	8.37
2028 T	0.238	0.0210	8.82

show that, which ever antibody combination was used, semi-quantitative detection of *A. lolii* in grass samples was highly reproducible indicating that relative *A. lolii* levels in experimental samples could be quoted with a reasonable degree of confidence. It is interesting to note that reproducibility was lowest when IgG 'Cs' was used as the source of both capture and conjugate antibody (Fig. 2.20b). This may be due to the lower affinity which this antibody appears to have for *A. lolii*.

The significant correlations observed in endophyte-free samples in all but one antibody combination (Figs. 2.20a, b and d) and Table 2.4), are a concern, as this could mask an endophyte effect. Such an effect could be due to the presence of other fungi in the sample. However, it is more likely to be a real grass effect as results from section 2.3.6 indicate that fungi other than those of the genus *Acremonium* (section *Albo-lanosa*) are not detected, even at extremely high concentrations. As a precautionary measure, it could be advantageous to use IgG '4p' as the capture antibody and IgG 'Cs' as the conjugate, as the grass effect was least apparent with this combination (Fig. 2.20c). However, a decision to stay with IgG 'Cs' as the capture antibody and IgG '4p' as the conjugate was made, as absorbances in endophyte-free samples using this combination were so low as to make any grass effect negligible. Absorbances in endophyte-free samples using IgG '4p' as both capture and conjugate antibody (Fig. 2.20a) were higher than would perhaps be desirable in an assay. Of particular concern was the grass sample which registered absorbances greater than 0.1 absorbance units in both assays. On re-examination (by staining and alkaloid analyses), the grass was confirmed as being endophyte-free, and when used as a negative control, the absorbance registered by this grass never exceeded absorbances registered by any endophyte-infected sample.

Results indicated (Fig. 2.21) that assay reproducibility was also high when *A. lolii* concentrations were calculated by converting sample absorbances to a percentage of

an *A. lolii* standard included on each plate. *A. lolii* concentrations were calculated by relating the percentages to a dilution curve similar to that in Figure 2.22. This result is important as it enables inter-plate comparisons to be made when these are necessary. It is interesting to note however, that concentrations in six out of the seven ryegrass samples were slightly higher in the ELISA performed on 28/4/92 than they were in the 3/5/92 assay. This may be due to chance. However, it could also be due to slight differences in the conditions between the two assays and/or due to a lack of complete homogeneity in the *A. lolii* standard used. Whatever the reason, this effect could perhaps be circumvented by including, when possible, all ryegrass samples to be compared, on the same plate.

The precision profile (Fig. 2.22) is a means of visualizing the performance of an immunoassay over a range of antigen concentrations. Results from Figure 2.22 show that between *A. lolii* concentrations of 625µg/ml to 0.095µg/ml, CV% were below 6%. As is commonly seen in immunometric assays (Feldkamp 1992), CV% rose drastically at the lower antigen concentrations. A CV% of 10% or less should be obtainable in most assays at most concentrations (Feldkamp 1992). With a CV% cutoff point of 10%, the working range of the current assay begins at an *A. lolii* concentration of approximately 0.06µg/ml, below which the precision of the assay might be questioned (Fig. 2.22). However, by standards commonly used in plant ELISA's (Sutula *et al.* 1986), the limit of detection of the assay appears to lie close to the lowest antigen concentration of 0.011µg/ml, as the absorbance registered at this concentration was more than the mean absorbance plus three standard deviations of the negative control wells. This is only an approximate limit of detection however, as it is difficult to decide upon a precise limit of detection of an assay, even with the aid of statistics (Sutula *et al.* 1986).

The CV% of the three replicated *A. lolii*-infected plant samples (Table 2.5) all lay below the cutoff point of 10%, indicating that well to well variation within the plate was

acceptably low.

Results from ELISA's 9-13 indicate that the assay is sufficiently reproducible and precise above an antigen concentration of 0.06µg/ml, to be used in the routine analysis of *A. lolii* in perennial ryegrass samples.

2.3.6 Assay specificity and the effect of host grass matter on *A. lolii* detection.

Introduction:

Antibodies to fungi have been described as being notoriously non-specific, cross reacting widely with both closely and distantly related fungi, as well as with host molecules (Mohan and Ride 1982, Gendloff *et al.* 1983, Kough *et al.* 1983, Gleason *et al.* 1987, Dewey and Brasier 1988, Dewey *et al.* 1989). This is certainly true when the specificity achieved in fungal immunoassays is compared with that achieved against viruses (Kelly *et al.* 1978, Koenig 1978).

Species specificity has never been achieved in assays against endophytic fungi using polyclonal antibodies. Musgrave (1984) found that his *A. lolii* based ELISA did not serologically distinguish between isolates of *A. lolii*, *A. coenophialum* and *Epichloe typhina*. At high endophyte concentrations, Johnson *et al.* (1985b) also found that *A. lolii* and *A. coenophialum* were serologically indistinguishable using antibodies raised against *A. coenophialum*. There was also a considerable amount of cross reactivity at lower endophyte concentrations. Considerable modification of the ELISA has also failed to eliminate cross reactivity at the species level, although it was reduced (Reddick and Collins 1988). However, in all endophyte assays, antibody specificity has been sufficient to overcome the problems posed by fungal contaminants (Johnson *et al.* 1982, Musgrave 1984, Musgrave *et al.* 1986, Arese *et al.* 1990).

In the context of the present study, developing a highly species specific assay is not the aim, as *A. lolii* will be the only *Acremonium* species present in the experimental

grasses (as determined microscopically). However, like previous assays against endophytes, the antibodies must be specific enough to distinguish between *A. lolii* and the other fungal contaminants which are likely to be present. Cultures of fungi from several genera were therefore subjected to the ELISA test to investigate the extent of the specificity of the *A. lolii* antibodies. In the case of the *Gliocladium*-like endophyte, potentially the most serious contaminant, this aspect was also tested in grass samples. Species specificity was further investigated by testing the reactivities of two other endophytic *Acremonium* species (*A. starrii* and *A. coenophialum*) , both in pure culture and in grass samples. As an important aspect of specificity, the effect of host grass material on detection of *A. lolii* was also investigated.

Methods:

ELISA 14: The specificity of the *A. lolii* antibodies were tested using ELISA, by comparing the reactivities of fungal cultures of *A. lolii* (from cv. Yatsyn), *A. coenophialum* (two lines from tall fescue (*Festuca arundinacea*), *A. starrii* (two lines from *Bromus anomalus*), *A. strictum*, *Gliocladium*-like sp., *Pithomyces chartarum* and *Penicillium paxilli*. Cultures of all of the above fungi were maintained and filtered according to the description in section 2.2.1. After filtration, the mycelial fractions of each fungus were placed into separate petri dishes, freeze-dried and ground to a fine powder by mortar and pestle. The finely ground mycelium from each fungus was then extracted in sample buffer (10mg/ml) for 6 hours at room temperature. A three-fold dilution series (from 10000µg/ml to 0.17µg/ml) was made from the supernatant of each extract, which was then subjected to the ELISA test using protocol 4, and IgG 'Cs' as the capture antibody and IgG '4p' as the conjugate.

ELISA 15: The reactivity of *B. anomalus* both with and without its natural endophyte, *A. starrii*, and the reactivity of tall fescue with and without *A. coenophialum*, was compared to that of *A. lolii*-free and infected perennial ryegrass.

Perennial ryegrass infected with only *Gliocladium*-like endophyte was also tested. Material from the basal 8cm region of three different plants from each category (with the exception of the *Gliocladium*-like infected plants where only two such individuals were available), was harvested, freeze-dried and finely ground. These were extracted (20mg/ml) for 6 hours at room temperature and subjected to the ELISA test using IgG 'Cs' as the capture antibody and IgG '4p' as the conjugate, with protocol 4.

ELISA 16: The effect of host grass constituents on detection of *A. lolii* was tested. A three-fold dilution series of *A. lolii* was prepared as described for ELISA 14. A second three-fold dilution series of *A. lolii* was prepared using the same method except that endophyte-free perennial ryegrass extract (20mg/ml) was used in place of pure sample buffer. The two serial dilutions were subjected to the ELISA using protocol 4, and IgG 'Cs' as the capture antibody and IgG '4p' as the conjugate.

Results:

ELISA 14: Results for the four endophytic fungi (*A. lolii*, *A. coenophialum*, *A. starrii* and *Gliocladium*-like) and the three other fungi (*P. chartarum*, *P. paxilli* and *A. strictum*), are presented in Figures 2.23 and 2.24 respectively. Reactivities are expressed as percentages which were calculated by dividing the absorbance obtained for *A. lolii* at the highest concentration (which is therefore automatically assigned a value of 100%), by the absorbance of the sample. This was to standardize the results as several plates were used for the experiment. Additionally, absorbances for the two *A. starrii* strains were meaned as they were virtually identical. This was also the case with *A. coenophialum*.

The highest absorbances were recorded by *A. lolii* at all antigen dilutions (Figs. 2.23 and 2.24). Isolates of *A. starrii* and *A. coenophialum* also reacted significantly with the antibodies. Up to a mycelial concentration of approximately 2000µg/ml, the antibodies were more reactive against *A.coenophialum* than *A. starrii*. However, the

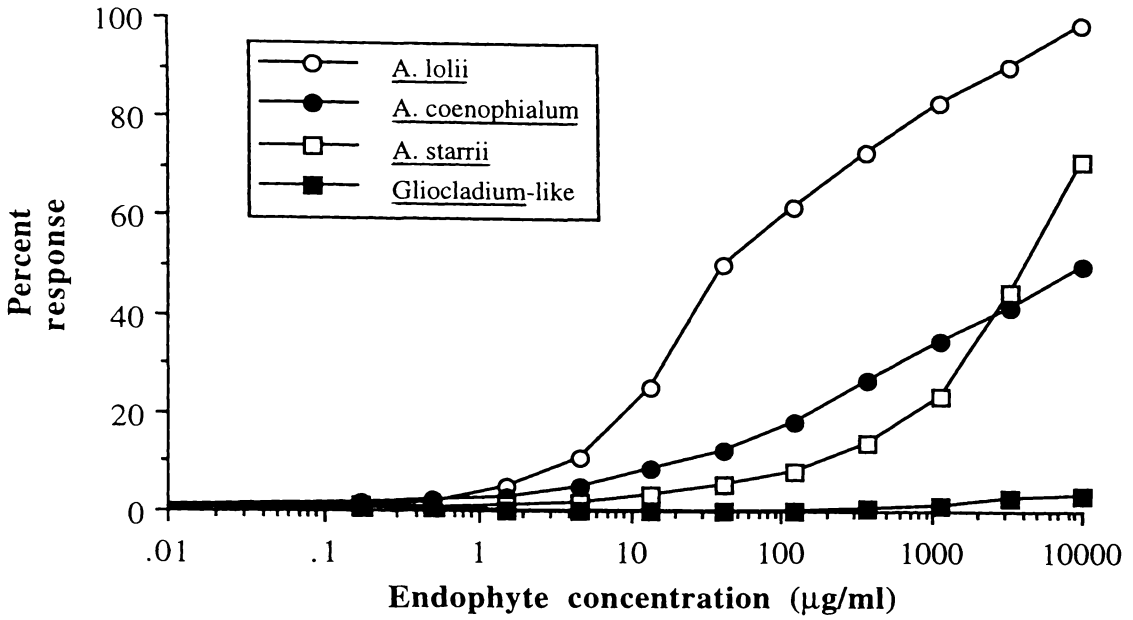


Figure 2.23 Absorbance curves for detection of A. lolii, A. starrii, A. coenophialum and Gliocladium-like endophytes, using a double antibody sandwich ELISA (protocol 4) with IgG 'Cs' (1.4µg/ml) as the capture antibody and IgG '4p' (1.0µg/ml) as the conjugate. Freeze-dried mycelium was extracted (10mg/ml) for 6 hrs at room temperature.

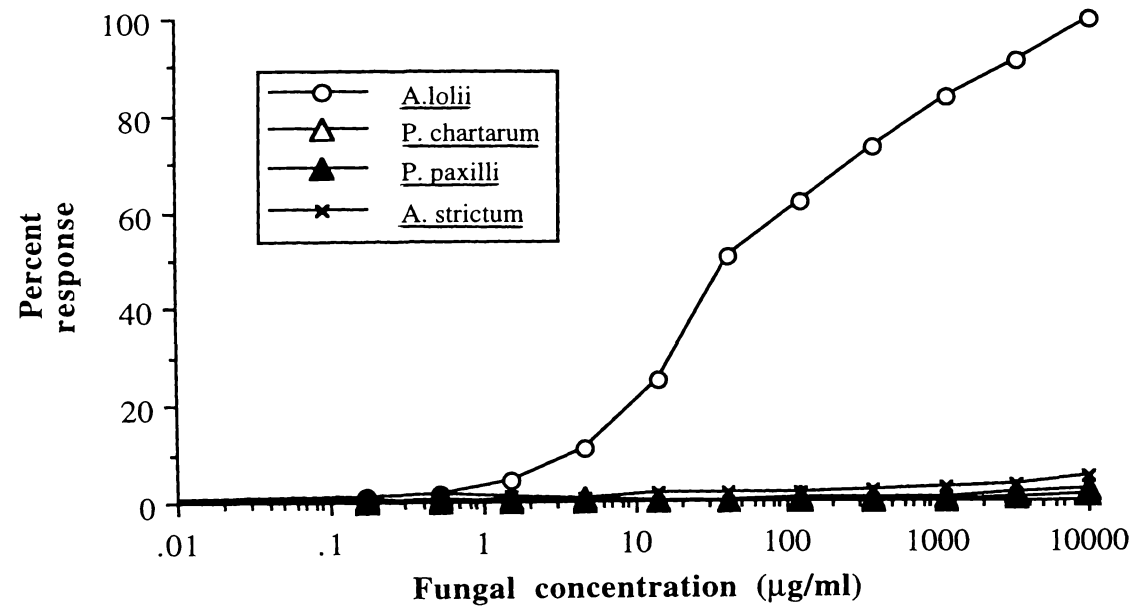


Figure 2.24 Absorbance curves for detection of A. lolii, A. strictum, P. chartarum and P. paxilli. Sample preparation and extraction, and the ELISA conditions used were as described in Figure 2.22.

dilution curves were not parallel and the trend was reversed at higher mycelial concentrations. In comparison with A. *lolii* where 38µg/ml was required, approximately 10,000µg/ml of *A. coenophialum* mycelium and 3,500µg/ml of *A. starrii* mycelium was required to obtain a 50% response. The *Gliocladium*-like isolate was hardly reactive; in comparison with *A. lolii*, over 9000 times more *Gliocladium*-like mycelium was required to obtain an absorbance response of 3.2% (Fig. 2.23). The *A. strictum* isolate was fractionally more reactive than the *Gliocladium*-like fungus, while *P. chartarum* and *P. paxilli* were less so (Fig. 2.24).

ELISA 15: Absorbances in *A. lolii*-free ryegrass plants were low (0.011 - 0.029), irrespective of whether they were infected with the *Gliocladium*-like endophyte or not (Table 2.6). Relatively speaking, absorbances were high (0.105 - 0.369) in the *A. lolii*-infected ryegrasses. *A. starrii*-free *B. anomalus* grasses were more reactive than their endophyte-free ryegrass and tall fescue counterparts. Absorbances in *A. starrii*-infected samples (0.064 - 0.271) were higher than those recorded in *A. starrii*-free samples (0.033 - 0.037). *A. coenophialum*-free tall fescue was the least reactive grass (0.000 - 0.001). Although absorbances in *A. coenophialum*-infected samples were slightly higher (0.012 - 0.028), they were still within the range for *A. lolii*-free perennial ryegrass.

ELISA 16: The curve obtained for the *A. lolii* dilution series in endophyte-free ryegrass extract was similar, but not identical, to that obtained for the same dilution series in pure sample buffer (Fig. 2.25). Between *A. lolii* concentrations of zero - 4.57µg/ml, absorbances were slightly higher in the dilution series made from endophyte-free ryegrass extract. The reverse was true at higher concentrations.

Discussion:

Of all of the fungi tested, *A. lolii* showed the greatest reactivity with the antibodies (Figs. 2.23 and 2.24). This was expected as the antibodies were raised using

Table 2.6 **Absorbances from endophyte-infected and endophyte-free *Lolium perenne*, *Bromus anomalus* and *Festuca arundinacea*, determined using a double antibody sandwich ELISA (protocol 4) with lgG 'Cs' (1.4µg/ml) as the capture antibody and IgG '4p' (1.0µg/ml) as the conjugate. Grass material was extracted (20 mg/ml) for 6 hours at room temperature.**

Grass species	Endophyte status	Plant number	Absorbance (405nm)
L. perenne	endophyte-free	1	0.014
		2	0.011
		3	0.029
	Gliocladium-like infected	1	0.020
		2	0.009
	A. lolii-infected	1	0.105
		2	0.293
		3	0.369
B. anomalus	endophyte-free	1	0.034
		2	0.037
		3	0.033
	A. starrii-infected	1	0.064
		2	0.271
F. arundinacea	endophyte-free	1	0.001
		2	0.000
		3	0.001
	A. coenophialum-infected	1	0.008
		2	0.012
		3	0.028

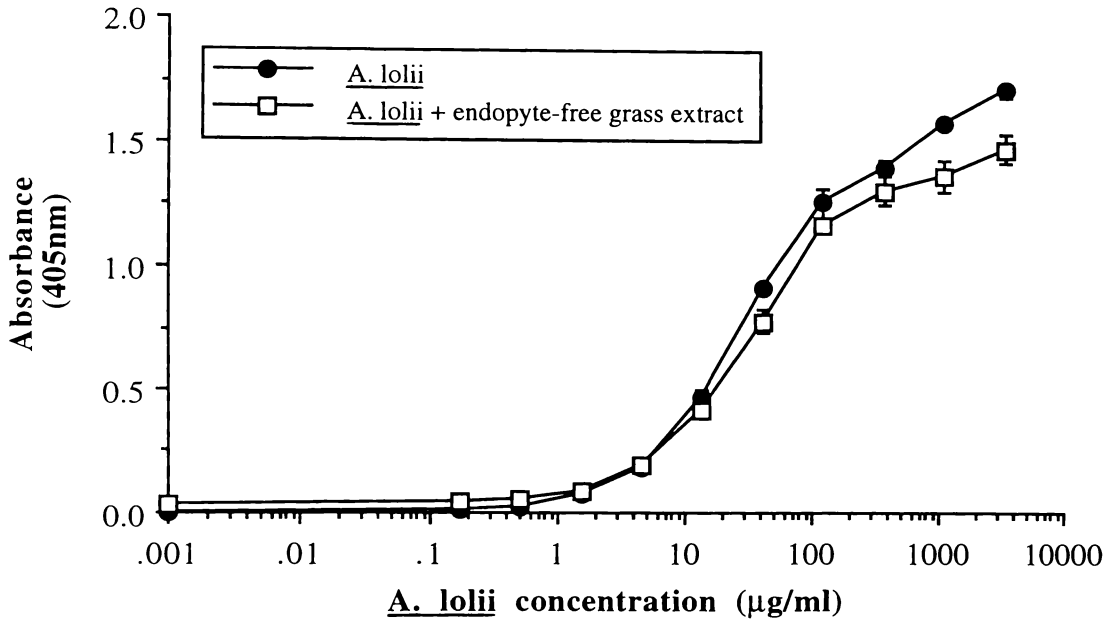


Figure 2.25 Absorbance curves for detection of A. lolii in sample buffer and sample buffer plus endophyte-free grass extract using a double antibody sandwich ELISA (protocol 4) with IgG 'Cs' (1.4µg/ml) as the capture antibody, and IgG '4p' as the conjugate. A. lolii mycelium was freeze-dried and extracted (10mg/ml) for 6 hrs at room temperature. Bars represent SEM.

A. lolii antigens. The only other fungi to react significantly with the antibodies were the *A. starrii* and *A. coenophialum* isolates (Fig. 2.23). The antibodies were therefore not specific to *A. lolii* at the species level. This was also not unexpected as species specificity has not been achieved in previous endophyte immunoassays using polyclonal antibodies (Johnson *et al.* 1982, Johnson *et al.* 1985b, Musgrave *et al.* 1986). Species specificity in such a system is probably only attainable either by using monoclonal antibodies (Dewey 1988) or by raising antisera to single specific fungal molecules (Dickerson and Pollard 1982, Benhamou *et al* 1985, Svircev *et al.* 1988).

Although the antibodies were not specific to *A. lolii* at the species level, cross reactivities with isolates of *A. coenophialum* and *A. starrii* were only moderate. In this context, the antibodies are surprisingly specific to *A. lolii*. Although direct comparisons with results from other studies are difficult due to the different nature of the assays, it is interesting to note that this degree of species specificity was not obtained by Musgrave *et al.* (1986) who could not serologically distinguish between isolates of *A. lolii* and *A. coenophialum*. Using antibodies raised against *A. coenophialum*, Johnson *et al.* (1985b) were also unable to distinguish between the two species at the highest antigen concentration. Although some cross reactivity against *A. lolii* was observed, a greater degree of species specificity was achieved by Reddick and Collins (1988) in their ELISA test which was developed against *A. coenophialum*. Following their investigation into the antigenic nature of *A. lolii* and *A. coenophialum*, Musgrave *et al.* (1986) concluded that by using purified soluble antigens, it may be possible to produce species specific antibodies to these endophytes. It is therefore interesting to note that the antibodies raised by Reddick and Collins (1988), and IgG 'Cs' raised in the present study, were, within the context of their own assay, more species specific than those produced by Johnson *et al.* (1982) and Musgrave (1984), and were produced using the extracellular antigen fraction described by Musgrave (1986), rather than the mycelial

fraction. Gerik *et al.* (1987) also concluded that the specificity of their antibodies to *Verticillium dahliae* was enhanced by using the soluble protein fraction, rather than the mycelial fraction, as the immunogen.

Cross reactivities of the antibodies with isolates of *A. strictum*, *Gliocladium*-like, *P. chartarum* and *P. paxilli* were so low as to be negligible (Figs. 2.23 and 2.24), indicating that antibody specificity is almost certainly sufficient to prevent false positive recordings due to contaminating organisms in grass samples. An inert response was particularly important in the case of the *Gliocladium*-like fungus, as perennial ryegrass is often infected with this endophyte. Considering that over 9000 times more *Gliocladium*-like mycelium than *A. lolii* mycelium would be needed to cause the same 3.2% response in the assay, a false positive result due to the presence of the *Gliocladium*-like endophyte would be extremely unlikely. Musgrave *et al.* (1986) also observed minimal cross reactivity of the *Gliocladium*-like endophyte with anti-*A. lolii* antiserum.

The lack of any appreciable reactivity of *A. strictum* is interesting as this fungus is a member of the genus *Acremonium*, and the antiserum is active against three other members of this genus. However, *A. strictum* is not a member of the section *Albo-lanosa* and so is only thought to be distantly related to the endophytic *Acremoniums*. Using antibodies prepared against *A. coenophialum*, Johnson *et al.* (1985b) also found that absorbances with *A. strictum* were very low.

The degree of reactivity of an antigen with antibodies prepared against a different antigen, largely reflects the phylogenetic relationship between the two antigens (Aldwell *et al.* 1985). The more closely two organisms are related, the more likely it is that they share a greater number of similar or identical epitopes. Using this principle, immunoassay techniques have been used to investigate phylogenetic relationships among many groups of organisms (Beverly and Wilson 1985, O'Brien *et al.* 1985), including fungi (Fitzell *et al.* 1980, Shelby *et al.* 1987, Pollonelli and Morace 1989).

Immunological comparisons of different *Acremonium* endophytes both in the current study and in studies by Johnson *et al.* (1985b), Musgrave *et al.* (1986), Shelby et *al.* (1987), and Reddick and Collins (1988), indicate that they are closely related, and that *Acremonium* endophytes themselves are closely related to the fungus *Epichloe typhina.* Results from the present study and those of Johnson *et al.* (1985b), also suggest that *A. strictum* is only distantly related to the other *Acremonium* species. This serological difference is also reflected in the very different characteristics of their life history; *A. strictum* is a free-living fungus while all of the others are endophytic.

From the results in Figure 2.23, it is difficult to ascertain whether *A. coenophialum* is more or less cross reactive than *A. starrii,* as the dilution curves for these two fungi were not parallel. Thus it is impossible to state which of these two endophytes is more closely related to *A. lolii.*

In accordance with the results obtained using fungal cultures, infection of perennial ryegrass with the *Gliocladium*-like endophyte had no effect on absorbance (Table 2.6). However, *A. starrii* infection in *B. anomalus* was detected. The significantly higher absorbances recorded in *A. starrii*-free *B. anomalus* samples also suggests a grass effect. The ability of the antibodies to detect *A. coenophialum* in tall fescue was minimal suggesting that *A. starrii* may be more closely related to *A. lolii* than *A. coenophialum* is, although endophyte concentration differences in the grasses may also explain this. Johnson *et al.* (1985b) found that their ELISA to *A. coenophialum* also easily detected *A. lolii* in situ, indicating the possible greater species specificity of the present assay.

Results from Figure 2.25 (and results from section 2.3.5) show that the antibodies were not immune from the effects of endophyte-free grass extract. The presence of endophyte-free grass extract decreased absorbances at higher *A. lolii* concentrations, and increased them at the lower concentrations. The reduced signal

observed with high *A. lolii* concentrations may have been due to a masking effect of the grass antigens, preventing complete capture of the available *A. lolii* antigen pool. On the other hand, the greater reactivity observed at the lower *A. lolii* concentrations may have been caused by non specific binding of grass antigens. In general however, the similarity between the two dilution curves in Figure 2.25 indicates that the extent of this grass effect was small. Additionally, this grass effect should be similar in all *A. lolii*-infected samples. Importantly therefore, the presence of the grass extract component in samples can be ignored when interpreting results.

2.4 CONCLUDING SUMMARY.

The extent of the immune responses in the nine rabbits were highly variable, and appeared to be independent of whether soluble or particulate *A. lolii* antigens were used. In rabbits 1-5, the secondary immune response had declined significantly by the time the second bleeding was taken 26 days after the final booster. The two most reactive antisera, those from rabbits 4 and C, were chosen for further development of the assay. IgG '4p' detected *A. lolii* down to a concentration of approximately 0.01µg/ml in a double antibody sandwich assay. The limit of detection of the assay using IgG '4p' decreased when a capture antibody concentration of 3µg/ml was used, probably because of steric hinderance of adsorbed antibody. Assay detection limits in both antibodies was improved by using higher conjugate concentrations.

Consistent detection of *A. lolii* in oven-dried grass samples was not possible, as absorbance differences between endophyte-free and *A. lolii*-infected samples were minimal. This situation was hardly improved by manipulation of protein blocking concentrations or sample extraction temperatures. Detection of *A. lolii* in perennial ryegrass was only possible when samples were freeze-dried rather than oven-dried. This was surprising as oven-drying has been used successfully in previous immunoassays against *Acremonium* endophytes. It was therefore concluded that the majority of antibodies from the two animals may have been effective only against heat sensitive *A. lolii* epitopes.

Manipulation of the ELISA conditions revealed that non specific binding in *A. lolii*-infected samples was reduced at higher BSA blocking concentrations. Tests also showed that using IgG 'Cs' as the capture antibody and IgG '4p' as the conjugate greatly enhanced *A. lolii* detection in ryegrass samples.

Aspects of the grass extraction also influenced the reactivity of samples. In

comparison with 40C, grass extraction at 250C increased absorbances in *A. lolii*-infected but not in endophyte-free samples. The relatively high Tween 20 concentration of 0.5% was considered optimal. At 250C, absorbances were higher with a 6 hour extraction compared to a 20 hour extraction. This may have been due to increased denaturation of the target antigens in the longer extraction. A 6 hour extraction at 250C was used for routine analysis as absorbances in *A. lolii*-infected samples were higher making errors less likely, and the 6 hour extraction was less time consuming. Finally, smaller extraction buffer volumes increased reactivities in endophyte-infected but not in endophyte-free samples.

Quantitative detection (using absorbance values) of *A. lolii* in *A. lolii*-infected samples was highly reproducible, no matter which antibody combination was used. A probable grass effect was also observed in endophyte-free samples, which, if strong enough, could mask an endophyte effect. This was considered unlikely as endophyte-free samples were so unreactive. Sample *A. lolii* concentrations (μg *A. lolii*/g of dry grass) were also reproducible from one plate to the next, but for the most accurate results, it was concluded that if possible, grass samples to be compared should be analyzed on the same plate.

The precision of the assay was acceptable (ie: CV% was below 10%) down to an *A. lolii* concentration of approximately 0.06μg/ml and well to well variability was low in the endophyte-infected grass samples tested.

As expected, the homologous antigen, *A. lolii*, showed the greatest reactivity of all of the fungi tested using the ELISA. *A. lolii* was easily distinguishable from *A. starrii* and *A. coenophialum* as the cross reactivity of the antibodies with these endophytes was only moderate. The use of soluble *A. lolii* antigens for producing IgG 'Cs', the first report of their use to produce antibodies against this endophyte, may explain the

increased species specificity of the assay. It was not possible to make a phylogenetic distinction between *A. starrii* and *A. coenophialum*, although the assay was able to detect the former but not the latter in grass samples. Isolates of *A. strictum*, *P. chartarum*, *P. paxilli* or *Gliocladium*-like endophyte were extremely unreactive. Absorbances were unaffected by the presence of the *Gliocladium*-like endophyte in ryegrass samples.

Grass constituents were shown to have an effect on detection of *A. lolii* antigens. In the presence of endophyte-free grass extract, detection was hindered at high *A. lolii* concentrations, while absorbances were higher at low *A. lolii* concentrations. However, the importance of grass constituents in the context of the assay was considered minimal, as the extent of this effect was small and should be the same for all *A. lolii*-infected samples.

In conclusion, the enzyme-linked immunosorbent assay developed in this study, was precise, sensitive and specific, and yielded highly reproducible results.

CHAPTER 3

SEASONAL LEVELS OF *ACREMONIUM LOLII*, PERAMINE AND LOLITREM B IN *A. LOLII*-INFECTED PERENNIAL RYEGRASS.

3.1 INTRODUCTION

Many aspects concerning the nature of the interactions of *A. lolii* with the host plant, invertebrate pest species and grazing livestock are not well understood. The purpose of this study was to measure concentrations of *A. lolii*, lolitrem B and peramine in *A. lolii*-infected perennial ryegrass on a seasonal basis. Apart from increasing our knowledge of the seasonality of these three parameters, this study could lead to a greater understanding of the environmental factors which may be responsible for *A. lolii* and alkaloid production and retention in the plant. Although seasonal concentrations of *A. lolii* and lolitrem B (but not peramine) have been monitored previously in composite pasture samples (Fletcher 1983, 1986, Mortimer *et al.* 1984, di Menna and Waller 1986, Prestidge and Gallagher 1988a, di Menna *et al.* 1992), they have not been previously monitored simultaneously in individual plants.

3.2 MATERIALS AND METHODS

3.2.1 Plant maintenance and sampling.

Twenty mature *A. lolii*-infected perennial ryegrass plants obtained from a variety of North Island habitats (Wedderburn *et al.* 1989) were grown outdoors at Ruakura, Hamilton. The plants were taken from pastures which were at least 20 years old and were selected for high or low summer levels of lolitrem B and *A. lolii*. Plants were

grown approximately 50cm apart in soil which was kept weed free by hand weeding. The plants were sampled on a monthly basis in two separate experiments. The first was from January 1990 to December 1990 and the second was from December 1991 to November 1992. The second experiment was considered necessary for several reasons. Firstly, alkaloid profiles for the two years could be compared to determine whether yearly differences occur, and if they do, to try to relate the differences to the environmental conditions at the time. Secondly, alkaloid concentrations in each plant could be compared to determine whether relative alkaloid levels are maintained from one year to the next, or whether they fluctuate randomly. Thirdly, all plant material harvested from the first experiment was oven-dried at 60^{0}C which, due to the nature of the ELISA, made analysis for *A. lolii* difficult. As certain aspects of the two experiments are different, they will be described separately.

Experiment 1: January 1990-December 1990. Samples of approximately 20 to 30 tillers were taken from each plant at monthly intervals by harvesting at ground level with a scalpel. Regrowth from the previous months harvest was not sampled and dead tillers were discarded. The plants were trimmed to 8cm above ground level immediately following sampling and were watered as necessary. Grass material was washed in cold tap water to remove soil debris, dried at 60^{0}C for 40 hours and finely ground in an Ika model MFC Culatti micro hammer mill. The samples were stored in glass scintillation vials at -20^{0}C before being analyzed for peramine and lolitrem B.

Experiment 2: December 1991-November 1992. Tillers were harvested at ground level at monthly intervals as described in experiment 1, except that immediately prior to sampling, the plants were trimmed to 8cm. The basal tiller material and regrowth from each plant sampled was washed, placed in separate plastic bags and

immediately frozen at -20^{0}C. The samples were then freeze-dried, and then ground and stored as before.

3.2.2 Peramine, lolitrem B and *A. lolii* analyses.

Peramine analysis: Peramine was analyzed by HPLC using a modification of a method supplied by B. A. Tapper (AgResearch, Palmerston North). This method differs considerably from that published by Tapper *et al.* (1989) and will therefore be described in detail.

Peramine standard and internal standard: 1mg of crystalline peramine sulphate (kindly provided by B. A. Tapper) was dissolved in 2.5ml of distilled water plus 7.5ml of methanol to make a 100µg/ml (ppm) standard. Homoperamine nitrate was used as an internal standard to monitor the performance of the assay. 1mg of homoperamine nitrate was dissolved in 10ml of methanol to make a 100µg/ml solution. A mixed 1µg/ml standard was used during the chromatography. This was made by mixing 1ml of the 100µg/ml peramine and 1ml of the 100µg/ml homoperamine solutions together in a 100ml volumetric flask and adding eluent (40% v/v methanol plus 5% v/v formic acid plus 55% v/v distilled water) to make a final volume of 100ml.

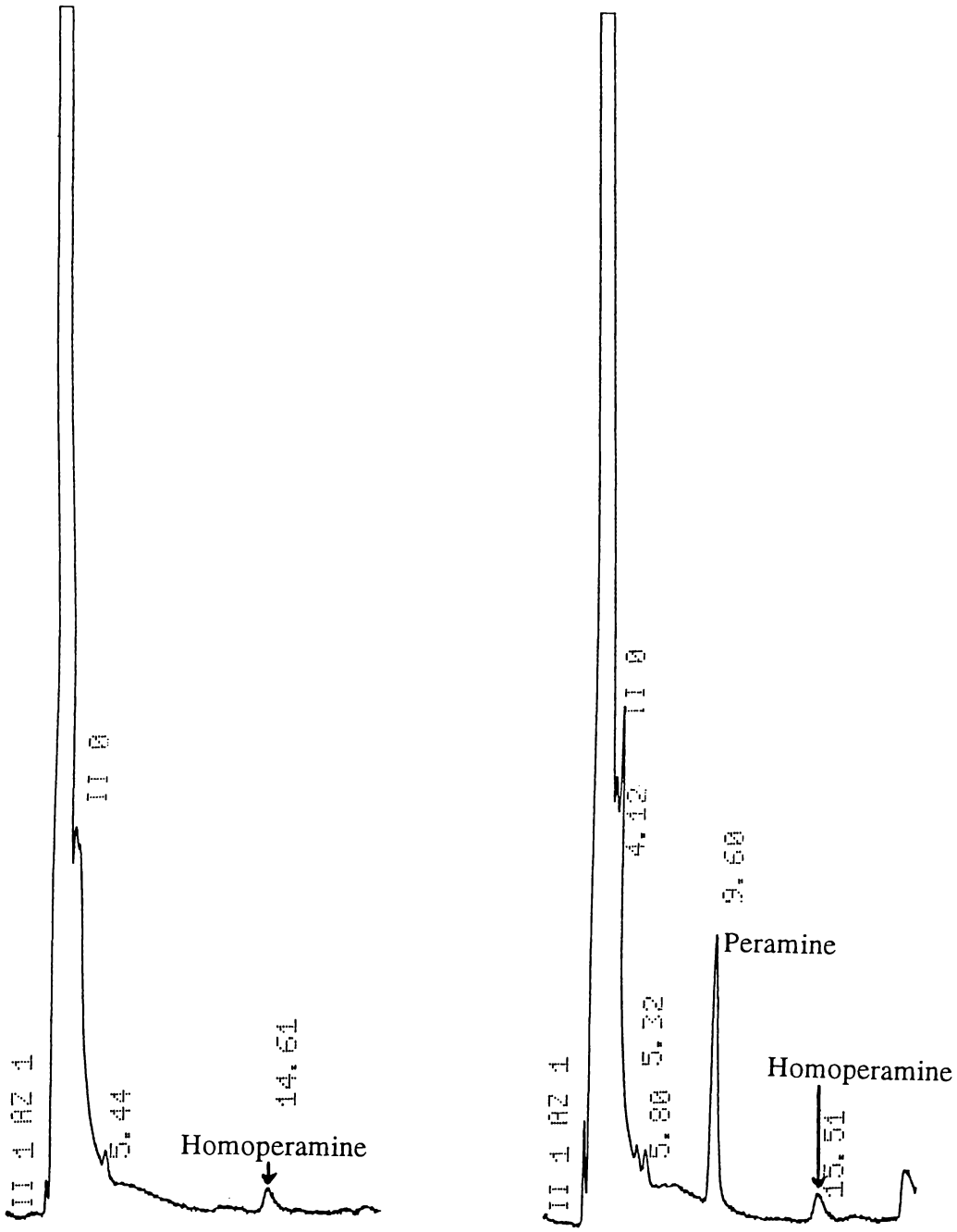
Extraction of plant material: Samples of dry ground grass were weighed (usually 100mg) into glass tubes into which 10µl of 100µg/ml homoperamine standard was added. 5ml of 30% propan-2-ol was then added and after vortexing for 5 seconds, the tubes were placed in a 90^{0}C sand bath. After 30 minutes, the tubes were removed from the sand bath and cooled by partially submerging them in a cold water bath. While the tubes were cooling, 1cc Varian Bond Elut CBA columns packed with 100mg of adsorbent were fitted to a vacuum manifold and pre-conditioned. This was done by passing 500µl of a 40% (v/v) methanol, 5% (v/v) ammonia and 55% (v/v) distilled water solution followed by 1ml of a 40% (v/v) methanol and 60% (v/v) distilled water solution through each column. After the pre-conditioning phase, 1ml portions of grass

extract were passed through each column, followed by 1ml of 100% methanol. The peramine was eluted by passing 600µl of eluent through each column and collected in 1ml autosampler vials. 200µl of HPLC buffer (200ml methanol + 75ml stock buffer (18g guanidinium carbonate + 20ml formic acid + distilled water to 1l) + distilled water to 1l) was added to each vial which was then sealed and stored at 4^{0}C until analysis for peramine.

Measurement of peramine by HPLC: Peramine was measured by HPLC using a Spectra-Physics (model 740) liquid chromatograph fitted with an Alltech econosphere silica column, 5µm particle size, 150 x 4.6mm. Detection was with a Shimadzu variable wavelength spectrophotometric detector. The isocratic mobile phase was 100% HPLC buffer and the flow rate was 1ml/min. The pressure varied from approximately 900 to 1000 psi and the column was maintained at 35^{0}C during measurement.

The quantity of peramine in 20µl injection samples was determined by comparing the peak heights with those of 1µg/ml peramine in the peramine and homoperamine mixed standard. Peramine eluted approximately 7 to 10 minutes after injection, while homoperamine eluted after approximately 13 to 16 minutes. Typical elution patterns from an *A. lolii*-free and an *A. lolii*-infected perennial ryegrass sample are shown in Figure 3.1. Several unidentified UV absorbing substances were also often present in the samples although these did not interfere with the detection of peramine (Figs. 3.1 and 3.3). Samples were injected automatically every 25 minutes using a Micromeritics (model 725) automatic injector. Each sample was injected twice to ensure that the 20µl loop was completely filled. Peramine concentrations were therefore only calculated using results from the second injection.

In order to enhance the accuracy of the system, the effect of wavelength on peramine detection was investigated. Using a 5µg/ml mixed standard, the height of the peramine and homoperamine peaks were measured at a number of wavelengths between



a). b).

Figure 3.1 **Typical chromatograms of extracts from a) *A. lolii*-free and b) *A. lolii*-infected perennial ryegrasses, showing the peramine and homoperamine peaks.**

used per sample.To minimize the effects of interplate variation, each grass sample was analyzed twice for *A. lolii*. In each analysis, ELISA plates contained only the samples which were to be directly compared. In the first analysis, all plant material harvested in the same month was analyzed on the same ELISA plate enabling accurate inter-plant comparisons to be made. In the second analysis, all plant material harvested from the same plant during the entire year was analyzed on the same ELISA plate so that the seasonality of *A. lolii* could be accurately determined. 0.1mg/ml *A. lolii* standards were included on each plate enabling *A. lolii* concentrations to be calculated from a dilution series of the antigen.

3.2.3 Climate data. Mean monthly grass minimum temperatures and total monthly rainfall figures for January to December 1990 and December 1991 to November 1992 were obtained from a weather station situated approximately 200m from where the plants were being maintained. Mean monthly day lengths (photoperiods) were calculated from sun rise and sun set times in Hamilton over the period of the trial. These were supplied by the National Observatory of New Zealand, Wellington.

3.2.4 Statistical analyses. Mean *A. lolii*, peramine and lolitrem B concentrations plus standard errors of the means were calculated for each month. *A. lolii*, peramine and lolitrem B concentration changes over time were compared using paired difference *t* tests. Linear regression analysis was used to determine how closely *A. lolii*, peramine and lolitrem B concentrations were related to each other and to mean monthly grass minimum temperature, total monthly rainfall and day length. Multiple regression analysis was also used to examine the possible individual effects of mean monthly grass minimum temperature, total monthly rainfall and day length on *A. lolii*, peramine and lolitrem B concentrations.

3.3 RESULTS

Experiment 1: The seasonality of lolitrem B yielded a U shaped graph, with mean lolitrem B concentrations being highest during summer and early autumn (December to April), and lowest during winter and early spring (May to October) (Figure 3.4). More specifically, mean lolitrem B concentrations reached a maximum in February (4.48μg/g dry weight) and a minimum in August (0.44μg/g). Mean lolitrem B concentrations were significantly ($P<0.001$) correlated to mean monthly grass minimum temperatures ($r^2 = 79.3$) (Figs. 3.4 and 3.5). Also, mean lolitrem B concentrations peaked during periods of low rainfall (Figs. 3.4 and 3.5). During the summer and winter, lolitrem B levels in individual plants ranged from 0.91μg/g to 9.75μg/g and 0.09μg/g to 3.04μg/g respectively. Plants could be characterised by their lolitrem B concentration as they generally maintained relative levels throughout the year. It was therefore possible to identify high lolitrem B producing plants and low lolitrem B producing plants (Figure 3.6).

Inter-plant variation in seasonal peramine levels was considerable, resulting in a less discrete seasonal pattern. Summer and winter levels of peramine in individual perennial ryegrass plants ranged between 2.5μg/g-41.6μg/g and 2.9μg/g-36.1μg/g respectively. Large monthly fluctuations in peramine concentration within individual plants were also recorded. Mean peramine concentrations were relatively stable throughout the year although concentrations in winter were significantly ($P<0.01$) lower than summer and autumn levels (Fig. 3.4). Also there was a significant ($P<0.01$) decrease in the peramine concentration between January and March, followed by a significant ($P<0.05$) rise in April, resulting in a bimodal seasonality. Peramine concentrations in December were only slightly, but significantly ($P<0.05$) higher than in November, but were considerably lower ($P<0.01$) than in the previous January. Mean

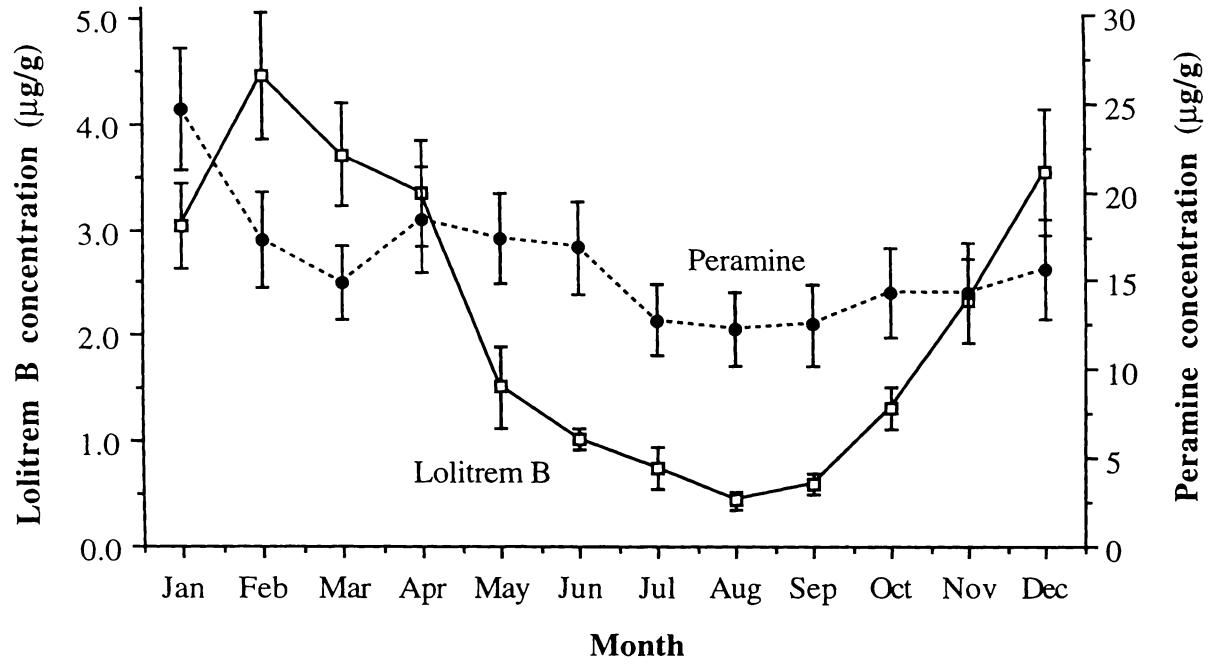


Figure 3.4 Mean monthly lolitrem B and peramine concentrations in A. lolii-infected perennial ryegrasses during 1990. Lolitrem B and peramine were measured using HPLC. Vertical bars represent SEM.

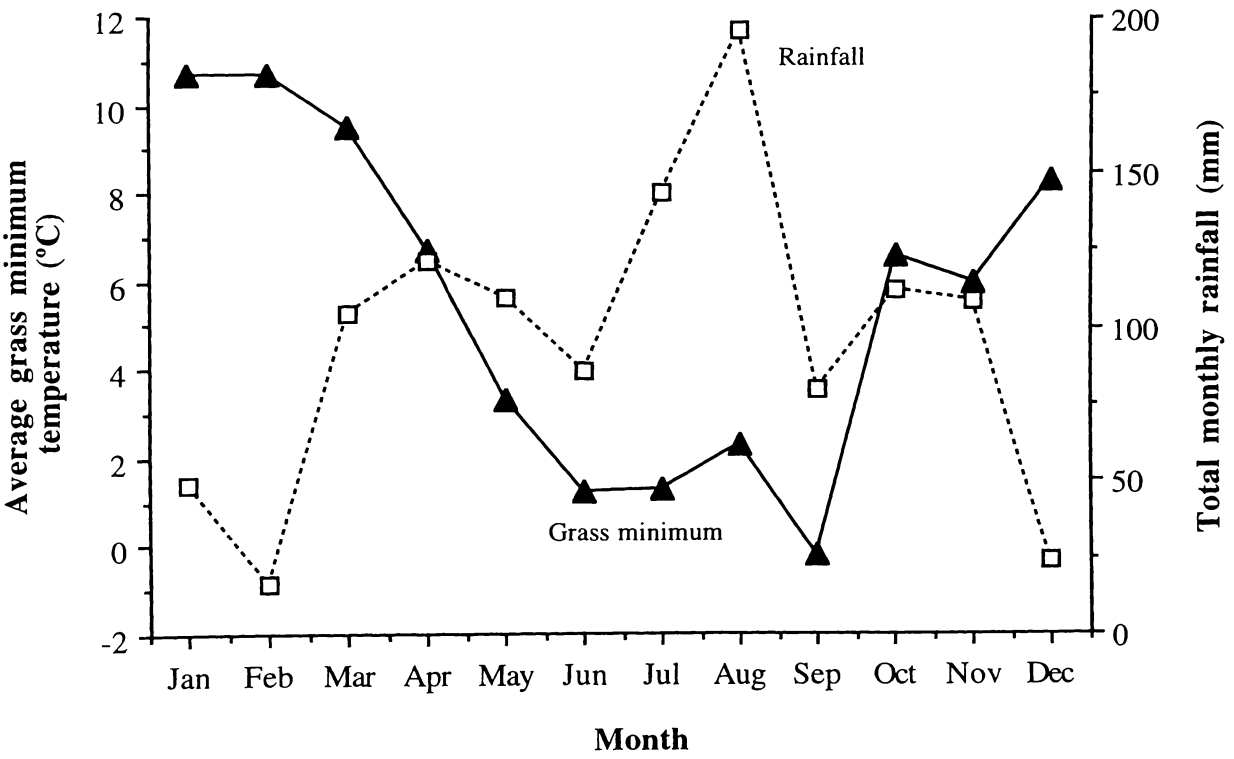


Figure 3.5 Mean monthly grass minimum temperature and total monthly rainfall measured at Ruakura during 1990.

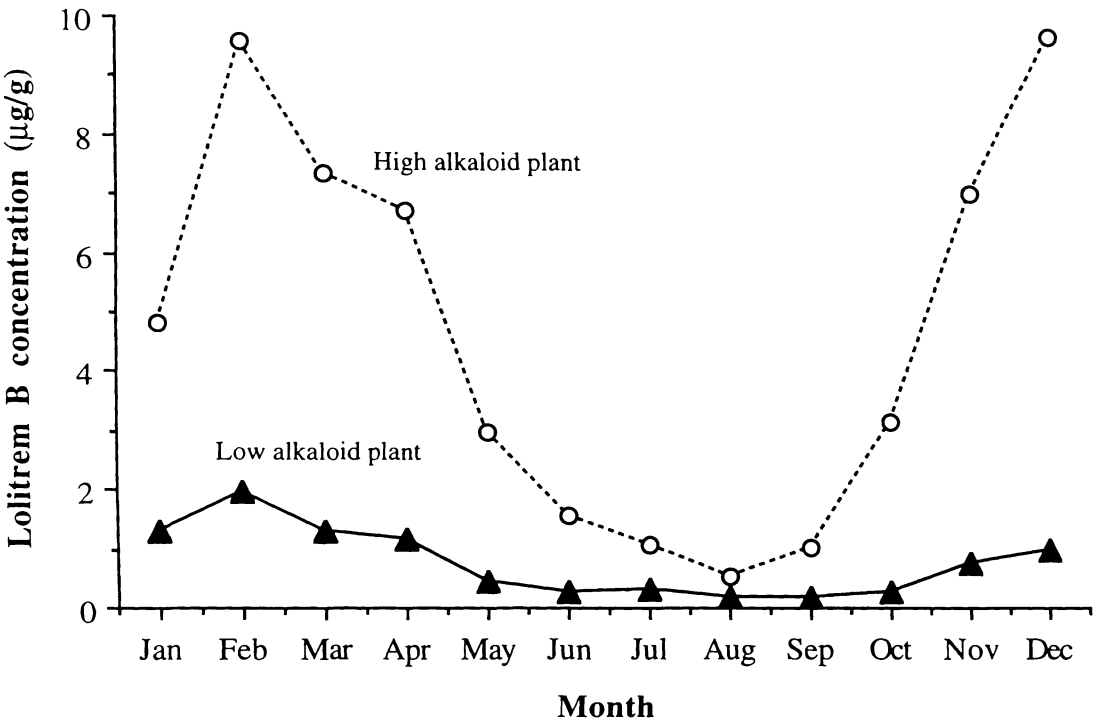


Figure 3.6 Monthly lolitrem B concentrations during 1990 from a representative high alkaloid plant and a representative low alkaloid plant.

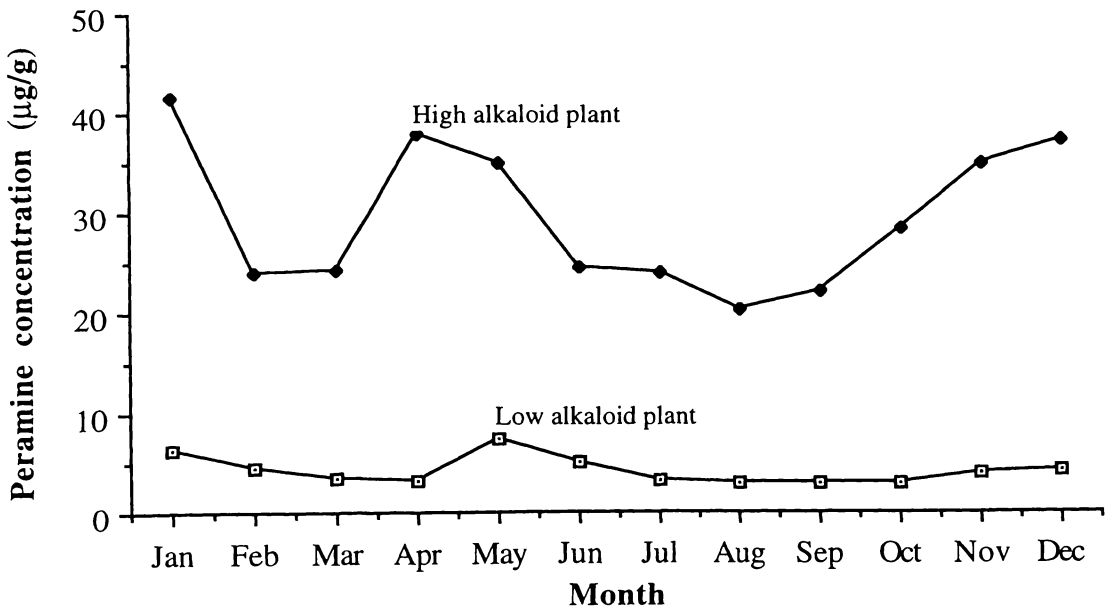


Figure 3.7 Monthly peramine concentrations during 1990 from a representative high alkaloid plant and a representative low alkaloid plant.

peramine concentrations were significantly ($P<0.05$) correlated with changes in mean monthly grass minimum temperature ($r^2 = 27.5$) (Figs. 3.4 and 3.5). Summer and winter concentrations of peramine in individual perennial ryegrass plants ranged between 2.5μg/g to 41.6μg/g and 2.9μg/g to 36.1μg/g respectively. Large monthly fluctuations in peramine concentration within individual plants were recorded. Relative concentrations of peramine in individual ryegrasses were maintained throughout the year enabling the identification of plants which were high or low in peramine (Fig. 3.7).

Lolitrem B and peramine concentrations in the test plants were significantly positively correlated in 9 out of the 12 months (Table 3.1). Mean lolitrem B and peramine concentrations for the entire year were also significantly ($P<0.001$) positively correlated ($r^2 = 72.3$) (Fig. 3.8).

Experiment 2: In the basal portion of the plant, mean *A. lolii* concentrations were highest in late spring/early summer (October to December) and lowest in winter and early spring (June to September) (Fig. 3.9). Specifically, *A. lolii* concentrations in the basal tissue decreased sharply between December 1991 and January 1992, after which levels rose slightly between February and March. Mean *A. lolii* concentrations in the basal tissues were relatively stable until June at which time they decreased sharply. *A. lolii* concentrations in this tissue continued to decline gradually and reached their lowest levels of the year in the August and September harvests. Between September and October, mean *A. lolii* concentrations increased considerably, and continued to increase from October to November.

The seasonal pattern of mean *A. lolii* concentrations in the regrowth component was similar to that recorded for the basal component (Fig. 3.9). Mean *A. lolii* concentrations in regrowth material were lower than levels in the basal tissues for every month of the year with the exception of January, at which time they were slightly higher. After a sharp decrease between January and February, *A. lolii* concentrations in

Table 3.1 **Relationship between peramine concentration and lolitrem B concentration in *A. lolii*-infected ryegrass plants for each month of 1990. (*P<0.05, ***P<0.001, NS = not significant).**

Month	Coefficient of determination (r^2)	Significance
January	30.7	*
February	30.6	*
March	7.0	NS
April	8.5	NS
May	28.9	*
June	72.4	***
July	28.8	*
August	16.7	NS
September	51.6	***
October	79.7	***
November	73.6	***
December	58.2	***

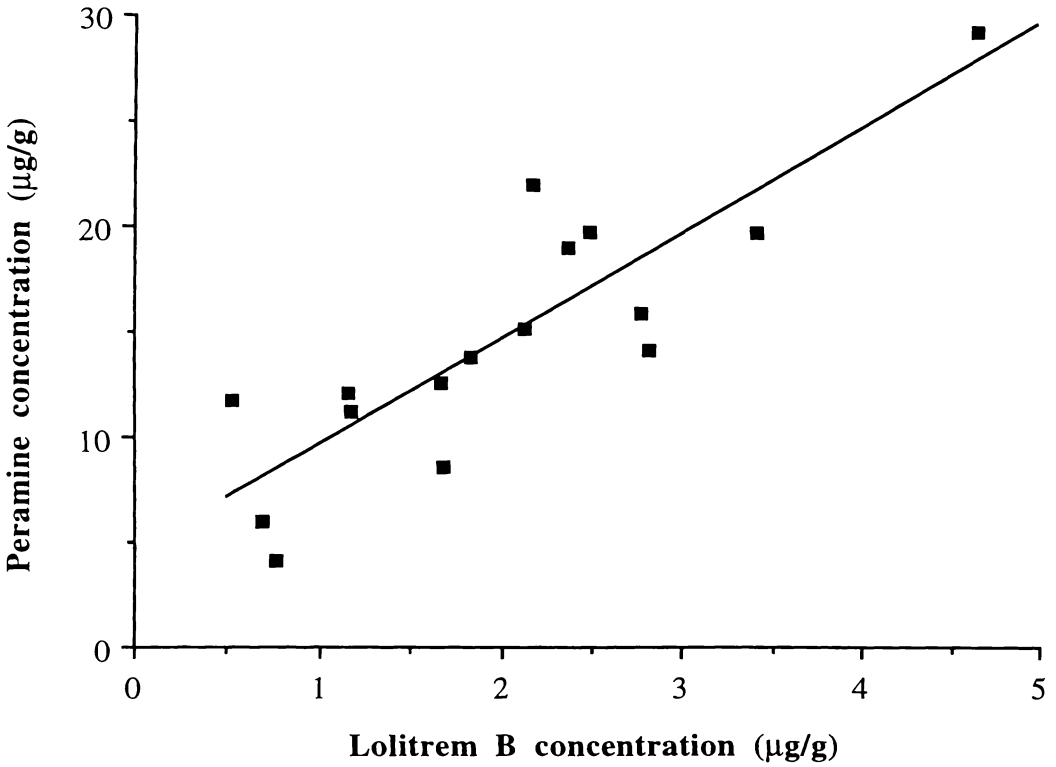


Figure 3.8 Relationship between the mean yearly concentrations of peramine and lolitrem B in A. lolii-infected perennial ryegrass during 1990.

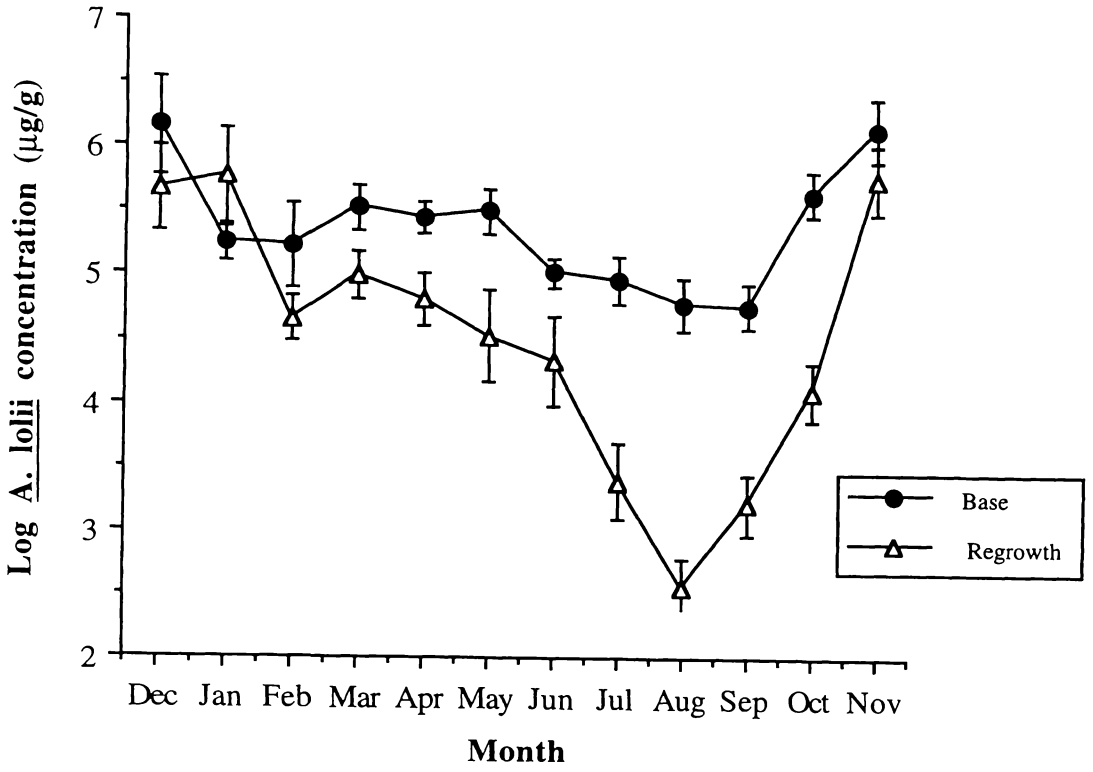


Figure 3.9 Mean monthly A. lolii concentrations in basal and regrowth components of A. lolii-infected ryegrass plants. Vertical bars represent SEM.

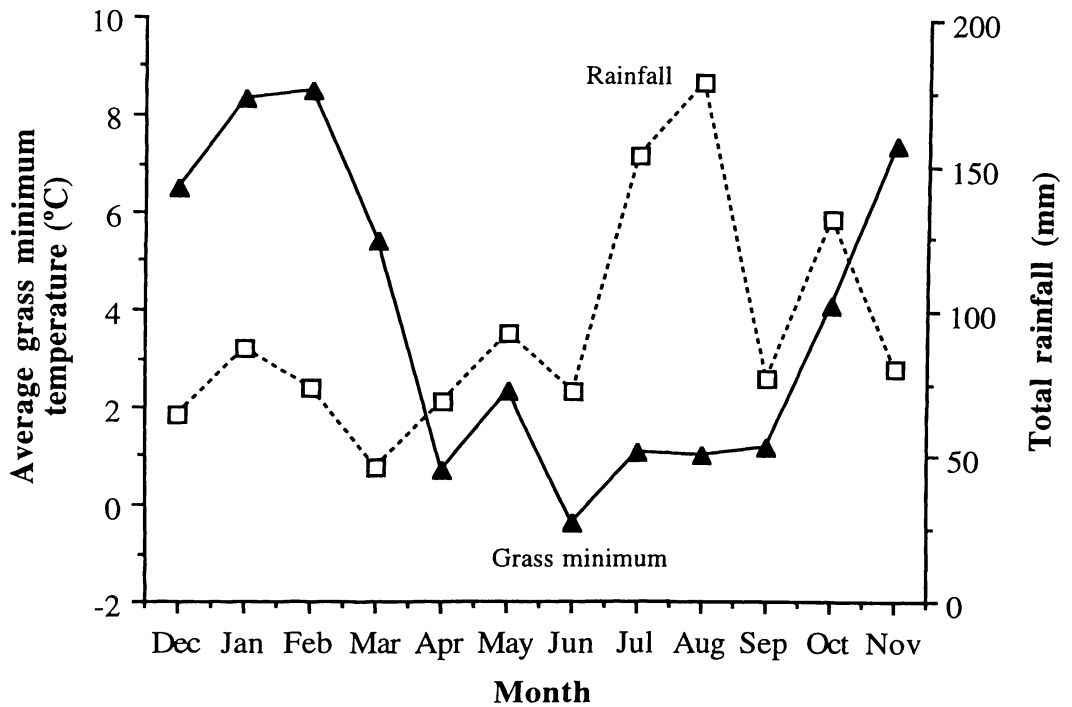


Figure 3.10 Mean monthly grass minimum temperature and total monthly rainfall measured at Ruakura from December 1991 to November 1992.

the regrowth component rose in March and then declined gradually until June. *A. lolii* concentrations then declined sharply between June and July, and then again between July and August. The lowest mean *A. lolii* concentration in the regrowth component for the year was recorded in August. *A. lolii* concentrations then increased sharply and steadily from August to November.

In the regrowth portion of the ryegrass plant, mean *A. lolii* concentrations were significantly correlated with changes in mean grass minimum temperature ($P<0.01$) (Figs. 3.9 and 3.10, Table 3.2) and total monthly rainfall ($P<0.05$) (Figs. 3.9 and 3.10, Table 3.3). Mean *A. lolii* concentrations in the basal component were not significantly correlated with changes in either grass minimum temperature (Figs. 3.9 and 3.10, Table 3.2) (although this was significant at the 10% level) or total monthly rainfall (Figs. 3.9 and 3.10, Table 3.3). Mean *A. lolii* concentrations in basal and regrowth portions of ryegrass plants were significantly ($P<0.05$) correlated with changes in day length (Figs. 3.9 and 3.10, Table 3.4). The results of the multiple regression showed that a significant amount of the variation (63.1%) in the *A. lolii* concentration of the regrowth component could be explained by changes in grass minimum temperature, monthly rainfall and day length combined (Table 3.5). This was not the case with *A. lolii* concentrations in the basal component (Table 3.5). Differences in the *A. lolii* concentration between different plants were relatively constant throughout the year, enabling high *A. lolii* producing plants and low *A. lolii* producing plants to be identified (Fig. 3.11).

Lolitrem B concentrations in the basal component peaked during summer and early autumn (January to April) and were lowest during winter and early spring (August to October) (Fig. 3.12). Lolitrem B concentrations in the basal portion increased sharply from December 1991 to reach the highest level for the year (4.62μg/g) in January 1992. After a small decline between January and February, lolitrem B concentrations decreased

Tables 3.2, 3.3 and 3.4 Results of linear regression analyses investigating individual relationships between mean monthly grass minimum temperature (GMT) (Table 3.2), total monthly rainfall (TMR) (Table 3.3) and mean monthly day length (MDL) (Table 3.4) with mean monthly log *A. lolii* concentration, mean monthly lolitrem B concentration and mean monthly peramine concentration, in basal and regrowth portions of *A. lolii*-infected perennial ryegrass plants from December 1991 to November 1992. (*P<0.05, **P<0.01, NS=not significant).

Table 3.2

Correlation	Coefficient of determination (r^2)		Significance	
	Basal	Regrowth	Basal	Regrowth
GMT v *A. lolii*	23.8	45.4	NS	**
GMT v Lolitrem B	27.2	38.4	*	*
GMT v Peramine	34.1	0.0	*	NS

Table 3.3

Correlation	Coefficient of determination (r^2)		Significance	
	Basal	Regrowth	Basal	Regrowth
TMR v *A. lolii*	8.4	43.2	NS	*
TMR v Lolitrem B	15.9	15.7	NS	NS
TMR v Peramine	44.2	23.1	*	NS

Table 3.4

Correlation	Coefficient of determination (r^2)		Significance	
	Basal	Regrowth	Basal	Regrowth
MDL v *A. lolii*	31.6	39.6	*	*
MDL v Lolitrem B	8.1	26.2	NS	NS
MDL v Peramine	23.8	0.0	NS	NS

Table 3.5 **Results of multiple regression analyses investigating the relationships between mean monthly grass minimum temperature (GMT), total monthly rainfall (TMR) and mean monthly day length (MDL) with mean monthly log *A. lolii* concentration, mean monthly lolitrem B concentration and mean monthly peramine concentration, in basal and regrowth portions of *A. lolii*-infected perennial ryegrass plants from December 1991 to November 1992. (*P<0.05, **P<0.01, NS=not significant).**

	Coefficient of determination (R^2)		Significance	
	Basal	Regrowth	Basal	Regrowth
A. lolii v GMT, TMR, MDL	20.1	63.1	NS	*
Lolitrem B v GMT, TMR, MDL	32.8	33.4	NS	NS
Peramine v GMT, TMR, MDL	54.4	70.1	*	**

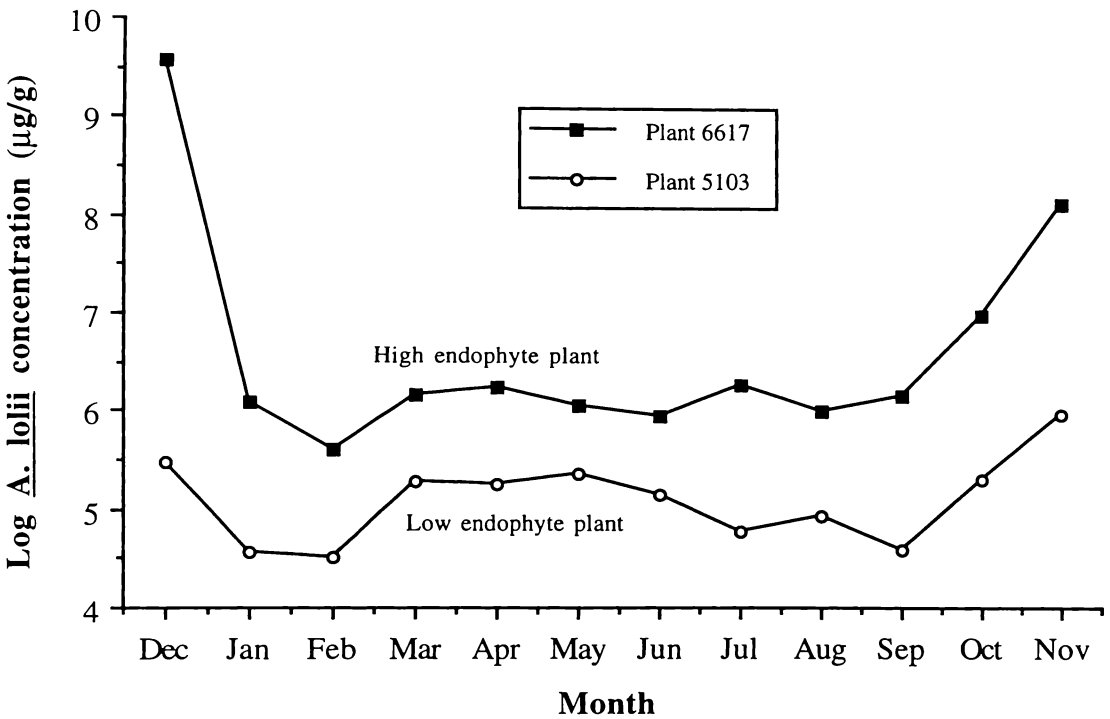


Figure 3.11 Monthly log A. lolii concentrations during 1991-1992 from a representative high endophyte plant and a representative low endophyte plant.

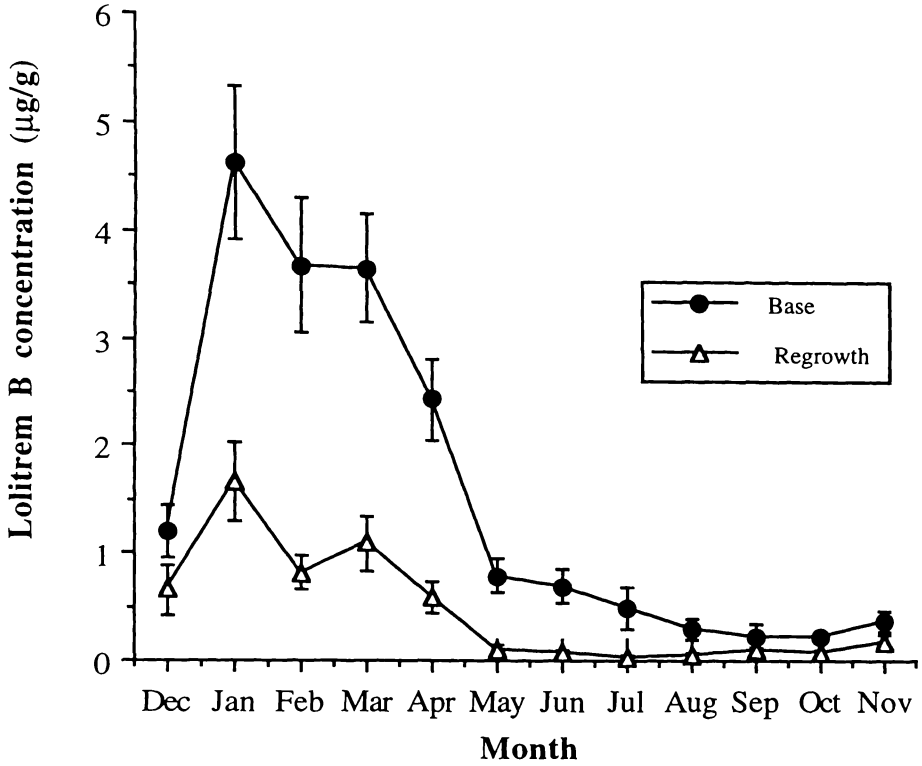


Figure 3.12 Mean monthly lolitrem B concentrations in basal and regrowth components of A. lolii-infected ryegrass plants during 1991-1992. Vertical bars represent SEM.

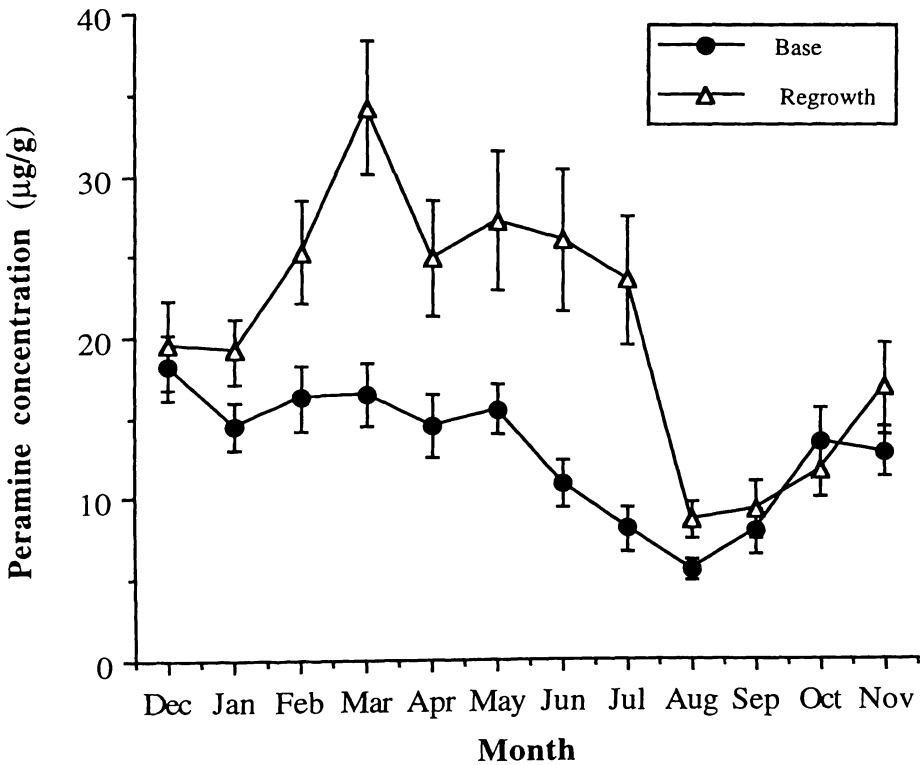


Figure 3.13 Mean monthly peramine concentrations in basal and regrowth components of A. lolii-infected ryegrass plants during 1991-1992. Vertical bars represent SEM.

markedly between March and May. Lolitrem B concentrations in the basal component continued to decline gradually and reached their lowest level (0.21μg/g) in the October harvest after which there was a slight increase between October and November.

Lolitrem B concentrations in the regrowth component were lower than those in the basal component for all harvests (Fig. 3.12). The seasonal pattern of lolitrem B concentrations in the regrowth component was similar to that in the basal component with mean lolitrem B concentrations in the regrowth component being highest during summer and early autumn (December to April) and lowest during winter and early spring (June to October). Specifically, the highest mean lolitrem B concentration in the regrowth portion (1.67μg/g) was recorded in January, while the lowest (0.025μg/g) was recorded in July.

In the basal and regrowth components, mean lolitrem B concentrations were significantly ($P<0.05$) correlated with changes in mean monthly grass minimum temperature (Figs. 3.10 and 3.12, Table 3.2). Mean lolitrem B concentrations were not correlated with changes in total monthly rainfall or day length (Figs. 3.10 and 3.12, Tables 3.3 and 3.4). Together, grass minimum temperature, rainfall and day length did not account for a significant amount of the variation in lolitrem B concentration in either basal or regrowth categories (Table 3.5).

Peramine concentrations in the basal component were relatively stable during summer and autumn (December 1991 to may 1992) and ranged from 14.5μg/g to 18.1μg/g (Fig. 3.13). After the May harvest, peramine concentrations declined steadily to reach the lowest level of the year (5.5μg/g) in August. After August, peramine concentrations in the basal component increased steadily and by October, were nearly as high as those recorded in January to May.

In the regrowth component, peramine concentrations were higher than those in the basal component for all months with the exception of October (Fig. 3.13). From the

January level, peramine concentrations in the regrowth component increased sharply to peak at 34.2μg/g in March. Following a decrease between March and April, peramine concentrations were relatively stable (23.5μg/g-27.2μg/g) between April and July. Peramine concentrations then declined sharply from July to reach their lowest level of 8.6μg/g in August. After August, peramine concentrations in the regrowth component increased steadily to reach 16.9μg/g by November.

Peramine concentrations in the basal portion were significantly ($P<0.05$) correlated with changes in mean monthly grass minimum temperature and total monthly rainfall (Figs. 3.10 and 3.13, Tables 3.2 and 3.3). Peramine concentrations in the regrowth component were not significantly correlated with changes in mean monthly grass minimum temperature, total monthly rainfall (although the relationship was significant at the 10% level) or photoperiod (Figs. 3.10 and 3.13, Tables 3.2, 3.3 and 3.4). Peramine concentrations in the basal portion were also not correlated to changes in photoperiod (Figs. 3.10 and 3.13, Table 3.4). Significant amounts of the variation in peramine concentrations in both basal and regrowth categories could be explained by the combined influences of grass minimum temperature, rainfall and photoperiod (Table 3.5).

Changes in mean monthly *A. lolii* concentration in basal tissues over time were not correlated to changes in mean monthly lolitrem B concentration (Table 3.6), whereas they were significantly ($P<0.05$) correlated in the regrowth component. Changes in mean monthly *A. lolii* concentration in the basal portion were significantly ($P<0.01$) correlated to changes in mean monthly peramine concentration over time (Table 3.6). There was no such correlation in the regrowth component.

Mean yearly lolitrem B and peramine concentrations were significantly ($P<0.01$ for basal tissues and $P<0.001$ for regrowth tissue) positively correlated ($r^2 = 40.9$ for basal tissues and 47.6 for regrowth tissues). Also, *A. lolii* concentrations in basal and

Table 3.6 **Relationship between log A. lolii concentration and lolitrem B and peramine concentrations in basal and regrowth components of all plants with time. (*P<0.05, **P<0.01, NS = not significant).**

Correlation	*Coefficient of determination (r^2)*		*Significance*	
	Basal	*Regrowth*	*Basal*	*Regrowth*
A. lolii v Lolitrem B	0.0	32.7	NS	*
A. lolii v Peramine	46.8	10.2	**	NS

regrowth tissues were significantly positively correlated with lolitrem B concentrations on 4 out of 12 and 5 out of 12 sampling occasions respectively throughout the year (Table 3.7) while *A. lolii* concentrations in basal and regrowth components were significantly positively correlated with peramine concentrations on 11 out of 12 and 7 out of 12 months respectively throughout the year (Table 3.8). Mean yearly *A. lolii* concentrations in basal and regrowth components were significantly ($P<0.001$) positively correlated with mean yearly lolitrem B concentrations (Figs. 3.14 ($r^2 = 59.4$) and 3.15 ($r^2 = 64.4$) respectively) and mean yearly peramine concentrations (Figs. 3.16 ($r^2 = 66.1$) and 3.17 ($r^2 = 51.4$) respectively).

Mean yearly lolitrem B and peramine concentrations from the 1990 trial were significantly ($P<0.001$) positively correlated with mean yearly lolitrem B and peramine concentrations ($r^2 = 86.5$ and 84.5 respectively) from the 1991/1992 trial.

3.4 DISCUSSION

ELISA technology and the hyphal count method have both been used previously to investigate seasonal changes of *A. lolii* concentration in endophyte-infected perennial ryegrass (Fletcher 1983, 1986, Mortimer *et al.* 1984, di Menna and Waller 1986, di Menna *et al.* 1992). However, in these studies *A. lolii* concentrations were monitored by taking samples of pasture which, over time, would not have been derived from the same plants. This could have introduced some unnecessary variability into the data. Also, in the studies in which an ELISA was used (Fletcher 1983, 1986), *A. lolii* concentrations were not monitored over a full 12 month period as the aim of these studies was to examine the seasonality of *A. lolii* with respect to the occurrence of ryegrass staggers (ie September to April). Finally, with the exception of the study by di Menna *et al.* (1992)

Table 3.7 **Relationship between log *A. lolii* concentration and lolitrem B concentration in basal and regrowth portions of *A. lolii*-infected perennial ryegrass plants for each month from December 1991 to November 1992. (*P<0.05, **P<0.01, NS = not significant).**

Month	Coefficient of determination (r^2)		Significance	
	Basal	Regrowth	Basal	Regrowth
December (1991)	33.4	52.0	*	**
January (1992)	29.1	45.7	*	**
February	9.7	44.2	NS	*
March	4.3	34.4	NS	*
April	28.7	45.4	*	**
May	15.2	4.5	NS	NS
June	0.3	0.1	NS	NS
July	0.0	0.0	NS	NS
August	42.0	3.2	**	NS
September	20.8	5.3	NS	NS
October	15.5	0.5	NS	NS
November	0.0	0.0	NS	NS

Table 3.8 **Relationship between log *A. lolii* concentration and peramine concentration in basal and regrowth portions of *A. lolii*-infected perennial ryegrass plants for each month from December 1991 to November 1992. (*P<0.05, **P<0.01, ***P<0.001, NS = not significant).**

Month	Coefficient of determination (r^2)		Significance	
	Basal	Regrowth	Basal	Regrowth
December (1991)	39.3	67.2	*	***
January (1992)	47.7	12.5	**	NS
February	40.9	70.7	*	***
March	55.6	73.8	**	***
April	86.3	76.6	***	***
May	28.3	62.2	*	***
June	51.4	16.7	**	NS
July	46.2	0.0	**	NS
August	0.0	0.0	NS	NS
September	47.4	0.8	**	NS
October	61.3	38.3	***	**
November	45.1	38.4	**	**

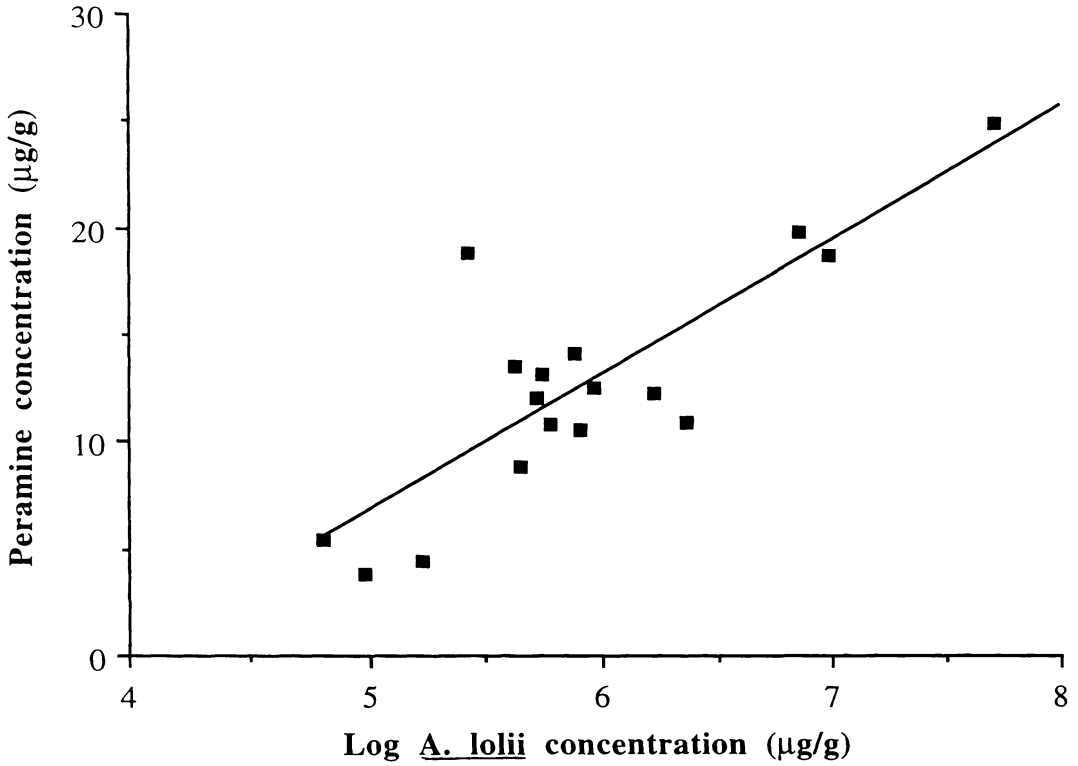


Figure 3.16 Relationship between the mean yearly concentrations of A. lolii and peramine in the basal portion of A. lolii-infected plants during 1991-1992.

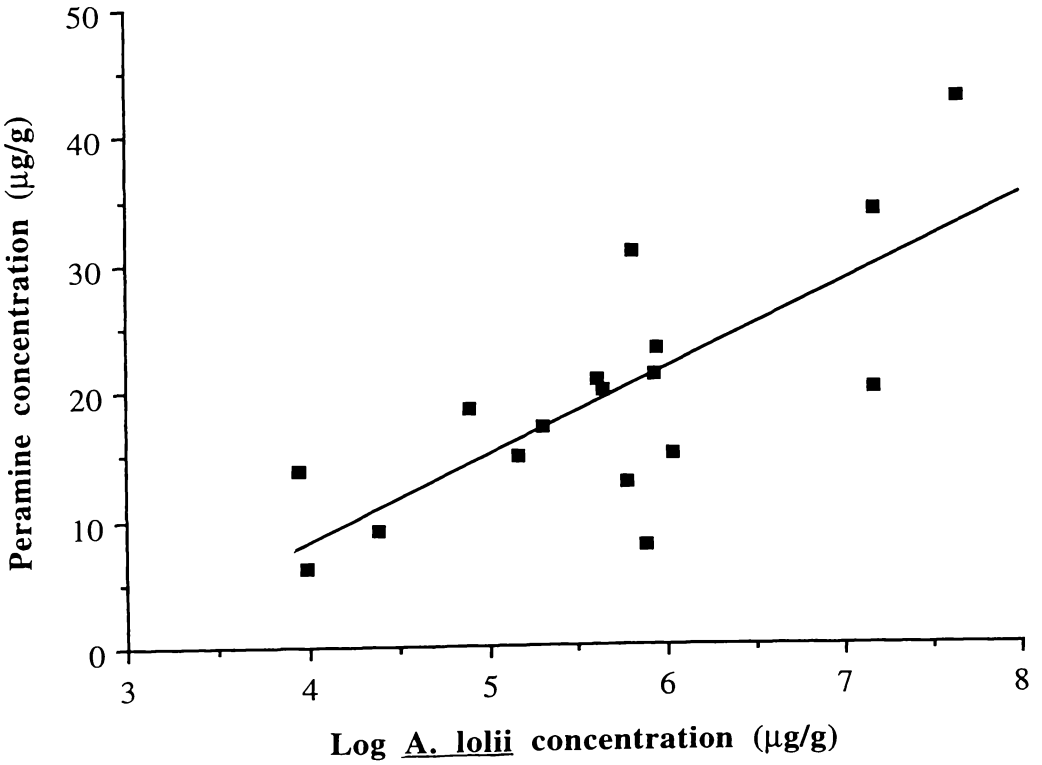


Figure 3.17 Relationship between the mean yearly concentrations of A. lolii and peramine in the regrowth portion of A. lolii-infected plants during 1991-1992.

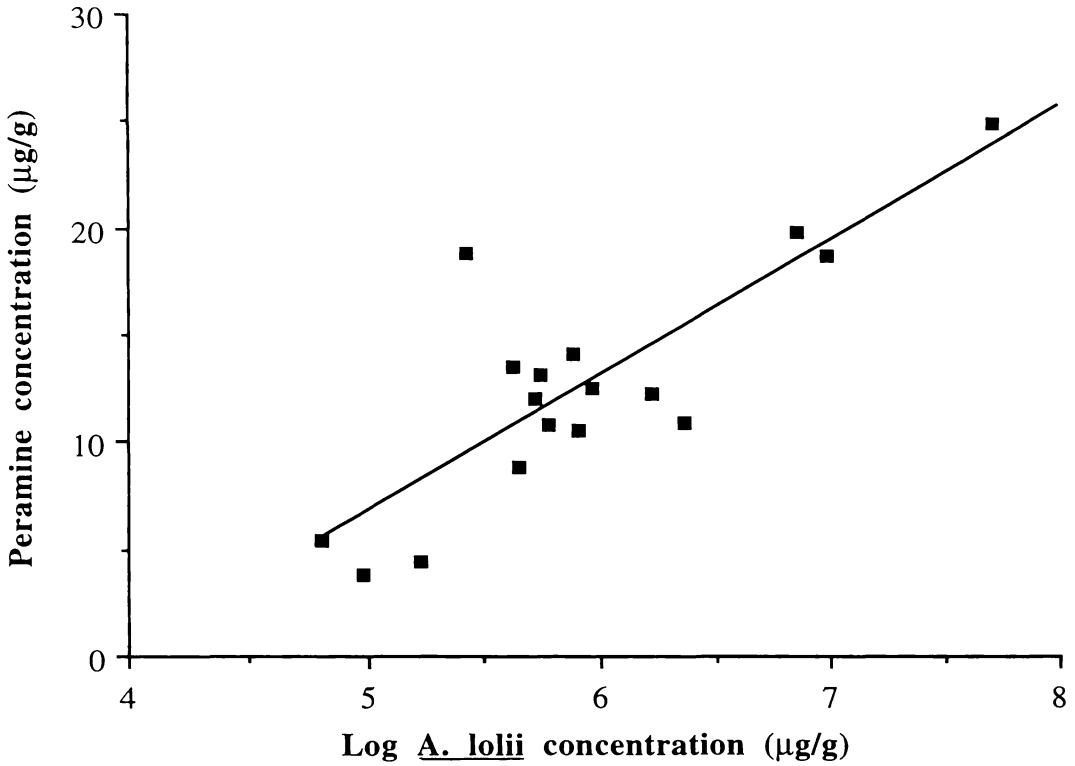


Figure 3.16 Relationship between the mean yearly concentrations of A. lolii and peramine in the basal portion of A. lolii-infected plants during 1991-1992.

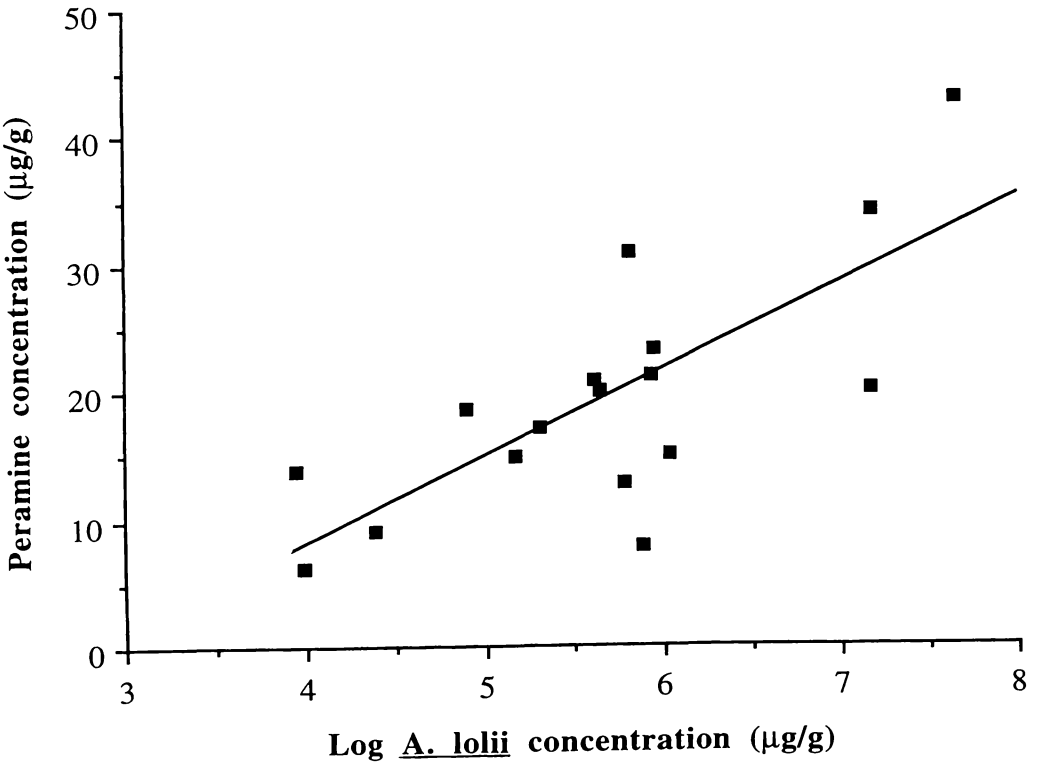


Figure 3.17 Relationship between the mean yearly concentrations of A. lolii and peramine in the regrowth portion of A. lolii-infected plants during 1991-1992.

who measured lolitrem B, alkaloid concentrations were not monitored simultaneously with *A. lolii* concentrations in previous trials.

In experiment 2 (1991-1992), the lowest mean *A. lolii* concentrations were recorded during winter and early spring (Fig. 3.9). Mean *A. lolii* concentrations then appeared to reach a peak in late spring and early summer with the highest concentration in the basal and regrowth components being recorded in December and November respectively. Similar seasonal changes in *A. lolii* concentration were recorded by Fletcher (1983, 1986) in Canterbury using ELISA and Mortimer *et al.* (1984), di Menna and Waller (1986) and di Menna *et al.* (1992) in the central North Island and waikato using the hyphal count method. These similar results were obtained despite the fact that the methods used to estimate *A. lolii* concentrations, the nature of the test samples and the time and place in which plant material was harvested differed widely. In the present study and in all of the studies previously discussed, *A. lolii* concentrations peaked either in early summer (November/December) (Fletcher 1983), summer/early autumn (January-March/April) (Mortimer *et al.* 1984), or both (di Menna and Waller 1986, Fletcher 1986). di Menna *et al.* (1992) found that at one site (Wairakei), *A. lolii* concentrations peaked in February, while at another site (Rukuhia) at the same time, they peaked from November to February. Also, a study performed in New Jersey USA, indicated that *A. lolii* hyphal counts peaked in spring (late May), fell in summer, and peaked again in autumn (mid October) (Breen 1990). Results from the basal portion in the current study also indicated the possible existence of two *A. lolii* peaks (ie a bimodal seasonality) between early summer and late autumn, as a small (though statistically insignificant) rise was recorded between March and May, after the large peak which occurred in November/December. Although by far the largest peak was recorded in the summer (November-January), the significant increase in *A. lolii* concentration between February and March in the regrowth component also indicated the presence of a late

summer/early autumn peak.

Without performing experiments under controlled conditions, it is very difficult to identify precisely the factors responsible for determining *A. lolii* concentrations in the host grass. Mortimer *et al.* (1984) and di Menna and Waller (1986) found that variations in *A. lolii* hyphal counts usually closely paralleled variations in mean monthly temperatures. However, di Menna and Waller (1986) found that this was not the case at one of the sites where *A. lolii* hyphal counts decreased over the hottest part of the year (January to March). This reduction in the *A. lolii* count was attributed by di Menna and Waller (1986) to summer moisture stress. Fletcher (1986) also found evidence suggesting that endophyte growth was greatest when rainfall was higher. Breen (1990 and 1992) suggested that *A. lolii* hyphal counts declined over summer in New Jersey as a result of temperatures exceeding the point at which maximal *A. lolii* growth occurs. Laboratory studies by Breen (1990 and 1992) showed that *A. lolii* hyphal counts were higher at moderate temperatures (14^{0}C and 21^{0}C) compared to cooler (7^{0}C) or warmer (28^{0}C) temperatures providing further evidence linking *A. lolii* concentration with temperature.

In the present study, *A. lolii* concentrations in the regrowth component were significantly positively correlated with mean monthly grass minimum temperature and significantly negatively correlated with total monthly rainfall (Tables 3.2 and 3.3) indicating the possible importance of temperature and moisture in determining *A. lolii* concentrations. The temperature correlation was the same as those observed in previous studies (ie the correlation was positive) (Mortimer *et al.* 1984, di Menna and Waller 1986). However, the negative rainfall correlation was surprising considering the previous suggestion of di Menna and Waller (1986) and Fletcher (1986) that higher rainfall increased *A. lolii* concentrations. Also, *A. lolii* concentrations in the basal component were not significantly correlated with either of these environmental

parameters, suggesting that either the effects of rainfall and temperature had a greater impact on *A. lolii* concentrations in the regrowth component compared to the basal component, or that the correlations in the regrowth component arose largely by chance and that temperature and moisture levels played only a small part in determining *A. lolii* concentrations anywhere in the plant. These inconsistent results illustrate the difficulty of describing the individual effects of environmental factors on *A. lolii* concentration using time sequence data, as many of the potentially important environmental and physiological factors (most of which were unrecorded in this study) are highly interrelated over time. Also, it is possible that factors such as temperature influenced *A. lolii* concentrations highly, but that due to the nature of the study, the two were out of phase.

Visual observation of Figure 3.9, rather than statistical comparisons may provide a clue as to one of the most important influences on *A. lolii* concentration. The short spring/early summer *A. lolii* concentration peak recorded in basal and regrowth tissues in experiment 2 (1991-1992) was observed to correspond closely with the growth spurt which is associated with the sexual reproductive development of the perennial ryegrass host, viz *A. lolii* concentrations in the basal and regrowth components of the ryegrass increased at the time when reproductive tiller development and seed production was at its height (October-January) (Fig. 3.9). Since the transmission of *A. lolii* is dependant upon the successful completion of the sexual reproductive cycle of the host grass, proliferation of *A. lolii* during flowering would appear to be advantageous to the endophyte, as it could increase the probability of *A. lolii* successfully infecting the ryegrass embryo and therefore being successfully disseminated. Being a long day plant (Bidwell 1979), perennial ryegrass will only flower when the day length is longer than a certain critical minimum, suggesting that photoperiod could, directly or indirectly (ie via plant hormones), influence *A. lolii* concentrations. It is therefore interesting to note that

the only environmental parameter which was significantly (if only at the 5% level) correlated with *A. lolii* concentrations in both regrowth and basal plant components, was mean monthly day length (ie photoperiod) (Table 3.4).

The results from the present study appear to indicate that none of the measured environmental parameters influenced *A. lolii* concentrations to the exclusion of the others. Increasing day length and temperature (which are obviously related themselves) were statistically related to increases in *A. lolii* concentration in most components, while increasing rainfall appeared to decrease *A. lolii* concentrations in the regrowth component. However, the results indicated that *A. lolii* concentrations increased markedly during the sexual reproductive phase of the host plant, suggesting that one or more of the factors influencing, or resulting from host plant reproductive development (ie photoperiod, temperature, plant hormone balance, plant nutrient status, plant cell structure, plant growth rate) could be important in determining *A. lolii* concentrations. This conclusion is supported by the findings of chapter 4 of this thesis. However, it is evident that other factors are also important in determining *A. lolii* concentrations in the host grass, as different plants contained consistently different concentrations of *A. lolii* (Fig. 3.11). The genetic characteristics of both the endophyte and the perennial ryegrass are probably therefore important factors in determining the relative levels of *A. lolii* in each plant. This conclusion is supported by the work of Breen (1992) who, studying four ryegrass genotypes, found that counts of *A. lolii* hyphae were highly dependant upon ryegrass line.

The results from Figure 3.9 show that *A. lolii* concentrations were higher in the base compared to the regrowth portion of the plant for all months of the year with the exception of January. These results are in complete agreement with those of Neill (1940), Musgrave (1984) and Musgrave and Fletcher (1984) and indicate that due to a number of possible factors (ie leaf structure, nutrient status, plant hormone balance),

growth of *A. lolii* in the leaf blade is limited, in comparison to growth in the leaf sheath.

The seasonal changes in mean lolitrem B concentrations recorded in experiments 1 (1990) and 2 (1991-1992) were similar, with concentrations peaking in summer and early autumn on both occasions (Figs. 3.4 and 3.12). A similar peak mean lolitrem B concentration of ca. 4.5µg/g was recorded on both occasions (using results from the basal component in experiment 2), with the peaks occurring in February in the 1990 experiment and January in the 1991-1992 experiment. The only notable difference between the two sets of results was the duration of the peak, which was more gradual and lasted longer in the 1990 trial. Although the plants monitored in the experiments were not present in a pasture situation precluding the making of any conclusions concerning the relationship between lolitrem B concentrations and specific outbreaks of ryegrass staggers, it is interesting to note that the time at which the lolitrem B peaks occurred, coincided well with the usual observed seasonality of the ryegrass staggers condition in stock (summer/autumn). It is also apparent that like *A. lolii*, lolitrem B concentrations in experiment 2 were consistently higher in the basal part of the plant compared to the regrowth portion (Fig. 3.12). This distribution of lolitrem B in the plant has been reported previously (Gallagher *et al.* 1987, di Menna *et al.* 1992, Davies *et al.* 1993, Keogh and Tapper 1993, chapter 4 present study) and probably explains the observation that serious ryegrass staggers outbreaks usually occur when stock are forced to graze pastures too close.

The results of the current study are in general agreement with those of Prestidge and Gallagher (1988a) and di Menna *et al.* (1992) although it is apparent that there may be considerable variation in the time of year at which lolitrem B peaks occur. di Menna *et al.* (1992) sampled ryegrass pastures at Rukuhia (near Hamilton) and Wairakei (near Taupo) and found that lolitrem B concentrations at the former site peaked in January while at the latter site, they peaked in April/May of the same year. Sampling ryegrass

pastures at Wairakei, Prestidge and Gallagher (1988a) also observed peak lolitrem B concentrations in April/May. Taken together, these results indicate that lolitrem B concentrations in ryegrass plants situated near Hamilton may peak 2-4 months earlier than lolitrem B concentrations in ryegrass plants maintained near Taupo. This may be due to differences in environmental conditions at the two sites. Although mean monthly grass minimum temperatures and lolitrem B concentrations in experiment 1 were highly correlated (r^2 = 79.3), the relationship was only just significant in experiment 2 (r^2 values of 27.2 and 38.4 for basal and regrowth portions respectively) indicating that the relationship was weaker than may have initially been thought. The fact that temperature profiles during experiments 1 and 2 of the present study, and during the study of Prestidge and Gallagher (1988a) were all very similar to each other, adds support to the suggestion that the relationship between temperature and lolitrem B concentration is not necessarily a strong one. However, temperature has been shown to influence the production of another alkaloid in *A. lolii*-infected perennial ryegrass. Huizing *et al.* (1991) showed that *A. lolii*-infected ryegrass only produced loline (N-acetyl and N-formyl loline) alkaloids at elevated temperatures (ie above 23^{0}C).

Relationships between temperature and alkaloid concentration have been observed in other grass/endophyte associations. Under controlled environment conditions, Kennedy and Bush (1983) observed that greater increases in loline alkaloid concentration occurred in *A. coenophialum*-infected tall fescue (*Festuca arundinacea*) plants maintained in a 21^{0}C/15^{0}C temperature regime compared to plants grown at 32^{0}C/27^{0}C or 16^{0}C/10^{0}C.

Prestidge and Gallagher (1988a) found that lolitrem B levels were highest during months with low rainfall. Lolitrem B concentrations in both years of the present study also peaked during periods of low rainfall (Figs. 3.4, 3.5, 3.10 and 3.12). Whether this

was a direct effect of moisture stress in experiment 1 is uncertain however, as the plants were irrigated over summer and did not appear to be stressed. Also, in experiment 2, mean monthly lolitrem B and total monthly rainfall were not correlated for the year (Table 3.3), suggesting that if rainfall, and therefore moisture stress, did have an effect, it was masked by changes in other factors which occurred at the same time. Under controlled conditions, Barker *et al.* (1993) found that water deficit had no effect on lolitrem B concentration.

Moisture stress has been shown to influence concentrations of other alkaloids in grass/endophyte associations. Barker *et al.* (1993) found that ergovaline concentrations were significantly higher in unwatered plants of one *Acremonium*-infected line of perennial ryegrass. Kennedy and Bush (1983) found that loline (N-acetyl loline and N-formyl loline) alkaloid concentrations in *A. coenophialum*-infected tall fescue increased significantly during periods of water stress. Belesky *et al.* (1989b) showed this to be an inconsistent effect although the total loline alkaloid yield in leaf tissue increased in all water stressed isolines compared to adequately watered isolines. Belesky *et al.* (1989b) also found that this was the case for ergopeptine alkaloids in leaf tissue.

Instead of being under the direct control of environmental factors such as temperature and moisture level, it would be reasonable to suspect that changes in lolitrem B concentration over time could be governed by similar changes in the *A. lolii* concentration. Although the relationship was weak, this appeared to be the case in the regrowth component (Table 3.6). However, linear regression analysis between mean monthly log *A. lolii* concentration and mean monthly lolitrem B concentration over the twelve month period of experiment 2, indicated that lolitrem B concentration changes in the basal part of the plant over time were not a function of *A. lolii* concentration changes over time (Table 3.6). In deciding whether *A. lolii* concentration changes over time play a large part in determining lolitrem B concentrations, it may be more important to take

into account the result from the basal component as the nature of the regrowth component changed each month depending upon the growth rate of the plant, whereas the composition of the basal component was more consistent over time.

Prestidge and Gallagher (1985) reported that adult Argentine stem weevil were not deterred from feeding on artificial diets with lolitrem B concentrations of 5μg/g (wet weight); this equates to approximately 40-50μg/g dry weight (D.D. Rowan, pers. comm.). In the current study, a lolitrem B concentration of 5μg/g (dry weight) for individual plants was considered high during the summer. It is unlikely therefore that lolitrem B has a major role in deterring natural infestations of adult Argentine stem weevil. However, stem weevil larval growth rates and survival are significantly reduced at 5μg/g (wet weight) (Prestidge and Gallagher 1985), and Dymock *et al.* (1989) found that 2μg/g (wet weight) of lolitrem B incorporated into artificial diets acted as a feeding deterrent to Argentine stem weevil larvae. Lolitrem B concentrations recorded in this study, even in those in the basal portion of the plant, were therefore probably too low to adversely affect stem weevil larvae. However, the basal plant category was a composite sample, consisting of inner and outer leaf sheaths and leaf blades. Thus lolitrem B may still provide resistance against larval Argentine stem weevil attack as concentrations in the outer leaf sheaths are considerably higher than those in the inner leaf sheaths or leaf blades (Gallagher *et al.* 1987, Keogh and Tapper 1993, chapter 4 present study).

The present study is the first to report seasonal levels of peramine in *A. lolii*-infected perennial ryegrass. During winter, mean peramine concentrations declined by a factor of between 2-4 compared to summer levels (Figs. 3.4 and 3.13). This reduction was small compared to that observed for lolitrem B which declined by a factor of between 10-60 depending on the experiment and on the plant component. These results indicate either that peramine was metabolised more slowly, or that the production of peramine was maintained at a more constant level throughout the year than lolitrem B.

Also in complete contrast to what was observed with lolitrem B, mean peramine concentrations were consistently higher (apart from one month) in the regrowth component compared to the basal portion of the plant. As *A. lolii* concentrations were consistently lower in the regrowth compared to the basal component, this suggests that large amounts of peramine may have been translocated from the basal part of the plant into the regrowth portion. The high concentrations of peramine recorded in the regrowth component may be significant as there is speculation that peramine may be an important factor in causing faecal contamination in livestock (Fletcher and Sutherland 1993a, Pownall *et al.* 1993). Stock could therefore be obtaining much of this peramine from the regrowth component. Results presented in chapter 4 support this hypothesis. However, the results of previous reports concerning the vertical distribution of peramine in *A. lolii*-infected ryegrass have been inconsistent. Tapper *et al.* (1989) found that when cut 8-10cm from the crown, basal portions of *A. lolii*-infected ryegrass plants contained higher concentrations of peramine than the upper portions, whereas Fannin *et al.* (1990) found that the highest concentrations of peramine were present in the leaf blade. However, in these and other studies (ie Davies *et al.* 1993, Keogh and Tapper 1993) which also investigated the distribution of peramine in the plant, samples were only taken at one or two times in the year and it is evident from the results of the present study that the distribution of peramine between lower and upper parts of the plant is highly seasonally dependent.

Regression analyses were performed in an attempt to determine which, if any, of the measured environmental parameters (temperature, rainfall and photoperiod) were important in determining peramine concentrations in the plant. In both experiments, mean peramine levels were significantly correlated with changes in mean monthly grass minimum temperature (Figs. 3.4, 3.5, 3.10 and 3.13, Table 3.2), although this correlation was not strong. There was a slightly stronger correlation between mean

peramine concentration and total monthly rainfall in the basal part of the plant in experiment 2 (Figs. 3.10 and 3.13, Table 3.3), but there was no correlation between mean peramine concentration and photoperiod (Table 3.4). However, multiple regression analysis indicated that together, mean monthly grass minimum temperature, total monthly rainfall and mean monthly day length significantly influenced mean peramine concentrations, particularly in the regrowth component (Table 3.5). There was also considerable evidence indicating that seasonal changes in mean peramine concentration in the basal part of the plant were closely related to, and therefore possibly explained by, seasonal changes in the *A. lolii* concentration (Table 3.6).

Dymock *et al.* (1988) showed that Argentine stem weevil larval mortality was increased when feeding on diets containing 2μg/g (wet weight) of peramine. Adult argentine stem weevil are deterred from feeding by peramine levels as low as 0.1μg/g (wet weight) (Dymock *et al.* 1988) although Popay *et al.* (1990) found that 10μg/g (wet weight) was required to reduce feeding in a no choice situation. In these experiments, the concentration of peramine in the diet was calculated on a wet weight basis. Analysis of peramine concentrations in perennial ryegrass samples in the present study was performed on dry matter. It is therefore difficult to compare the effects on Argentine stem weevil of naturally occurring peramine in *A. lolii*-infected perennial ryegrass with peramine which has been artificially added to diets. However, peramine concentrations of 10μg/g (dry weight) in winter were common, indicating that a degree of weevil protection may be maintained throughout the year in *A. lolii*-infected perennial ryegrass. In the evolution of the *A. lolii*/perennial ryegrass symbiosis, peramine may have been more important in terms of insect resistance than lolitrem B, and maintaining a year round degree of insect resistance may also have been important. Selection pressure may therefore have favoured endophyte/grass associations which maintained higher year round levels of peramine, explaining the relatively minor winter lull in peramine

compared to that recorded for lolitrem B.

Seasonal fluctuations have been recorded for other endophyte-produced alkaloids. Results from the U.S.A., New Zealand and Australia show that ergovaline levels in *A. coenophialum*-infected tall fescue and *A. lolii*-infected perennial ryegrass are lowest in winter (Belesky *et al.* 1988, Rowan *et al.* 1990b, Rottinghaus *et al.* 1991, Woodburn *et al.* 1993). A similar seasonal pattern was observed with the loline alkaloids in *A. coenophialum*-infected tall fescue (Kennedy and Bush 1983, Belesky *et al.* 1987b). This is also similar to what was observed with lolitrem B and peramine in the present study. It is therefore probable that many, if not all alkaloids produced by the association of an *Acremonium* endophyte with a grass, undergo seasonal fluctuations in their production, and reach their lowest concentrations in winter.

As well as seasonal variations in lolitrem B and peramine concentration, consistent plant to plant variations in alkaloid concentration (which were maintained over a two year period) were also recorded (Figs. 3.6 and 3.7). Thus it is evident that factors other than those related to season can also influence lolitrem B and peramine concentrations. Plant to plant differences in the levels of lolitrem B, peramine and ergovaline have been noted previously in perennial ryegrass (Rowan *et al.* 1990b, Ball *et al.* 1991, Davies *et al.* 1993). Similar observations concerning relative levels of loline and ergopeptine alkaloids in different *A. coenophialum*-infected tall fescue plants have also been made (Jones *et al.* 1985, Bacon 1988, Belesky *et al.* 1989b, Hill *et al.* 1991, Azevedo *et al.* 1993, Agee and Hill 1994). There is evidence indicating that the genetic characteristics of both the endophyte strain and the grass strain are important factors in determining the relative alkaloid levels in a plant. Christensen *et al.* (1991, 1993) found that endophyte strain could influence the presence and quantity of lolitrem B, peramine and ergovaline produced in perennial ryegrass, while Hill *et al.* (1991) and Agee and Hill (1994) found that tall fescue genotype influenced the amount of ergopeptine

alkaloids which accumulated. Davies *et al.* (1993) also presented evidence suggesting the existence of both endophyte genotype and grass genotype effects, and suggested among other possibilities, that differences in alkaloid content could relate to the vigour of the endophyte growth in the host plant. The highly significant degree of correlation between *A. lolii* concentration and lolitrem B and peramine concentrations observed in the present study (Figs. 3.14, 3.15, 3.16 and 3.17) indicate that this was certainly the case in the plants used. It is therefore not surprising that mean yearly lolitrem B and peramine concentrations for all of the plants were also highly correlated (Fig. 3.8). Thus it appears that *A. lolii* concentration is one of the most (if not the most) important factors which determine lolitrem B and peramine concentrations in *A. lolii*-infected perennial ryegrass plants derived from New Zealand pastures. These results therefore indicate that endophyte concentration may be an important consideration when selecting desirable grass/endophyte associations which leads to the question of whether alkaloid concentrations should be quoted as μg per g of endophyte as well as, or rather than, μg per g of dried grass.

3.5 CONCLUDING SUMMARY

Endophyte, peramine and lolitrem B concentrations in *A. lolii*-infected perennial ryegrass were lowest in winter. The highest *A. lolii* concentrations were recorded in early summer. This coincided with the time at which development of the plant sexual reproductive structures (ie reproductive stem, inflorescence, seed) was occurring. It was hypothesised that proliferation of *A. lolii* at this time would be advantageous to the endophyte as it may increase its chances of being successfully transmitted into the next plant generation via the seed. Lolitrem B concentrations peaked in summer/early autumn which coincided with the time at which ryegrass staggers outbreaks usually occur.

Lolitrem B concentrations were consistently higher in the basal part of the plant compared to the regrowth portion. This supports the findings of previous studies and explains the observation that serious ryegrass staggers outbreaks usually only occur when stock are forced to close graze pastures. Changes in lolitrem B concentration were only weakly correlated to changes in grass minimum temperature and were apparently not related to changes in photoperiod or rainfall. Peramine concentrations, particularly in the basal part of the plant, were relatively stable over the year compared to lolitrem B. It was suggested that during the course of evolution, plant/endophyte combinations producing relatively high amounts of peramine throughout the year may have been selected for as these individuals were probably resistant to insect attack for most or all of the year, and therefore at a selective advantage. Peramine concentrations were higher in the regrowth component compared to the basal portion of the plant. Multiple regression analysis indicated that changes in peramine concentration were closely related to changes in grass minimum temperature, monthly rainfall and photoperiod. However, seasonal peramine concentration changes in the basal part of the plant were also closely related to seasonal changes in the *A. lolii* concentration. Plants contained different levels of lolitrem B and peramine. These differences were generally maintained throughout the year, and from year to year. Mean yearly concentrations of lolitrem B and peramine in different plants were significantly correlated with, and therefore probably largely determined by, mean yearly concentrations of *A. lolii* in the same plants. This is the first report of such a correlation.

CHAPTER 4

THE DISTRIBUTION AND PRODUCTION OF FUNGAL MYCELIUM AND THE ALKALOIDS PERAMINE AND LOLITREM B IN *A. LOLII*-INFECTED PERENNIAL RYEGRASS.

4.1 INTRODUCTION

Much of the biology regarding the *A. lolii*-perennial ryegrass symbiosis has yet to be resolved. A large part of this biology concerns the production of the various alkaloids and how this is related to the growth of the endophyte itself. The effect of time of year (examined in chapter 3), plant tissue and plant management on growth of *A. lolii* and on alkaloid production are of particular interest. Musgrave (1984) and Musgrave and Fletcher (1984) have examined the distribution of *A. lolii* in various tissues of perennial ryegrass. Also, the distribution of lolitrem B (Gallagher *et al.* (1982a, 1985, 1987), di Menna *et al.* (1992), Davies *et al.* (1993)) and peramine (Tapper *et al.* (1989), Fannin *et al.* (1990), Davies *et al.* (1993)) in *A. lolii*-infected perennial ryegrass plants has been studied. However, from these investigations, it is difficult to reconcile causes and effects, as alkaloid and *A. lolii* concentrations were monitored independently of each other. Keogh and Tapper (1993) have performed the only study where *A. lolii*, lolitrem B and peramine were monitored in the same plant tissues at the same time. Although much useful information was gained from their study, many questions regarding the growth of *A. lolii* and production of associated alkaloids were not answered. For example, how does the growth of *A. lolii*, and production of peramine and lolitrem B change over time? How concentrated are *A. lolii* hyphae, and peramine and lolitrem B in *all* parts of the plant, including the reproductive structures (in many of the previous

studies, some plant tissues were not analyzed)? What effect does plant management have on the growth of *A. lolii* and on alkaloid production?

With answers to the above questions, it should be possible to define more clearly under which circumstances livestock are at risk from ingesting significant quantities of toxin and therefore suffering from *A. lolii* associated illnesses. Also, it could provide valuable information on how invertebrate resistance could be maximized in *A. lolii*-infected plants. The aim of the work presented in this chapter was to investigate the effect of time, plant part, plant reproductive development and plant management on the growth of *A. lolii* and on the concentration and total production of peramine and lolitrem B.

4.2 MATERIALS AND METHODS

4.2.1 Plant maintenance and sampling.

Eight mature and genetically different *A. lolii*-infected perennial ryegrass plants (1-8), plus one *A. lolii*-free plant, were each split into five clones. The *A. lolii*-infected plants were the progeny of pair crosses performed between ryegrass plants described in chapter 3. All of the clones were planted approximately 50cm apart into Horotiu sandy loam soil on 7/7/91, and maintained outdoors at the Ruakura Agricultural Research Centre, Hamilton. Plants obtained all of their water from rainfall and did not receive additional irrigation.

On 16/9/91, all of the plants were trimmed to 8cm. A regular clipping regime was then continued for three of the five clones of each line. These three clones were termed 'vegetative' plants as the trimming regime prevented the plants from becoming fully reproductive. Depending upon their harvest date, the 'vegetative' plants were trimmed down to 8cm on 2/10/91, 14/10/91, 24/10/91, 30/10/91, 14/11/91, 22/11/91,

2/12/91, 17/12/91 and 31/12/91. After 16/9/91, the remaining two clones of each plant were left untrimmed and were termed 'reproductive' plants.

On 1/10/91, 6/11/91 and 14/1/92, one 'vegetative' clone of each of the nine lines was removed from the soil and washed thoroughly under cold tap water. After trimming the harvested plants to a height of 8cm, the tillers on each plant were separated, counted and dissected into the following components: 4th leaf sheath, 4th leaf blade, 3rd leaf sheath, 3rd leaf blade, 2nd leaf sheath, 2nd leaf blade, 1st leaf, senescent (dead) leaf, stem tissue, crown and root. The 4th leaf refers to the oldest (outermost) leaf and the 1st leaf refers to the youngest (innermost). Due to the nature of the sample, there was no 4th leaf blade and 4th leaf sheath component in the 14/1/92 harvest.

On 6/11/91 and 14/1/91, one 'reproductive' clone of each of the nine lines was harvested as described for the 'vegetative' plants. Tillers from the plants harvested on 6/11/91 were separated and classed as being either vegetative, intermediate or reproductive. Vegetative tillers were classed as such if they showed no, or only extremely early signs, of developing reproductive structures. Tillers were classed as being reproductive if the inflorescences had fully emerged. Stems which were neither fully vegetative or reproductive were classed as intermediate. There were no intermediate tillers in the 'reproductive' plants harvested on 14/1/92.

Vegetative tillers from the 6/11/91 harvest were counted and a large proportion of them dissected into 4th leaf sheath, 4th leaf blade, 3rd leaf sheath, 3rd leaf blade, 2nd leaf sheath, 2nd leaf blade, 1st leaf, senescent leaf, stem tissue, crown and root. The root components from all three classes of tiller from the 'reproductive' plants, were bulked to form one root category. The vegetative tillers which were not dissected, were trimmed to 8cm to form two further categories called lower vegetative tiller material (ground level to 8cm) and upper vegetative tiller material (leafy material from 8cm upwards). Vegetative

tillers from the 14/1/92 harvest of the 'reproductive' clones were dissected into the same components as the vegetative tillers from the 6/11/91 harvest except that there were no 4th leaf sheath and 4th leaf blade categories.

Intermediate tillers harvested on 6/11/91 were not dissected, but were counted and then trimmed to 8cm to form two categories called lower intermediate tiller material (ground level to 8cm) and upper intermediate tiller material (material from 8cm upwards).

The reproductive tillers harvested on 6/11/91 were counted and a large proportion of them were dissected into the following categories: 3rd leaf sheath, 3rd leaf blade, 2nd leaf sheath, 2nd leaf blade, 1st leaf sheath, 1st leaf blade, inflorescence, senescent leaf, lower reproductive stem (reproductive stem minus leaf sheath material up to the end of the second to last internode), upper reproductive stem (the last internode before the inflorescence) crown and root. Like the vegetative and intermediate tillers, the remaining undissected reproductive tillers were trimmed to a height of 8cm and incorporated into lower reproductive tiller material (ground level to 8cm) and upper reproductive tiller material (8cm and above) categories. Reproductive tillers harvested on 14/1/92 were dissected into seed, seed head (minus seed), lower reproductive stem (culm up to the end of the second to last internode), upper reproductive stem (the last internode before the seed head), senescent leaf, crown, lower reproductive tiller and upper reproductive tiller categories. By 14/1/92, there was no green leaf material left on the reproductive tillers, accounting for the absence of the leaf sheath and blade categories. Senescent leaf material which could not be assigned to vegetative or reproductive tillers was included in a separate category (mixed dead leaf).

All of the harvested plant material, including the trimmings (regrowth), was immediately frozen at -20^{0}C, after which it was freeze-dried, weighed and ground to a

fine powder in an Ika model MFC Culatti micro hammer mill. The samples were stored in glass scintillation vials at -20^{0}C before being analyzed for *A. lolii*, peramine and lolitrem B.

4.2.2 *A. lolii*, peramine and lolitrem B analyses.

Plant samples were analyzed for *A. lolii*, peramine and lolitrem B content using the methods outlined in section 3.2.2. *A. lolii*-free samples were not analyzed for peramine or lolitrem B.

4.2.3 Statistical analyses and calculations.

Data from *A. lolii*-infected samples were log transformed and analyzed using analysis of variance. Most of the data are presented in bar chart form. Least significant ratios (LSR) are presented with each bar chart. The LSR was calculated by multiplying the mean standard error of the differences by the value of *t* at the 5% level and back transforming the resulting figure. Comparisons between two values on the same bar chart are statistically significant at the 5% level if a figure greater than the LSR is obtained when the larger value is divided by the smaller value. The total quantities of *A. lolii*, peramine and lolitrem B in each plant part, were estimated by multiplying the component concentration by the dry weight of the plant part. The dry weight of the plant component was first 'corrected' by proportionally incorporating the dry weight of any undissected material into each component. Total quantities in the regrowth component of 'vegetative' plants were calculated by summing the quantities of *A. lolii*, peramine and lolitrem B removed from the plants during the entire trimming regime (including the clippings taken on the day of harvest).

The total amount of *A. lolii*, peramine and lolitrem B contained in each plant, excluding that present in the roots, was also calculated. This was done by multiplying the component concentration by the dry weight of the plant part, and summing the results for each plant. Using these results a concentration for the entire plant (with the

exception of the roots) could be calculated. The roots were excluded from the calculations as concentrations of *A. lolii*, peramine and lolitrem B in this tissue were so low.

4.3 RESULTS

4.3.1 Analysis of the *A. lolii*-free clones by ELISA.

Over 70% of the samples from the *A. lolii*-free clones registered absorbances which were below that registered by the blank wells. A mean absorbance equivalent to 5.5μg/g (standard deviation 10.1μg/g) of *A. lolii* was recorded for all of the samples. Consistently higher than average '*A. lolii* concentrations' were recorded in the root (12.2-18.76μg/g) and senescent leaf (11.1-52.36μg/g) components.

4.3.2 'Vegetative' plants.

1/10/91 harvest: The highest *A. lolii* concentrations were present in the reproductive stem tissue (182μg/g) and the crown (130μg/g) (Fig. 4.1). Compared to root (26μg/g), 1st leaf (22μg/g), leaf blade (15-20μg/g) and regrowth (10μg/g) tissues, *A. lolii* concentrations were also high in the leaf sheaths (68-117μg/g). The datum point for the senescent leaf component is not presented as there was insufficient material to analyze for *A. lolii* in some of the plants.

Compared to other components, peramine concentrations were highest in the leaf sheaths (21.1-24.2μg/g) (Fig. 4.3). The lowest concentrations of peramine were present in the roots (0.04μg/g), senescent (dead) leaf (4.1μg/g) and 4th blade (5.5μg/g).

Lolitrem B concentrations were comparatively low in root (0.1μg/g), regrowth (0.17μg/g) and leaf blade (0.4-0.6μg/g) components, while concentrations were high in the 4th sheath (2.9μg/g), 3rd sheath (1.9μg/g) and senescent leaf (1.4μg/g) (Fig. 4.5).

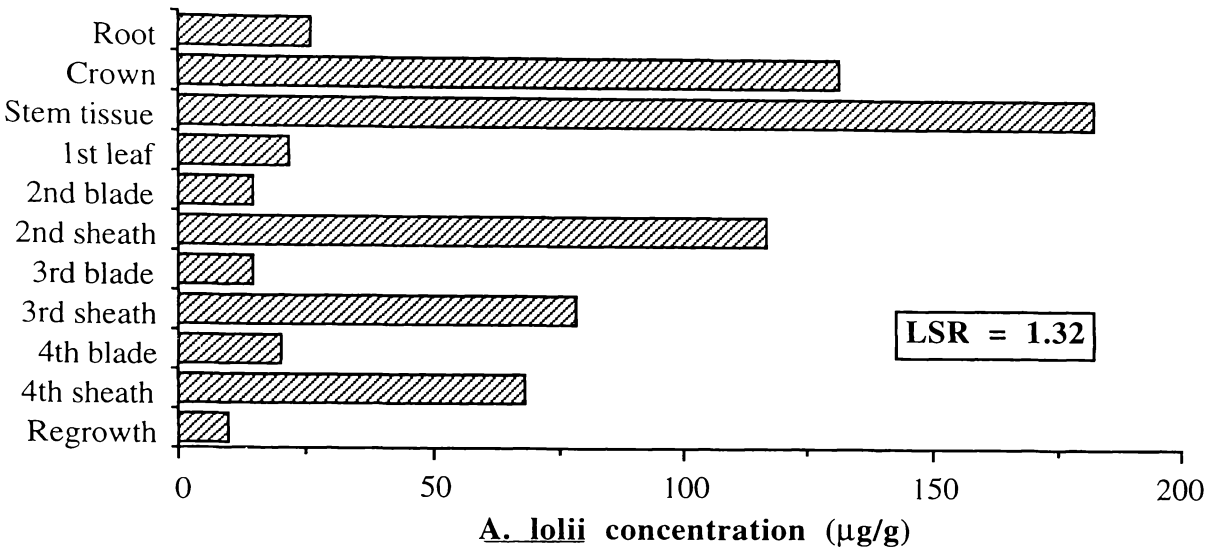


Figure 4.1 Concentration of A. lolii (µg of freeze-dried mycelium per g of dry grass material) measured by ELISA in different components of 'vegetative' perennial ryegrass plants sampled on 1/10/91. Values are a mean of eight different A. lolii-infected plants.

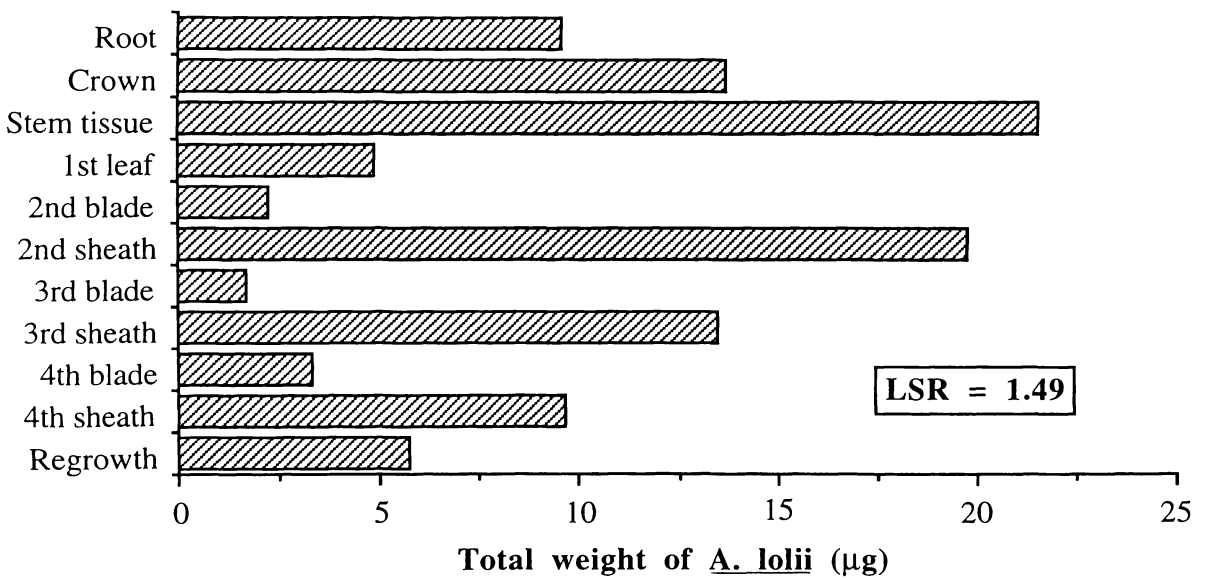


Figure 4.2 Total amount of A. lolii (µg of freeze-dried mycelium per tissue) in different components of 'vegetative' perennial ryegrass plants sampled on 1/10/91. Values are a mean of eight different A. lolii-infected plants.

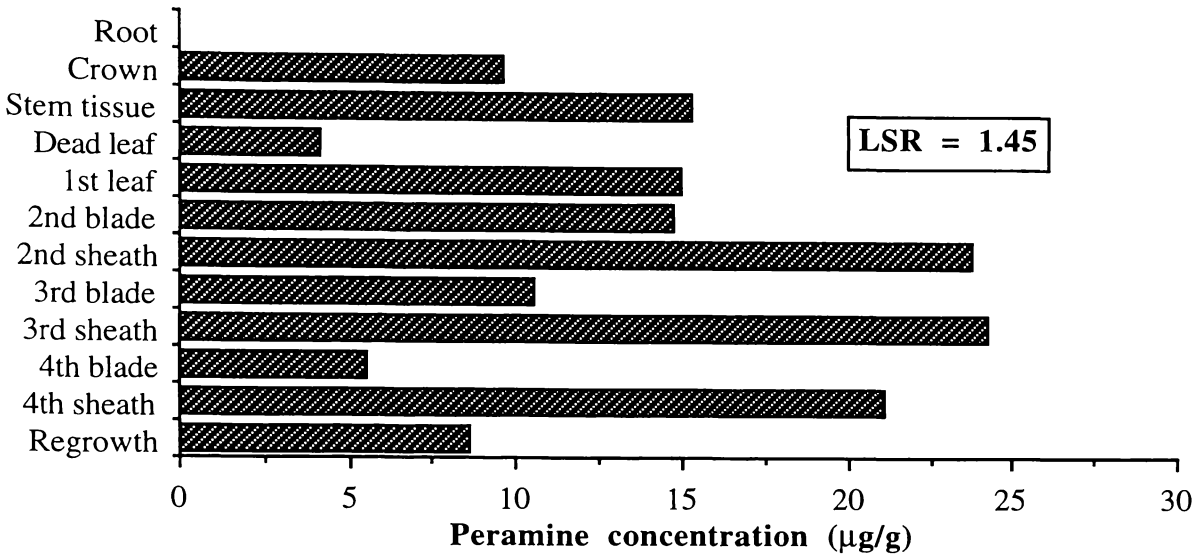


Figure 4.3 Peramine concentration (µg of peramine per g of dry grass material) measured by HPLC in different components of 'vegetative' perennial ryegrass plants sampled on 1/10/91. Values are a mean of eight different A. lolii-infected plants.

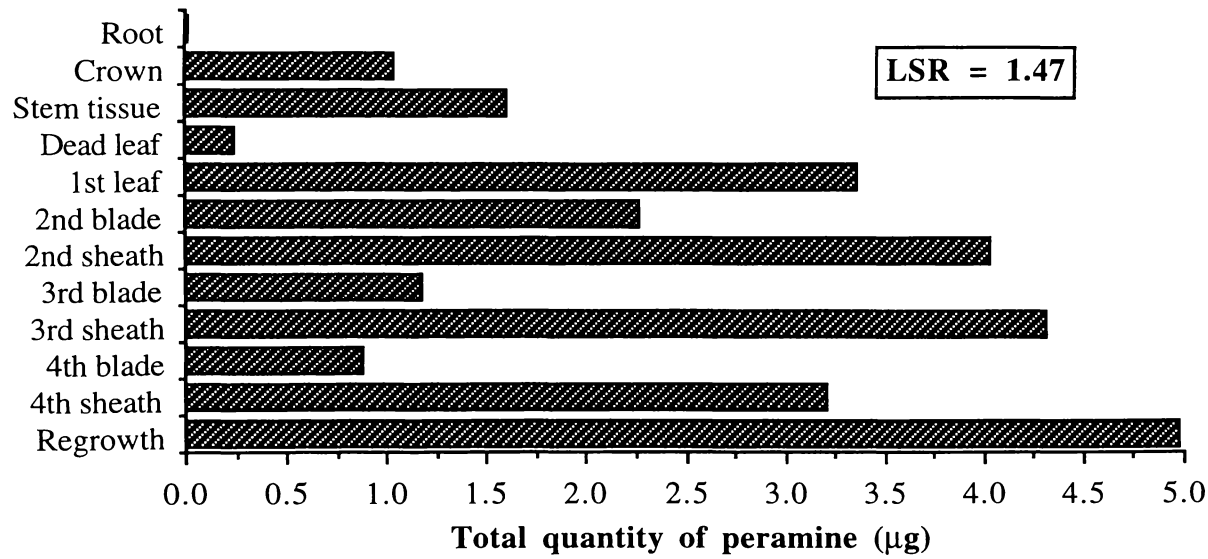


Figure 4.4 Total amount of peramine (µg of peramine per tissue) in different components of 'vegetative' perennial ryegrass plants sampled on 1/10/91. Values are a mean of eight different A. lolii-infected plants.

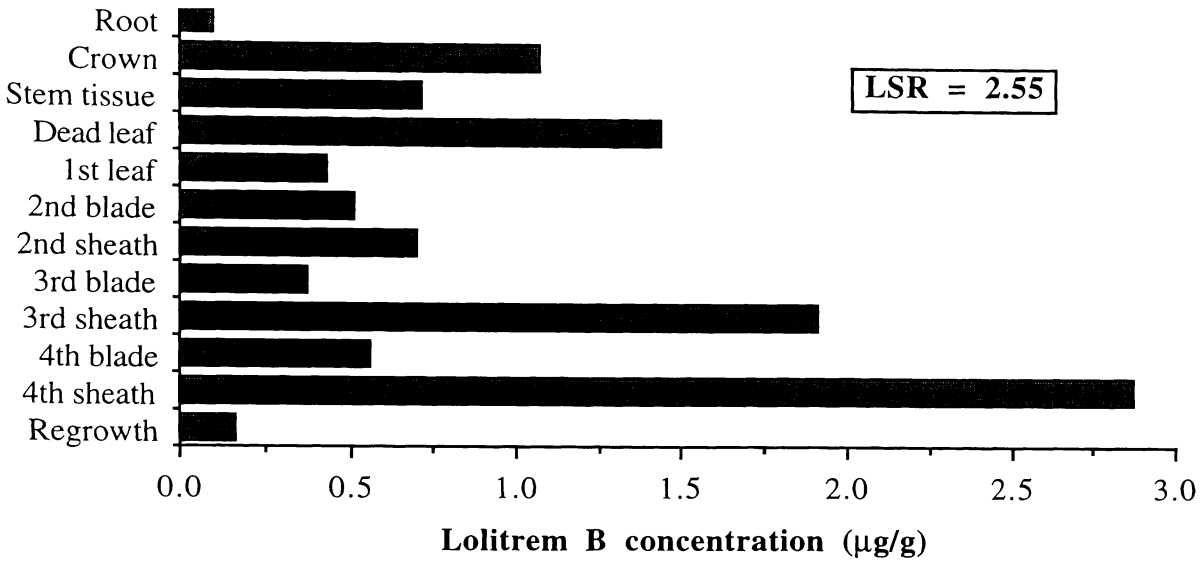


Figure 4.5 Lolitrem B concentration (µg of lolitrem B per g of dry grass material) measured by HPLC in different components of 'vegetative' perennial ryegrass plants sampled on 1/10/91. Values are a mean of eight different A. lolii-infected plants.

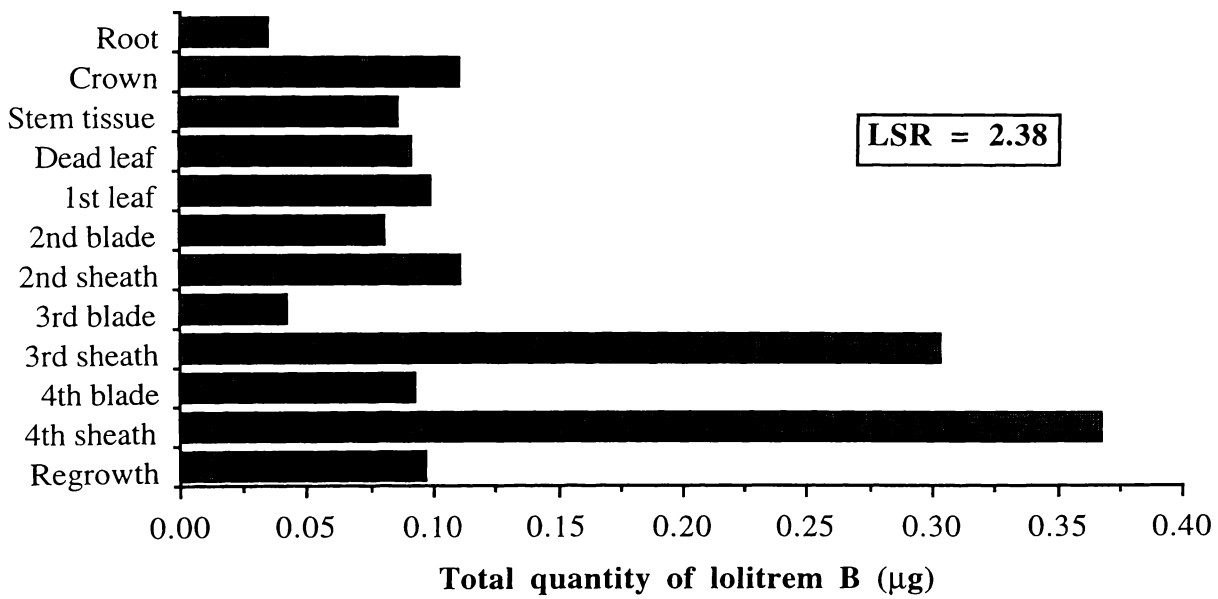


Figure 4.6 Total amount of lolitrem B (µg of lolitrem B per tissue) in different components of 'vegetative' perennial ryegrass plants sampled on 1/10/91. Values are a mean of eight different A. lolii-infected plants.

A. lolii, peramine and lolitrem B concentrations were significantly ($P< 0.001$) higher in the leaf sheaths than in the leaf blades (Figs. 4.1, 4.3 and 4.5 respectively). The difference in the *A. lolii* concentration between the sheath and the blade increased as leaf number decreased (Fig. 4.1). The opposite was true of peramine (Fig. 4.3). *A. lolii* concentrations increased as leaf sheath number decreased ($P< 0.01$) (Fig. 4.1). Peramine concentrations, particularly in the blade component, also increased as leaf number decreased ($P< 0.001$) (Fig. 4.3), while lolitrem B concentrations were significantly ($P< 0.05$) higher in sheath 4 compared to sheath 2 (Fig. 4.5).

The highest quantities of *A. lolii* were recorded in the reproductive stem tissue, 2nd leaf sheath, 3rd leaf sheath and crown (Fig. 4.2). The plant parts containing the largest amounts of peramine included the regrowth, 3rd leaf sheath, 2nd leaf sheath, 1st leaf and 4th leaf sheath (Fig. 4.4). Little peramine was present in the roots or senescent leaf material. The largest quantities of lolitrem B were contained in the 3rd and 4th leaf sheaths (Fig. 4.6).

6/11/91 harvest: *A. lolii* concentrations were lowest in the root (46µg/g) and regrowth (39µg/g) components, while the highest concentrations were recorded in the crown (225µg/g), reproductive stem tissue (193µg/g) and senescent leaf (127µg/g) (Fig. 4.7). *A. lolii* concentrations in the 1st leaf and the 2nd to 4th leaf sheath and blade categories had intermediate values ranging from 49µg/g to 107µg/g. With the exception of the 2nd and 3rd sheath, *A. lolii* concentrations were higher on 6/11/91 than 1/10/91.

Peramine concentrations were below the level of detection in the root category and were low in the senescent leaf material (2.7µg/g) and crown (7.6µg/g) (Fig. 4.9). The highest peramine concentrations were registered in the 1st leaf (50.6µg/g), 2nd leaf sheath (44.9µg/g) and 2nd leaf blade (49.4µg/g). Peramine concentrations on 6/11/91 had increased substantially in most plant components compared to 1/10/91.

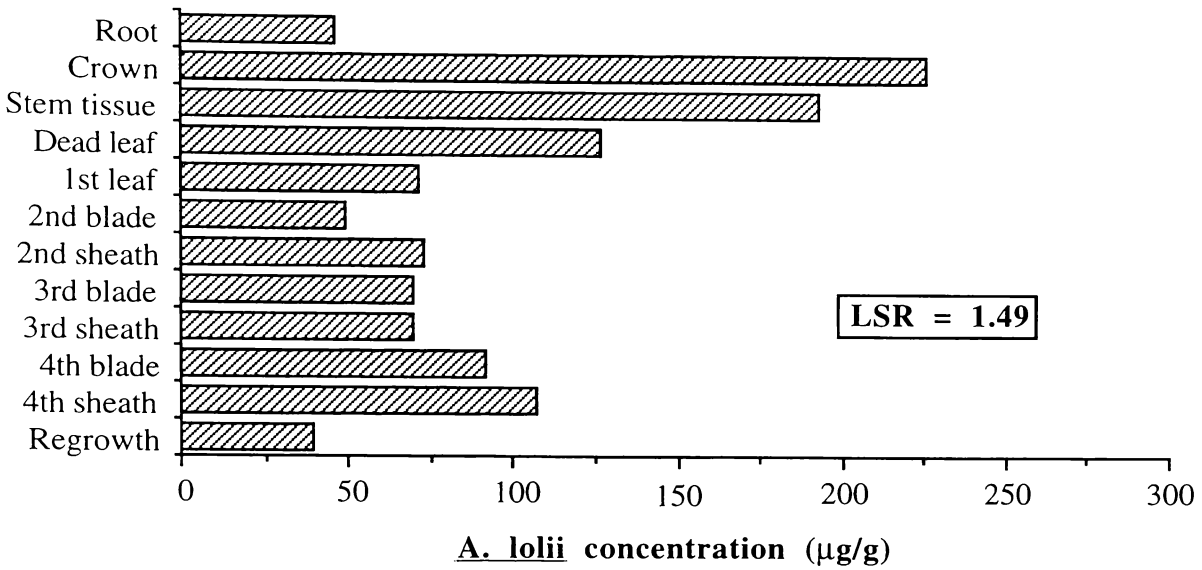


Figure 4.7 Concentration of A. lolii (µg of freeze-dried mycelium per g of dry grass material) measured by ELISA in different components of 'vegetative' perennial ryegrass plants sampled on 6/11/91. Values are a mean of eight different A. lolii-infected plants.

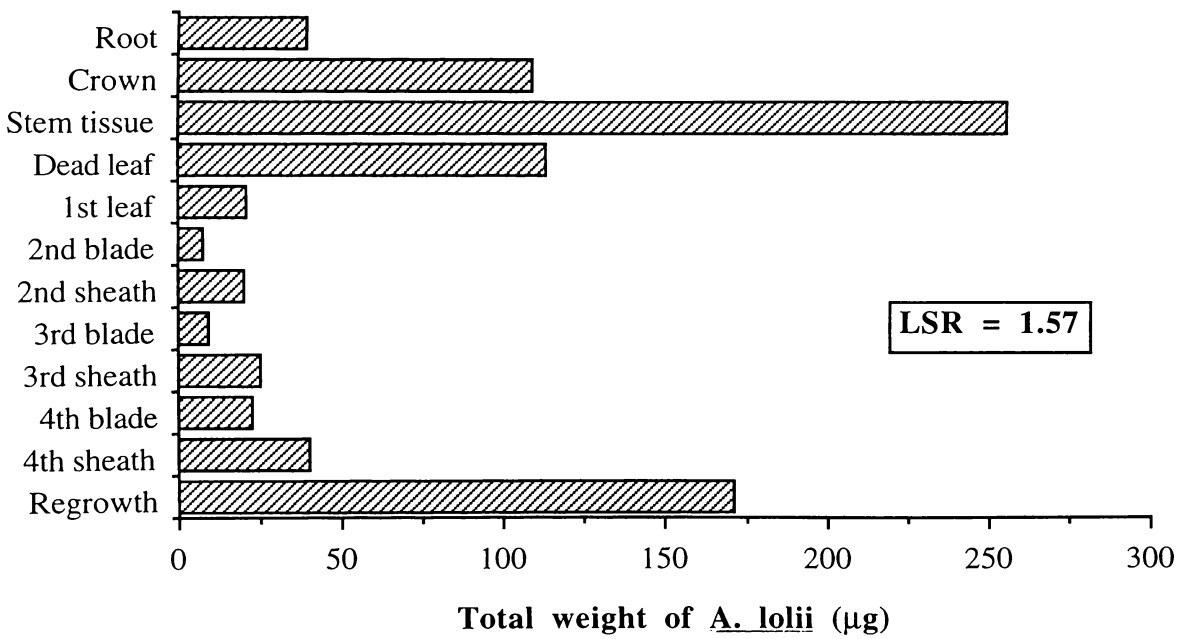


Figure 4.8 Total amount of A. lolii (µg of freeze-dried mycelium per tissue) in different components of 'vegetative' perennial ryegrass plants sampled on 6/11/91. Values are a mean of eight different A. lolii-infected plants.

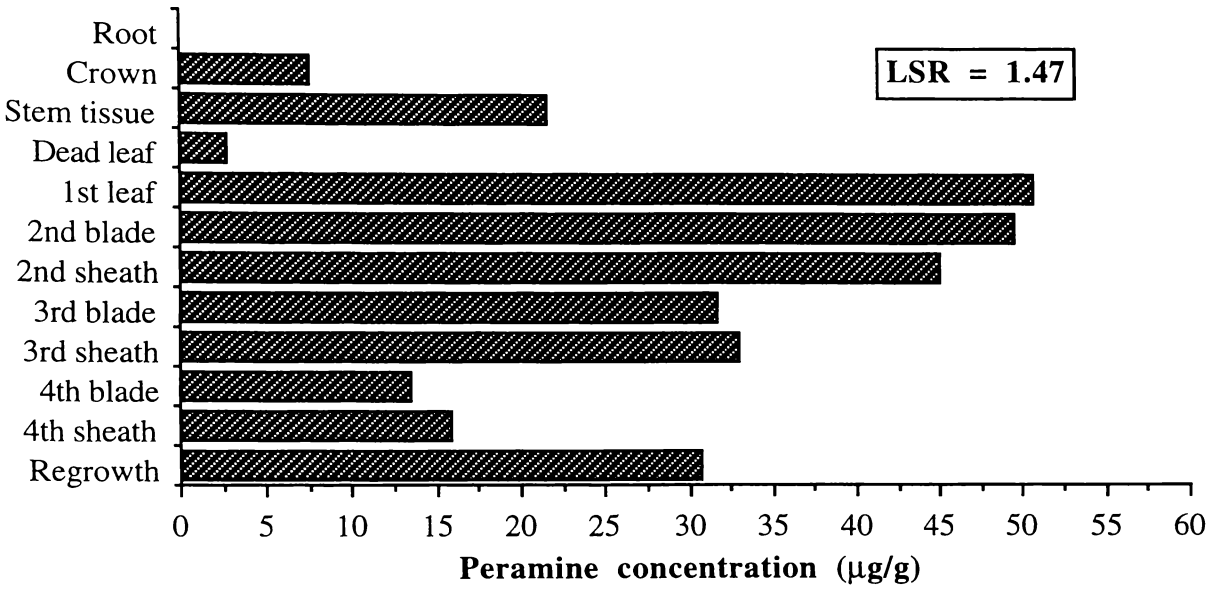


Figure 4.9 Peramine concentration (µg of peramine per g of dry grass material) measured by HPLC in different components of 'vegetative' perennial ryegrass plants sampled on 6/11/91. Values are a mean of eight different A. lolii-infected plants.

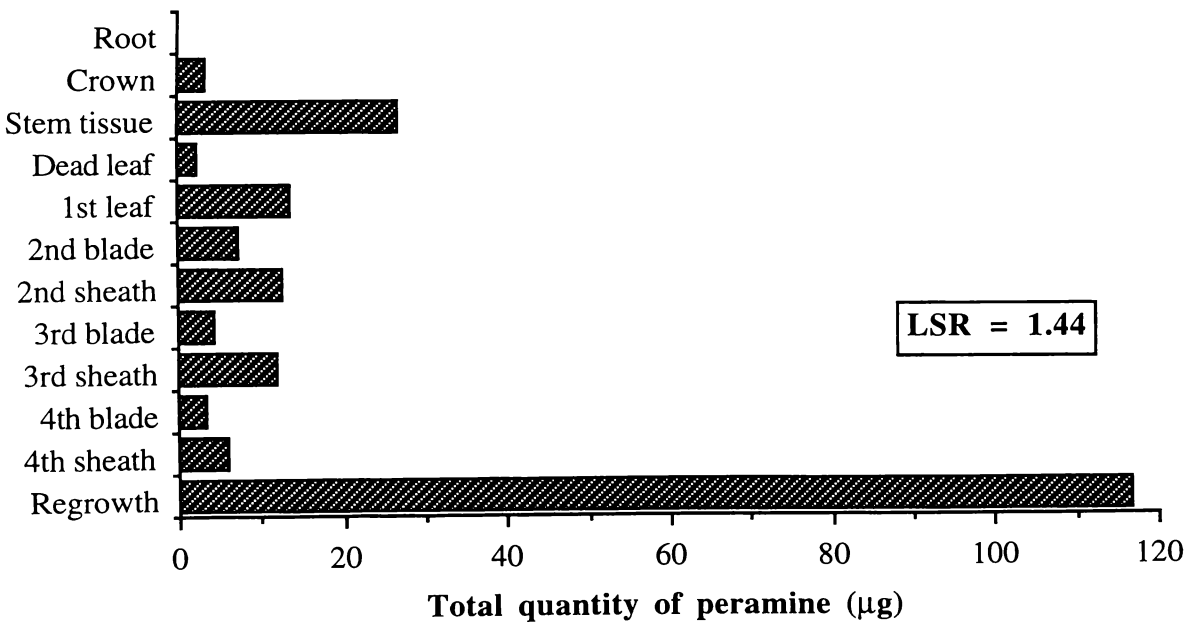


Figure 4.10 Total amount of peramine (µg of peramine per tissue) in different components of 'vegetative' perennial ryegrass plants sampled on 6/11/91. Values are a mean of eight different A. lolii-infected plants.

The highest concentrations of lolitrem B were recorded in the 4th leaf sheath (10.4μg/g), 3rd leaf sheath (5.7μg/g) and senescent leaf (4.6μg/g) (Fig. 4.11). Lolitrem B concentrations were lowest in the root (0.2μg/g) and regrowth (0.7μg/g) categories. Lolitrem B concentrations in all plant components increased considerably between 1/10/91 and 6/11/91.

A. lolii and lolitrem B concentrations were significantly ($P< 0.05$ and 0.001 respectively) higher in the leaf sheath tissue compared to the leaf blade (Figs. 4.7 and 4.11 respectively). Also, *A. lolii* and lolitrem B concentrations increased as leaf number increased ($P< 0.01$ and 0.001 respectively). Conversely, peramine concentrations increased as leaf number decreased ($P< 0.001$) (Fig. 4.9).

The greatest quantities of *A. lolii* were recorded in the reproductive stem tissue and regrowth components (Fig. 4.8). The crown and senescent leaf material also contained relatively high amounts of *A. lolii*. By far the largest amount of peramine was present in the accumulated regrowth category (Fig. 4.10). Of the total peramine content of the plant (excluding that present in the roots), 56.8% was contained in the regrowth. Compared to other plant parts, the quantity of peramine present in the reproductive stem tissue was also relatively high. The highest quantities of lolitrem B were recorded in the senescent leaf component (Fig. 4.12). The regrowth, reproductive stem tissue, 3rd and 4th leaf sheath components also contained relatively high quantities of lolitrem B.

14/1/92 harvest: As the quantity of material from some of the samples was only just sufficient for chemical analysis, data for *A. lolii* are incomplete. However, all of the plant components from two of the grasses were analyzed (plants 1 and 3), and a mean of these two is presented in Figures 4.13 and 4.14. *A. lolii* concentrations were highest in the reproductive stem tissue and crown and lowest in the roots, 1st leaf, leaf blades and regrowth.

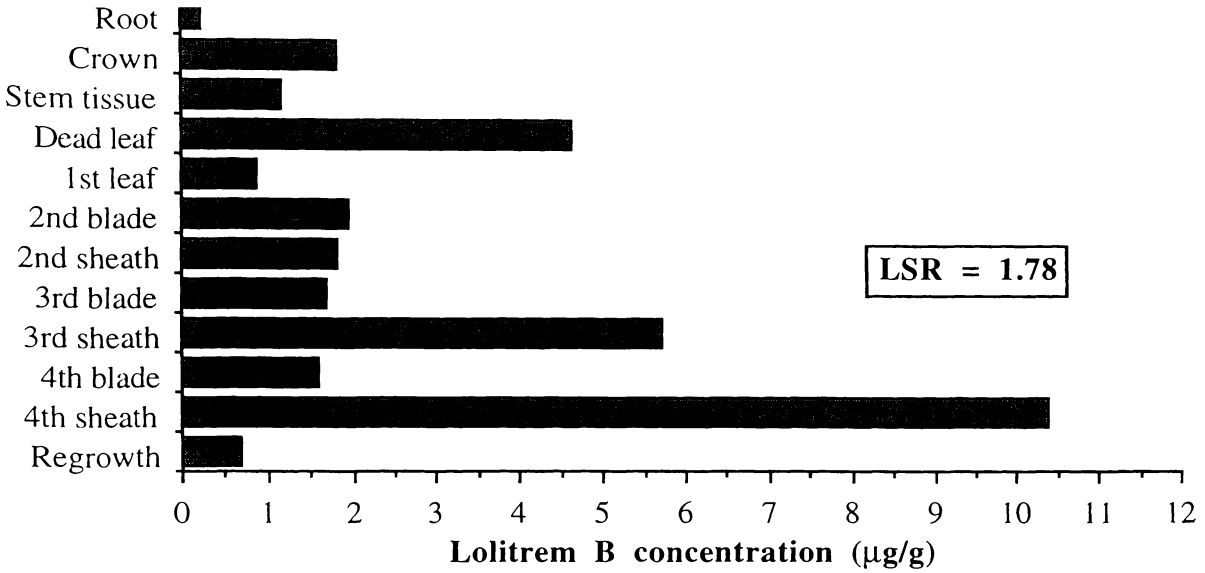


Figure 4.11 Lolitrem B concentration (µg of lolitrem B per g of dry grass material) measured by HPLC in different components of 'vegetative' perennial ryegrass plants sampled on 6/11/91. Values are a mean of eight different A. lolii-infected plants.

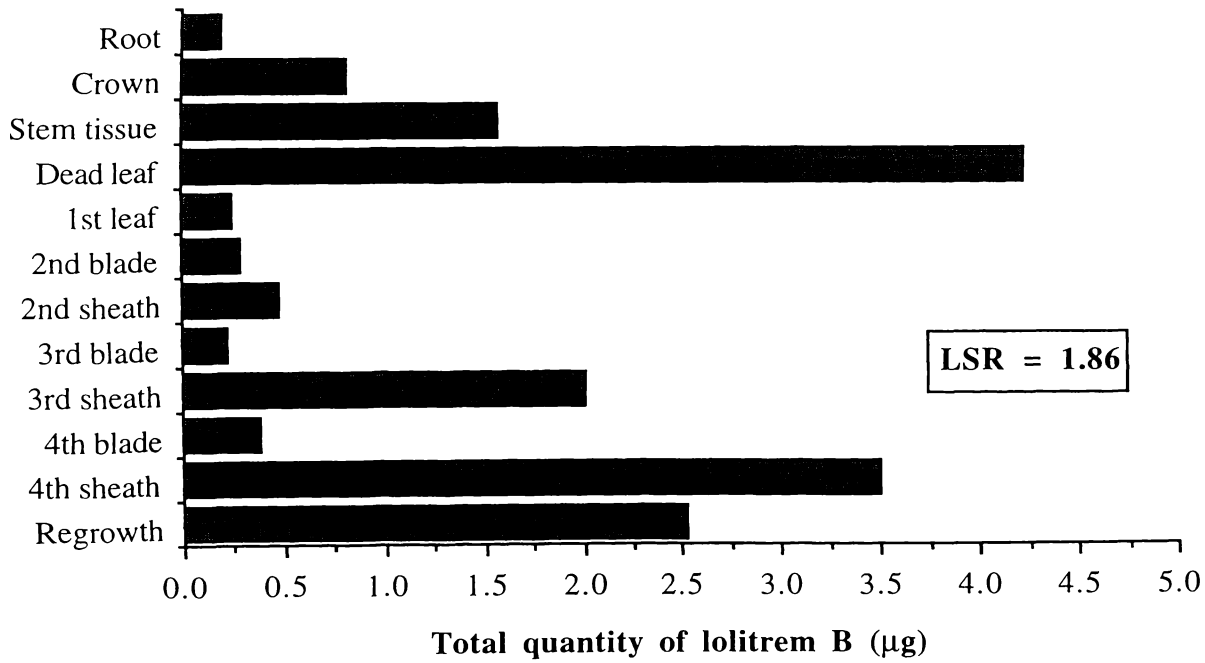


Figure 4.12 Total amount of lolitrem B (µg of lolitrem B per tissue) in different components of 'vegetative' perennial ryegrass plants sampled on 6/11/91. Values are a mean of eight different A. lolii-infected plants.

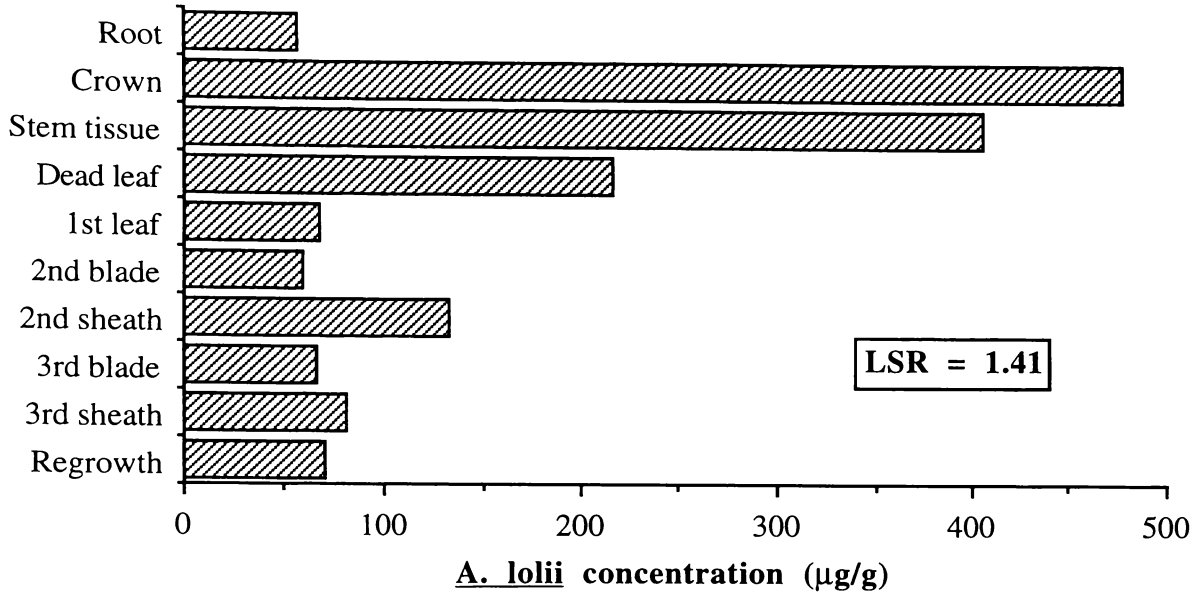


Figure 4.13 Concentration of A. lolii (µg of freeze-dried mycelium per g of dry grass material) measured by ELISA in different components of 'vegetative' perennial ryegrass plants sampled on 14/1/92. Values are a mean of two different A. lolii-infected plants.

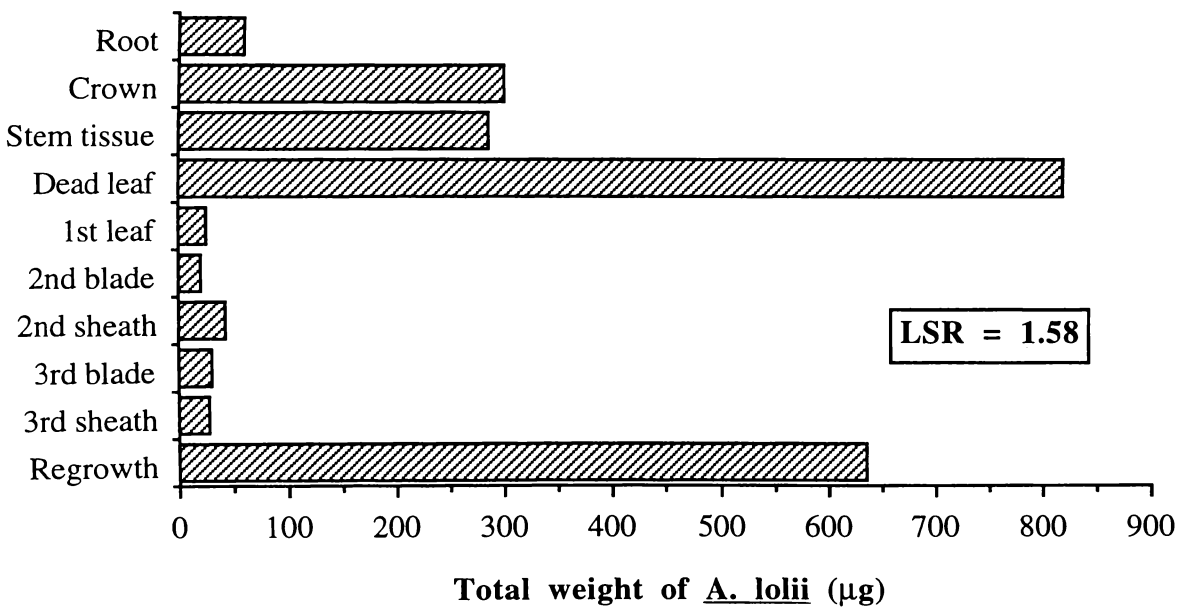


Figure 4.14 Total amount of A. lolii (µg of freeze-dried mycelium per tissue) in different components of 'vegetative' perennial ryegrass plants sampled on 14/1/92. Values are a mean of two different A. lolii-infected plants.

Peramine concentrations were highest in the 1st leaf (48.8μg/g), 2nd and 3rd leaf sheaths and blades (24.1-45.6μg/g) and regrowth (28.5μg/g), while in the senescent leaf and root categories, they were extremely low (Fig. 4.15). Apart from the crown tissue where there was a slight increase, peramine concentrations in all plant parts declined between 6/11/91 and 1/14/92 (Figs. 4.9 and 4.15).

Lolitrem B concentrations were low in the root (0.9μg/g), 1st leaf (0.8μg/g) and regrowth (1.3μg/g) categories (Fig. 4.17). The highest concentration of lolitrem B was found in the outermost (3rd) sheath (6.9μg/g). The senescent leaf component also contained a high lolitrem B concentration (6.0μg/g). Between 6/11/91 and 14/1/92, lolitrem B concentrations increased in the senescent leaf, reproductive stem, crown and root tissues (Figs. 4.11 and 4.17). Lolitrem B concentrations in all other tissues were similar at the two dates.

A. lolii and lolitrem B (Figs. 4.13 and 4.17) concentrations were significantly ($P< 0.01$ and 0.001 respectively) higher in the sheath than the blade. As leaf number increased, lolitrem B concentrations increased ($P< 0.05$) (Fig. 4.17), whereas peramine concentrations decreased ($P< 0.001$) (Fig. 4.15).

The total amount of peramine in the accumulated regrowth tissue (Fig. 4.16) was very much higher than in any other plant component. On average, the quantity of peramine in the accumulated regrowth category accounted for 75.4% of the total amount of peramine present in the plant (excluding that present in the roots). The total amount of peramine present in the 1st leaf and 2nd and 3rd leaf sheath and blade tissues, was low even though concentrations in these components were comparatively high. Similarly, the total amount of lolitrem B in the 3rd sheath (Fig. 4.18) was low even though this component contained the highest concentration (Fig. 4.17). The largest amount of lolitrem B (49.5% of the total) was contained in the senescent leaf, although the

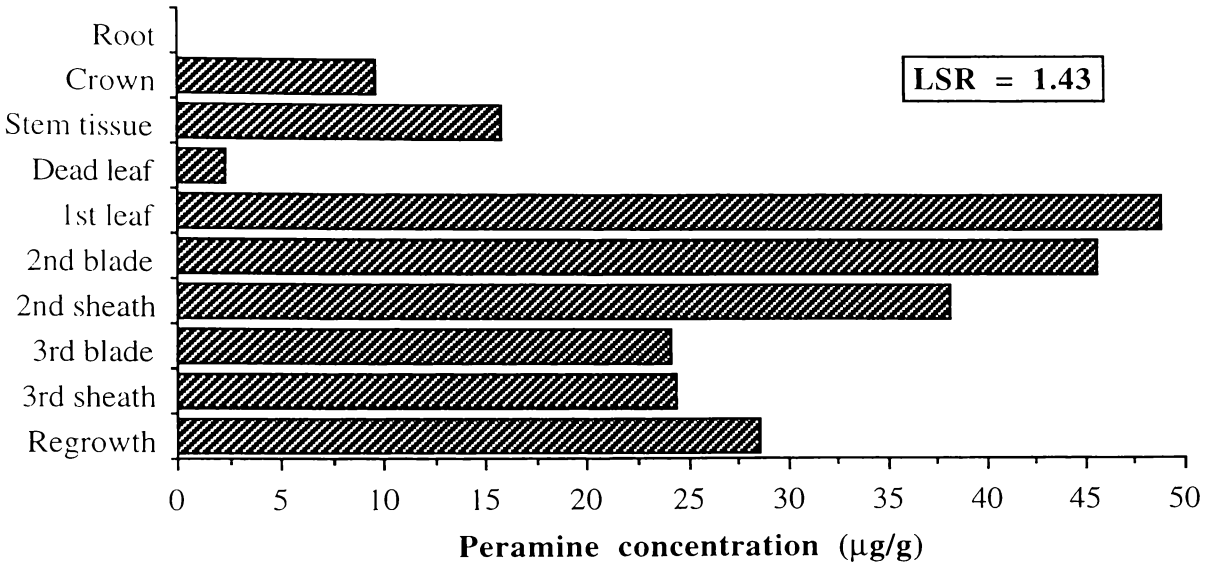


Figure 4.15 Peramine concentration (µg of peramine per g of dry grass material) measured by HPLC in different components of 'vegetative' perennial ryegrass plants sampled on 14/1/92. Values are a mean of eight different A. lolii-infected plants.

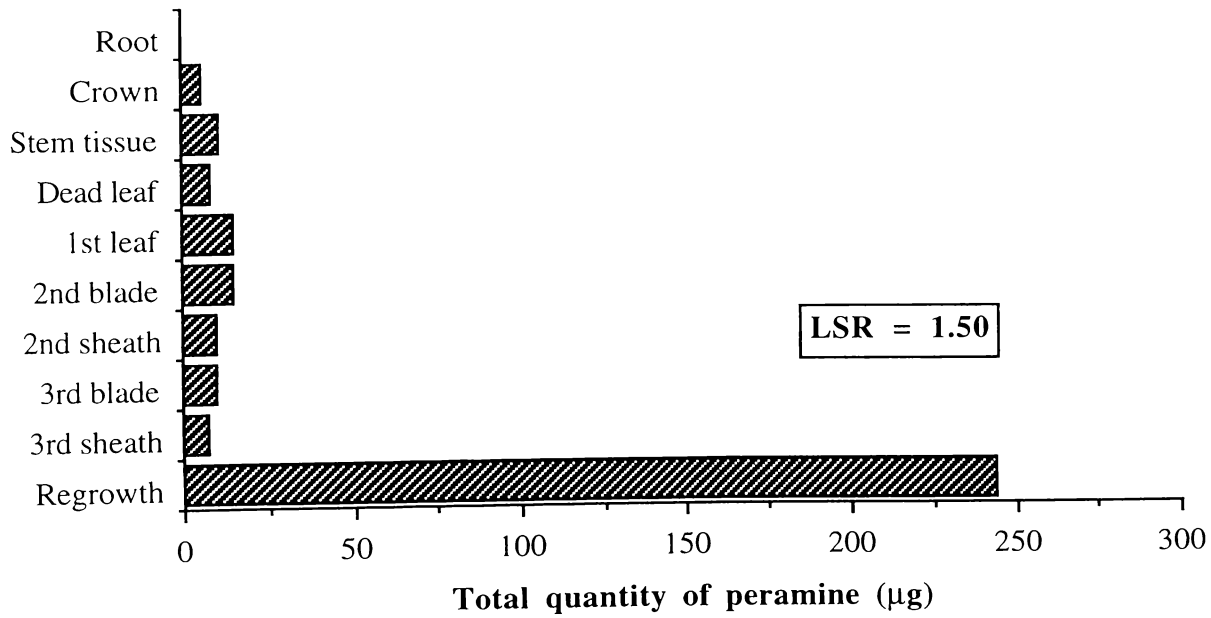


Figure 4.16 Total amount of peramine (µg of peramine per tissue) in different components of 'vegetative' perennial ryegrass plants sampled on 14/1/92. Values are a mean of eight different A. lolii-infected plants.

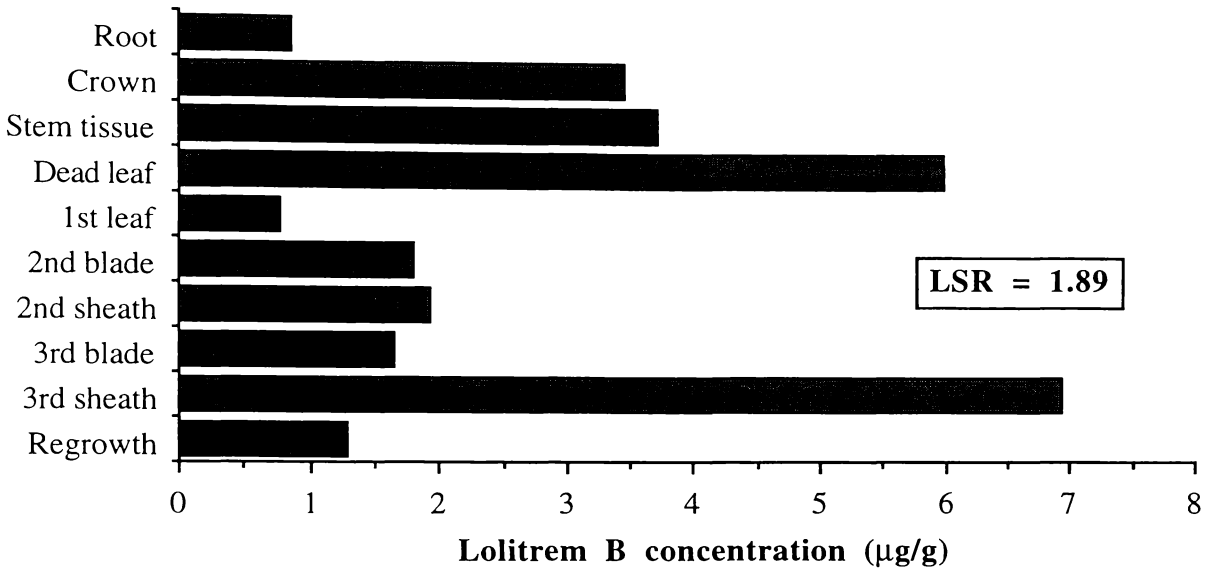


Figure 4.17 Lolitrem B concentration (µg of lolitrem B per g of dry grass material) measured by HPLC in different components of 'vegetative' perennial ryegrass plants sampled on 14/1/92. Values are a mean of eight different A. lolii-infected plants.

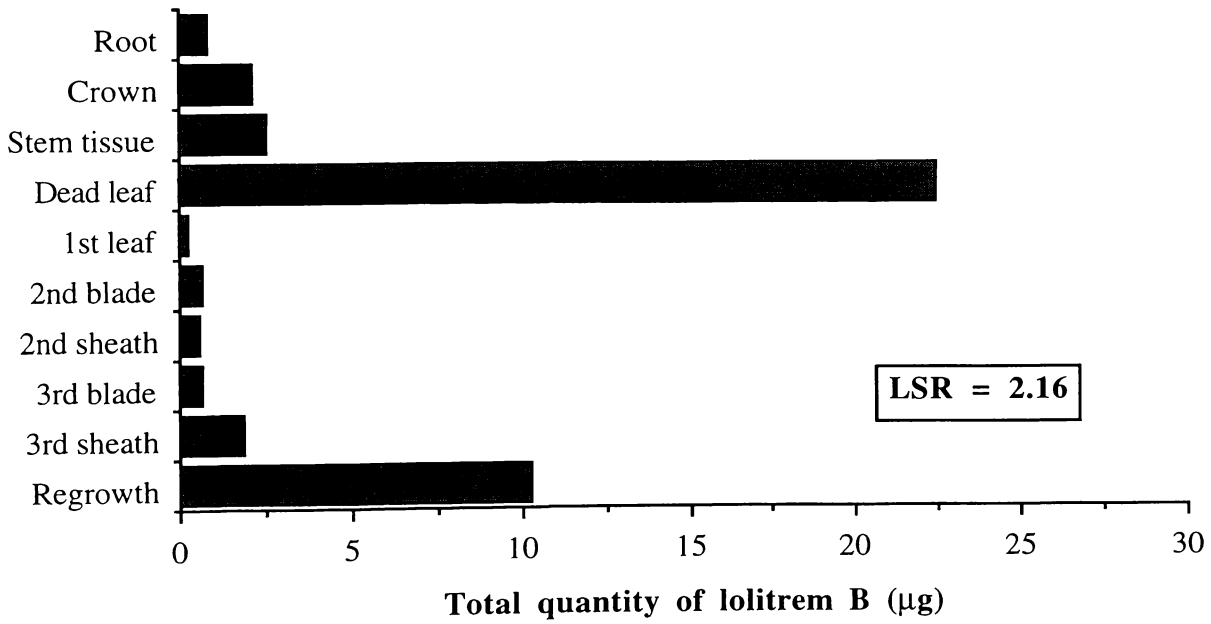


Figure 4.18 Total amount of lolitrem B (µg of lolitrem B per tissue) in different components of 'vegetative' perennial ryegrass plants sampled on 14/1/92. Values are a mean of eight different A. lolii-infected plants.

accumulated regrowth component also contained relatively high quantities (Fig. 4.18).

4.3.3 'Reproductive' plants.

6/11/91 harvest: The highest *A. lolii* concentrations were recorded in the inflorescence (334μg/g), reproductive stem tissue from vegetative tillers (228μg/g) and lower reproductive stem tissue from reproductive tillers (214μg/g) (Fig. 4.19). *A. lolii* concentrations were also relatively high in the crown tissues from vegetative and reproductive tillers, upper reproductive stem from reproductive tillers and the 2nd and 3rd leaf sheaths from vegetative tillers. The lowest *A. lolii* concentrations were found in the leaf blades from reproductive tillers (23-29μg/g), 1st leaf from vegetative tillers (31μg/g), 2nd leaf blade from vegetative tillers (36μg/g) and root (34μg/g). Comparing the bulked undissected herbage, *A. lolii* concentrations were highest in the reproductive tillers and lowest in the vegetative tillers (Fig. 4.31). In both vegetative and intermediate herbage, the lower part of the tiller contained the highest concentrations of *A. lolii*.

Peramine concentrations in the root were generally below the limit of detection (Fig. 4.21). Low concentrations of peramine were also registered in the senescent leaf material from reproductive tillers (1.8μg/g), leaf blades from reproductive tillers (3.9-5.1μg/g) and crown from reproductive (4.5μg/g). The highest concentrations of peramine were recorded in the 1st leaf from vegetative tillers (21.9μg/g) and the 2nd and 3rd leaf sheaths and blades from vegetative tillers (18.3-24.9μg/g). Peramine concentrations in the leaf sheaths from vegetative tillers were substantially higher on 6/11/91 in the trimmed ('vegetative') compared to the untrimmed ('reproductive') plants (Figs. 4.9 and 4.21 respectively). This was also the case for lolitrem B (Figs. 4.11 and 4.23). In the bulked undissected herbage, peramine concentrations were highest in vegetative tillers and lowest in reproductive tillers (Fig. 4.32). Peramine concentrations were highest in the upper part of the tiller in all three tiller categories.

Figure 4.19

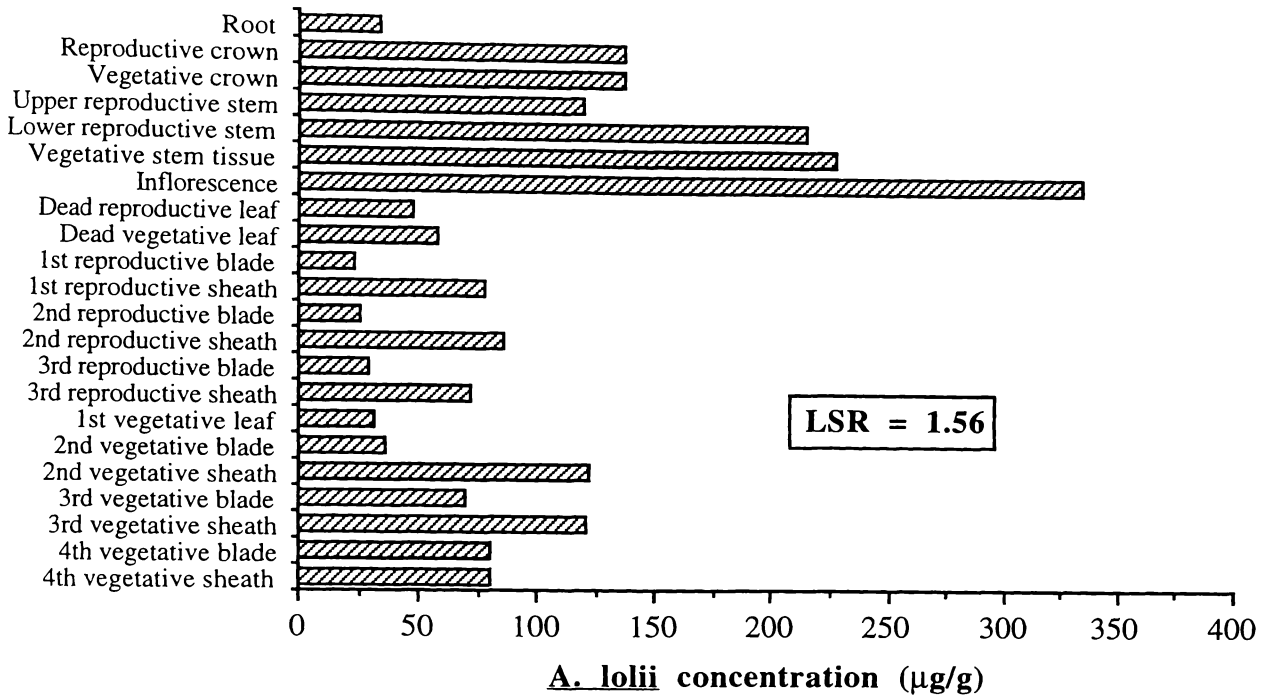
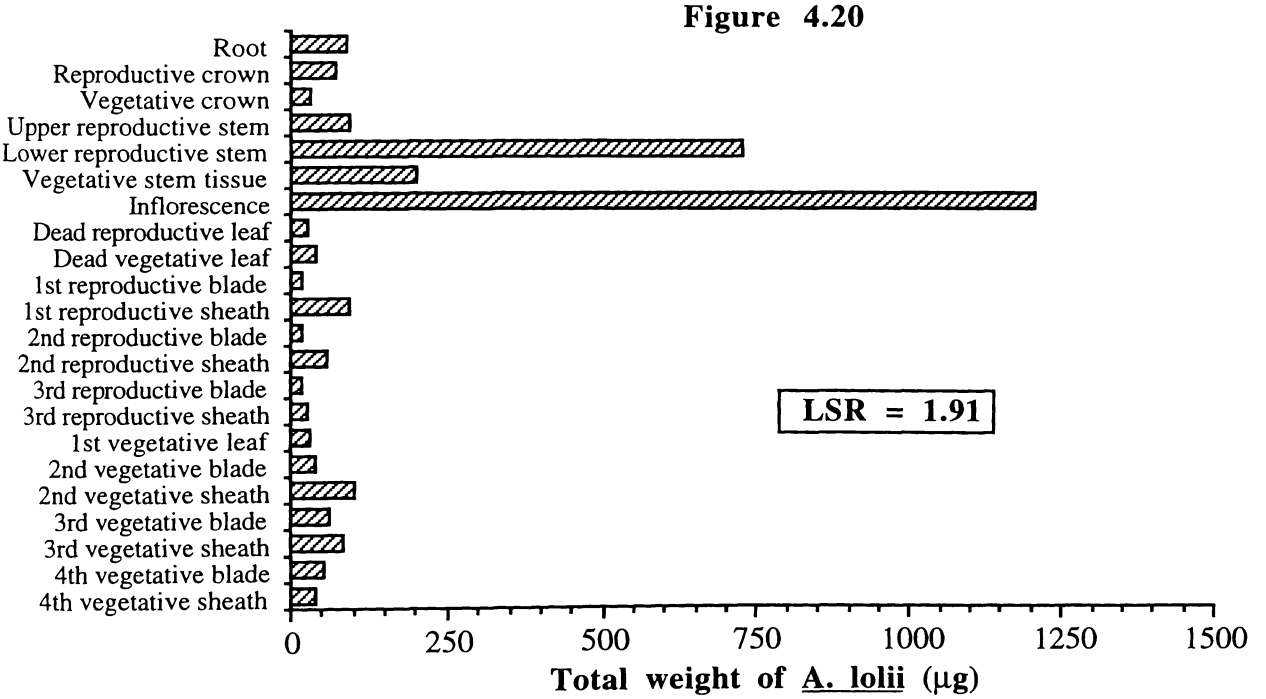


Figure 4.20



Figures 4.19 and 4.20 Concentration (Fig. 4.19) (µg of freeze-dried mycelium per g of dry grass material) and total amount (Fig. 4.20) (µg of freeze-dried mycelium per tissue) of A. lolii in different components of vegetative and reproductive tillers 'reproductive' perennial ryegrass plants sampled on 6/11/91. Values are a mean of eight different A. lolii-infected plants.

Figure 4.21

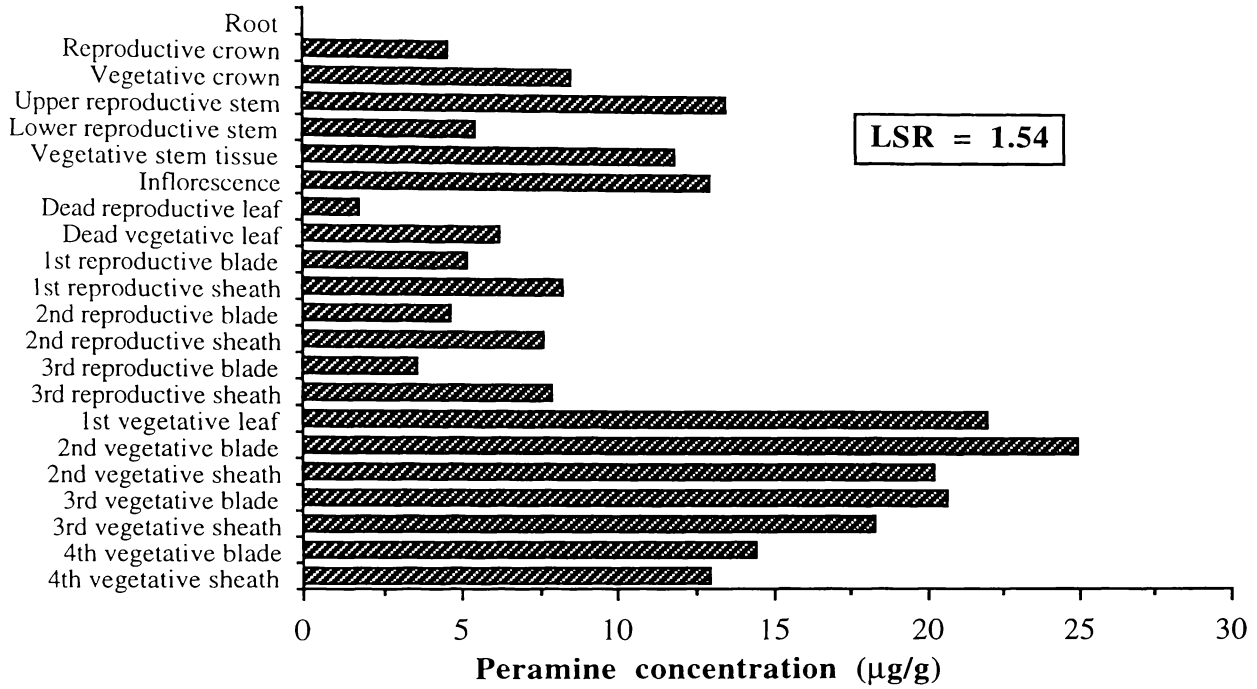
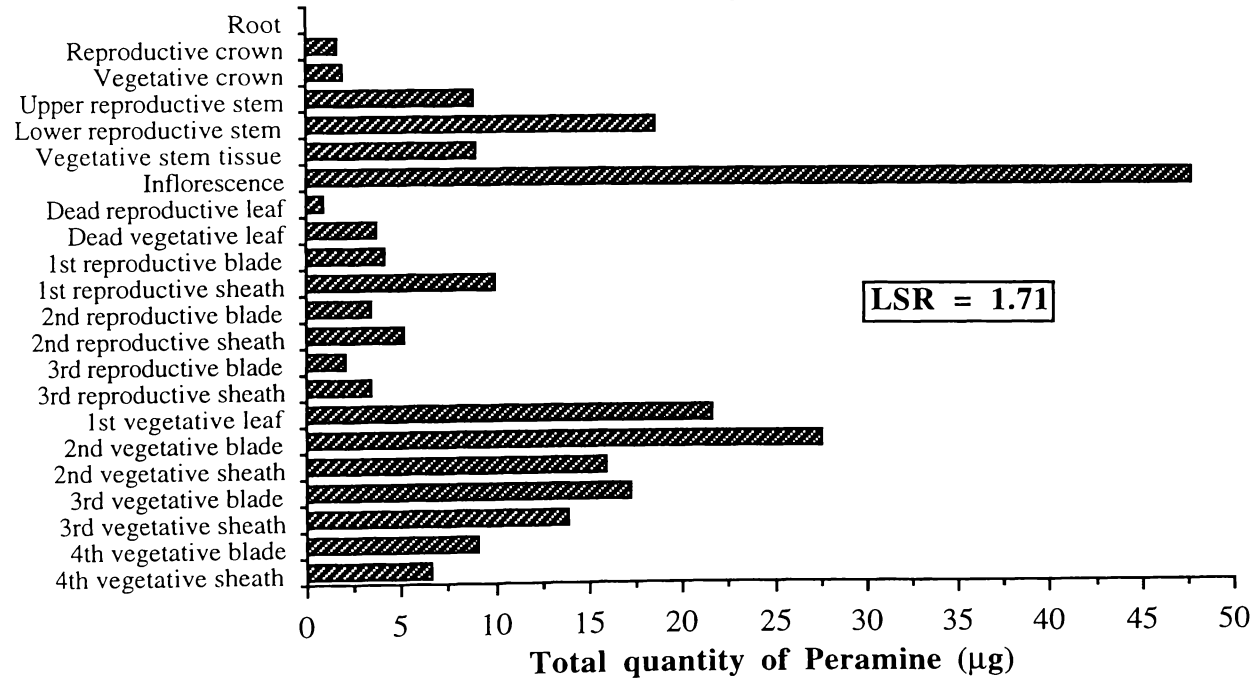


Figure 4.22



Figures 4.21 and 4.22 Concentration (Fig. 4.21) (µg of peramine per g of dry grass material) and total quantity (Fig. 4.22) (µg of peramine per tissue) of peramine in different components of vegetative and reproductive tillers from ryegrass plants sampled on 6/11/91. Values are a mean of eight different A. lolii-infected plants.

Figure 4.23

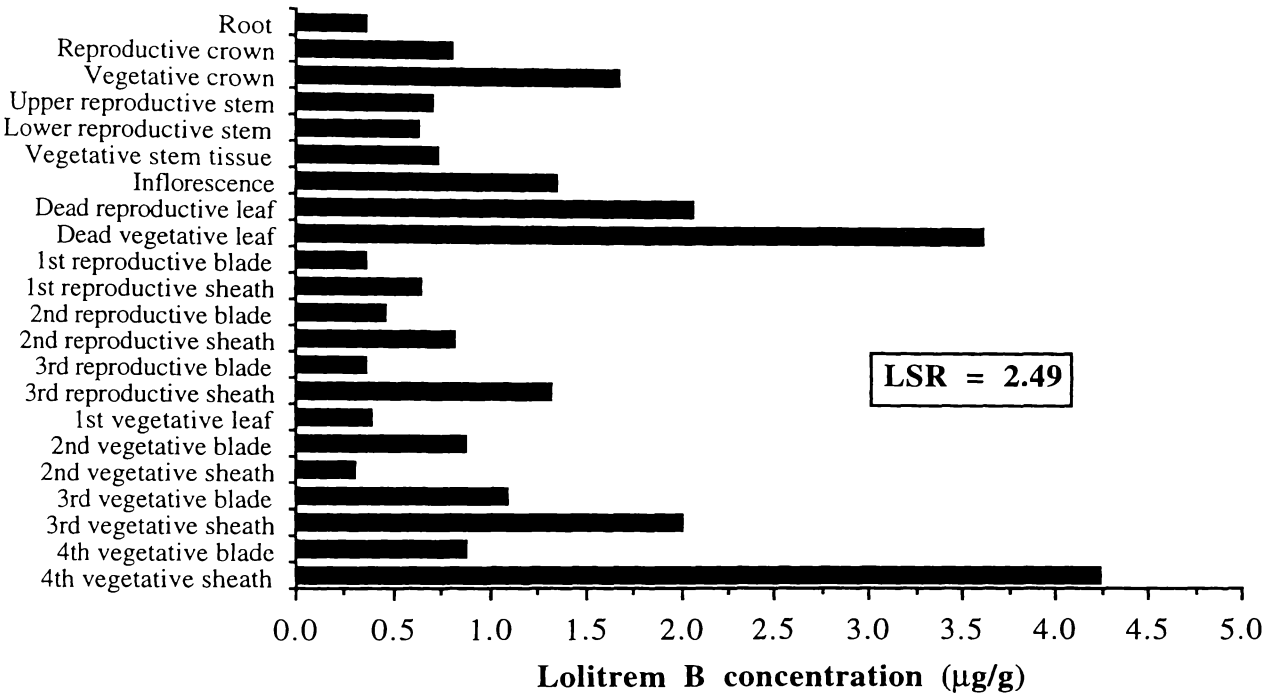
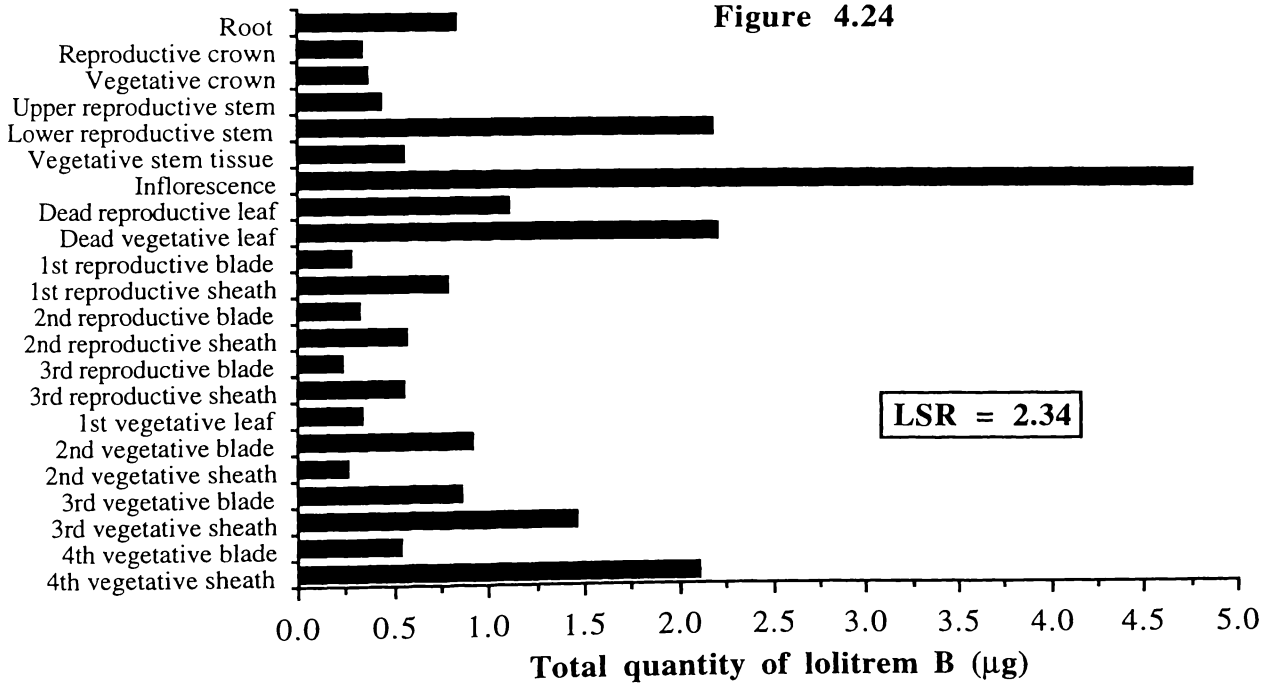


Figure 4.24



Figures 4.23 and 4.24 Concentration (Fig. 4.23) (µg of lolitrem B per g of dry grass material) and total quantity (Fig. 4.24) (µg of lolitrem B per tissue) of lolitrem B in different components of vegetative and reproductive tillers from 'reproductive' ryegrass plants sampled on 6/11/91. Values are a mean of eight different A. lolii-infected plants.

Lolitrem B concentrations were low in the root (0.4μg/g), leaf blades from reproductive tillers (0.4-0.5μg/g), 1st leaf from vegetative tillers (0.4μg/g) and 2nd leaf sheath from vegetative tillers (0.3μg/g) and high in the 4th leaf sheath from vegetative tillers (4.2μg/g) and senescent leaf material from vegetative tillers (3.6μg/g) (Fig. 4.23). Material from the lower part of whole vegetative, intermediate and reproductive tillers contained higher concentrations of lolitrem B than herbage from the upper portion of the tillers (Fig. 4.33).

Generally, concentrations of *A. lolii*, peramine and lolitrem B were higher in leaf sheaths and blades from vegetative tillers than leaf sheaths and blades from reproductive tillers (Figs. 4.19, 4.21 and 4.23 respectively). *A. lolii*, peramine and lolitrem B concentrations were not affected by leaf number (age) in leaves from reproductive tillers. This was not the case with leaves from vegetative tillers where peramine concentrations declined ($P< 0.001$) and lolitrem B concentrations rose ($P< 0.001$) with increasing leaf number (Figs. 4.21 and 4.23 respectively). Leaf sheaths from reproductive tillers contained higher concentrations of *A. lolii* ($P< 0.001$), peramine ($P< 0.001$) and lolitrem B ($P< 0.01$) than leaf blades from reproductive tillers (Figs. 4.19, 4.21 and 4.23 respectively). This was also the case with *A. lolii* ($P< 0.001$) and lolitrem B ($P< 0.05$) in leaves from vegetative tillers (Figs. 4.19 and 4.23). Peramine concentrations were significantly higher ($P< 0.001$) in the crown and senescent leaf tissue from vegetative tillers compared to concentrations in their reproductive counterparts (Fig. 4.21). Although not statistically significant, this also appears to be the case with lolitrem B (Fig. 4.23).

Most of the *A. lolii* mycelium was contained in only two tissues; the inflorescence (38.7% of the total) and the lower reproductive stem from reproductive tillers (23.3% of the total) (Fig. 4.20). The inflorescence also contained the greatest quantity of peramine (20.6% of the total) (Fig. 4.22). Leaf sheaths and blades from

vegetative tillers contained more peramine than leaf sheaths and blades from reproductive tillers. The plant component with the greatest amount of lolitrem B was again the inflorescence (Fig. 4.24) which accounted for 21.8% of the total lolitrem B content of the plant. Differences in the quantity of lolitrem B in other plant components were generally similar to differences in concentration.

14/1/92 harvest: As for the 14/1/92 harvest of the 'vegetative' plants, quantities of some of the samples from the 14/1/92 harvest of the 'reproductive' clones were insufficient for them to be analyzed for *A. lolii* by ELISA. However, it was possible to analyze the root, upper and lower stem from reproductive tillers, seed, seed head and all of the dead leaf material from all eight clones. Figures for the *A. lolii* concentration in these tissues appear in Figure 4.25. Data for the 1st vegetative leaf and the 2nd and 3rd sheaths and blades from vegetative tillers, also presented in Figure 4.25, are the mean of only two plants (4 and 5). The average concentration of *A. lolii* in the seed component was 13.074mg/g which was over 30 times higher than in any other plant part ($P< 0.001$) (Fig. 4.25). The concentration of *A. lolii* in the seed head (minus the seed) was slightly higher than in the inflorescence on 6/11/91 (Figs. 4.25 and 4.19 respectively). In whole undissected tillers, the mean *A. lolii* concentration of 2.854mg/g in the upper reproductive tiller category was considerably higher than the *A. lolii* concentration in any of the other three tiller categories (Fig. 4.31). Also, *A. lolii* concentrations were higher in the lower and upper vegetative and reproductive stem tissues on 14/1/92 than on 6/11/91.

Peramine concentrations were highest in the seed component (63.7µg/g) (Fig. 4.27). Excluding the senescent material, leaf tissues (both sheaths and blades) from vegetative tillers also had comparatively high concentrations of peramine (28.8-57.6µg/g). Peramine concentrations in all other plant parts were below 10µg/g. Roots and senescent leaf tissue from reproductive tillers had particularly low levels of

Figure 4.25

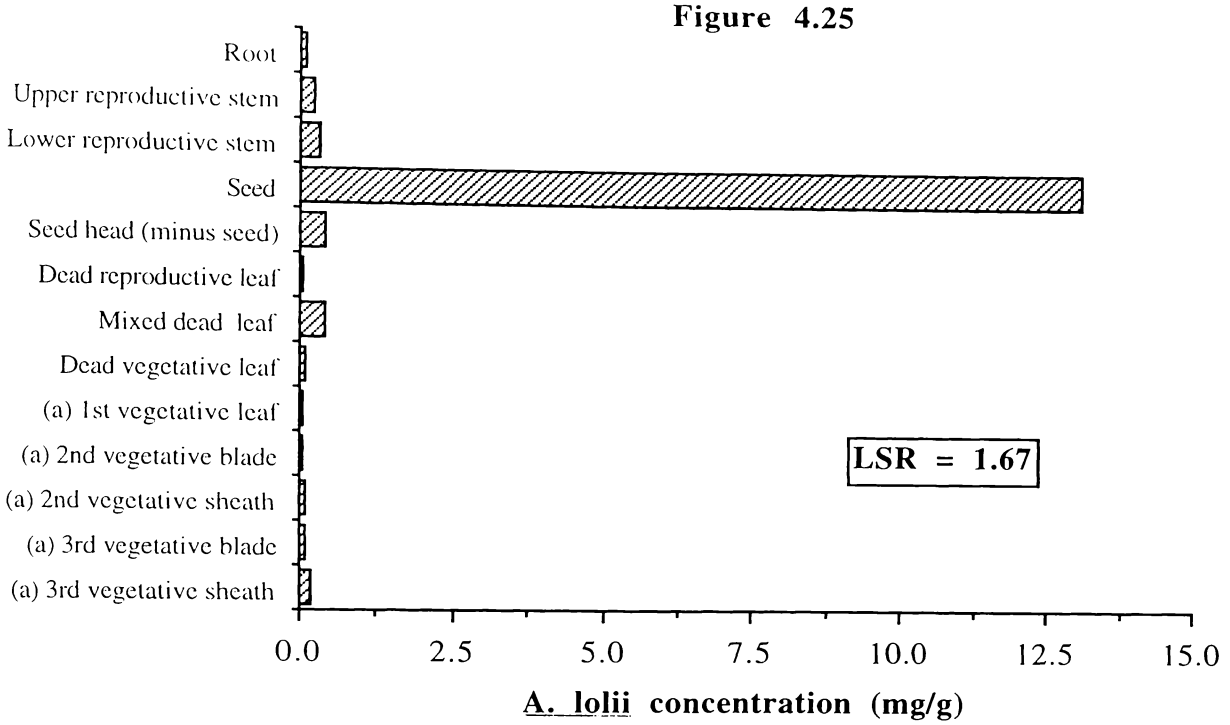
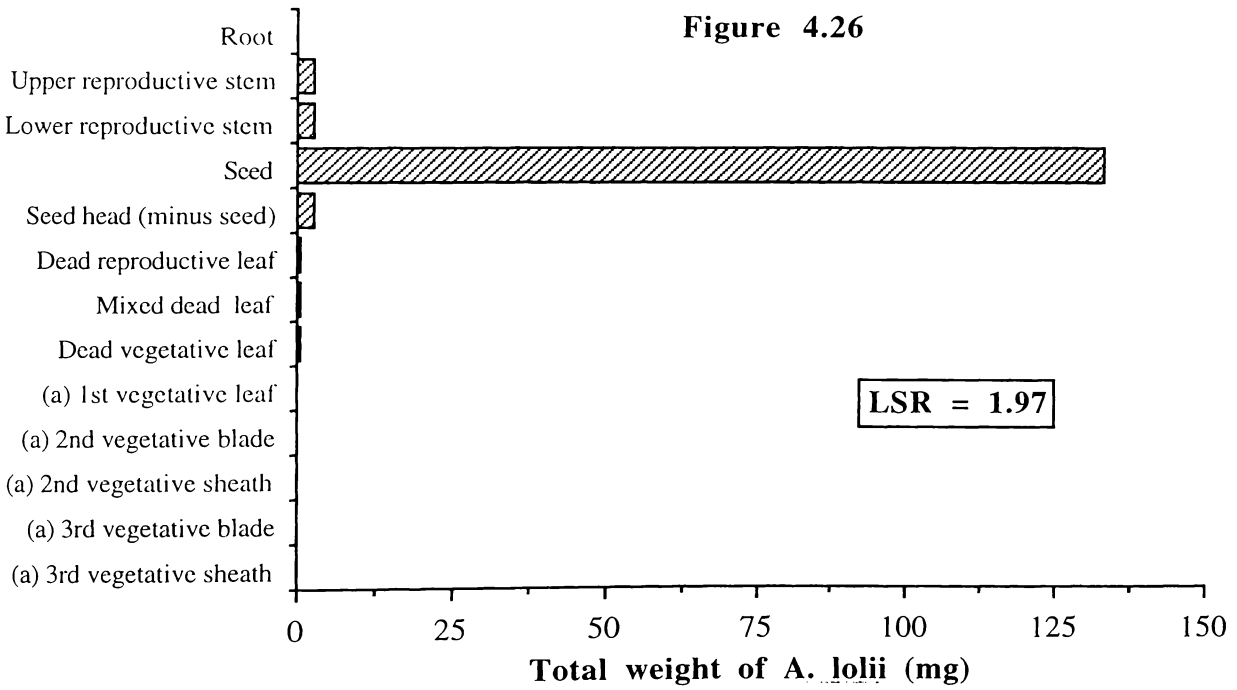


Figure 4.26



Figures 4.25 and 4.26 Concentration (Fig. 4.25) (μg of freeze-dried mycelium per g of dry grass material) and total amount (Fig. 4.24) (μg of freeze-dried mycelium per tissue) of A. lolii in different components of vegetative and reproductive tillers from 'reproductive' perennial ryegrass plants sampled on 14/1/92. Values are a mean of eight different A. lolii-infected plants. (a) denotes values which are a mean of only two plants.

Figure 4.27

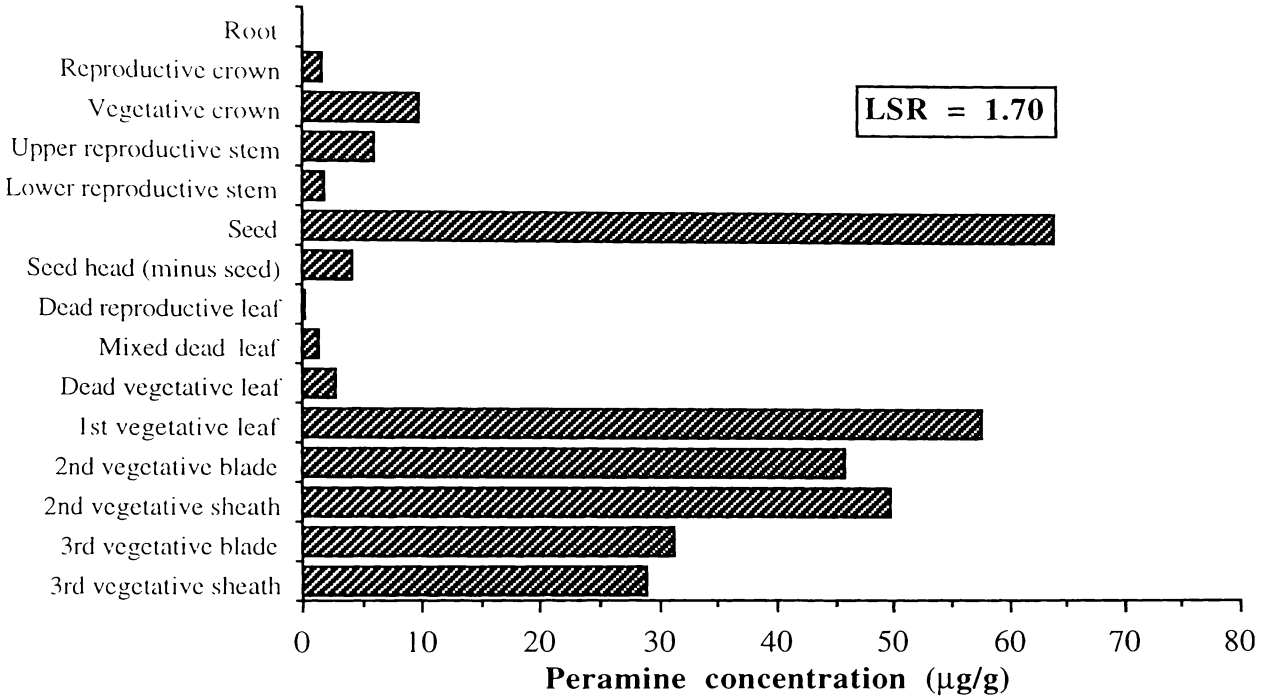
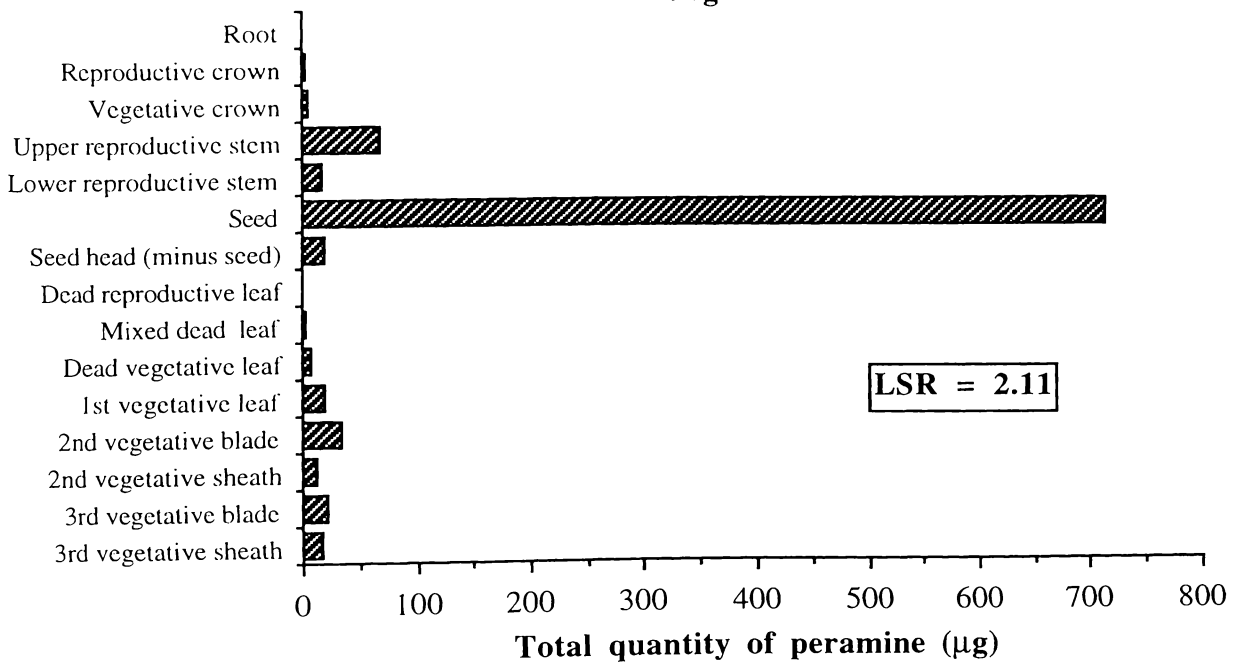


Figure 4.28



Figures 4.27 and 4.28 Peramine concentration (Fig. 4.27) (µg of peramine per g of dry grass material) and total amount of peramine (Fig. 4.28) (µg of peramine per tissue) in different components of vegetative and reproductive tillers from 'reproductive' ryegrass plants sampled on 14/1/92. Values are a mean of eight different A. lolii-infected plants.

peramine. Peramine concentrations were substantially higher in the 1st leaf and 2nd and 3rd leaf sheaths and blades from vegetative tillers on 14/1/92 than they were on 6/11/91 (Figs. 4.27 and 4.21 respectively). In undissected bulked herbage, peramine concentrations were higher in vegetative tillers compared to reproductive tillers (Fig. 4.32). Peramine concentrations were substantially higher in upper reproductive tillers compared to lower reproductive tillers. Peramine concentrations in the upper reproductive tiller component also increased considerably between the 6/11/91 and 14/1/92 harvests.

The highest concentration of lolitrem B (20.0μg/g) was contained in the seed component (Fig. 4.29). Lolitrem B concentrations in excess of 5.0μg/g were also recorded in the upper reproductive stem tissue from reproductive tillers, senescent leaf material from vegetative tillers and 3rd leaf sheath from vegetative tillers. The lowest lolitrem B concentrations were found in the roots (0.5μg/g), senescent leaf material from reproductive tillers (1.0μg/g), crown tissue from reproductive tillers (1.1μg/g) and 1st leaf from vegetative tillers (1.2μg/g). With the exception of the upper vegetative tiller component where only a small increase occurred, lolitrem B concentrations in undissected vegetative and reproductive tillers rose substantially between 6/11/91 and 14/1/92 (Fig. 4.33).

There were significantly higher concentrations of *A. lolii* ($P< 0.01$) and lolitrem B ($P< 0.05$) in the leaf sheaths compared to the leaf blades from vegetative tillers (Figs. 4.25 and 4.29 respectively). Lolitrem B concentrations were also significantly ($P< 0.05$) higher in leaf 3 (particularly in the sheath) than in leaf 2 from vegetative tillers (Fig. 4.29). Conversely, peramine concentrations were significantly ($P< 0.001$) higher in leaf 2 than in leaf 3 from vegetative tillers. *A. lolii*, peramine and lolitrem B concentrations were significantly higher ($P< 0.05$, 0.001 and 0.01 respectively) in crown tissue from

Figure 4.29

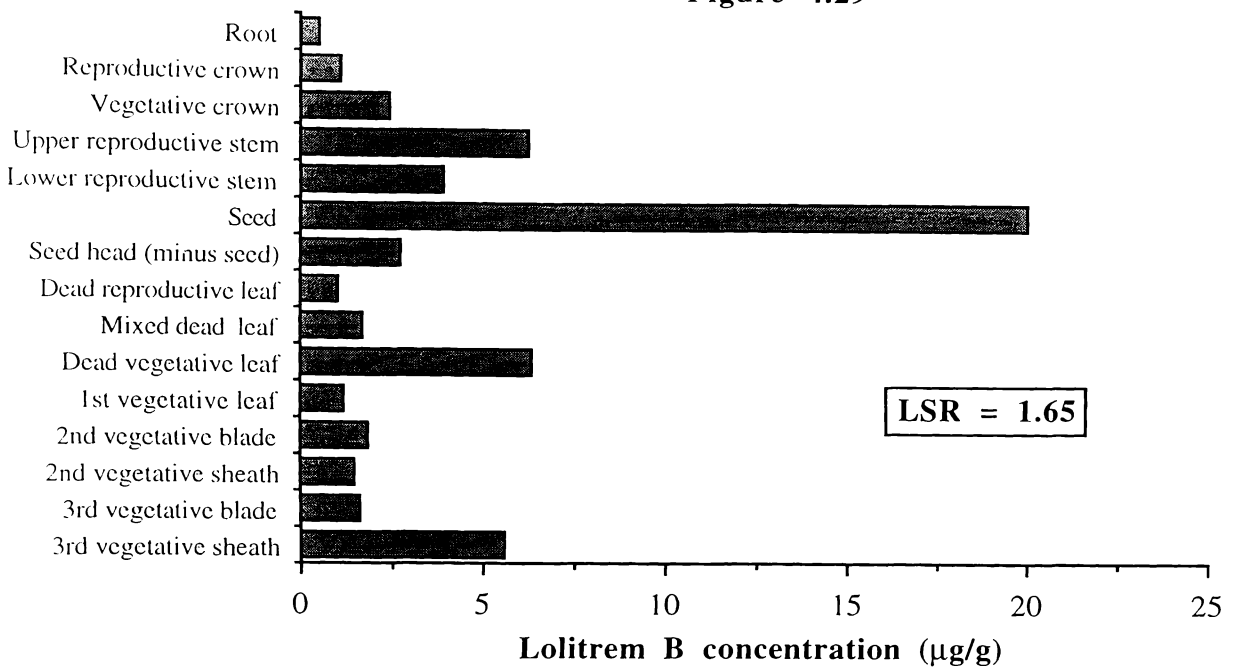
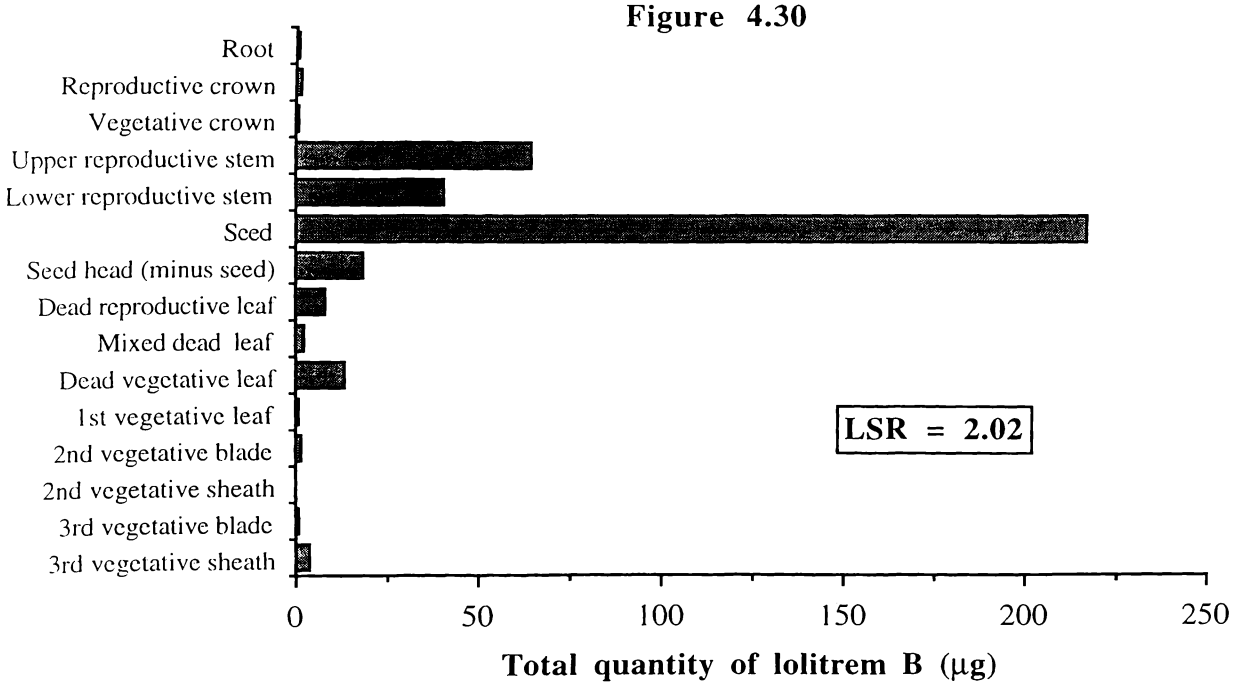


Figure 4.30



Figures 4.29 and 4.30 Lolitrem B concentration (Fig. 4.29) (µg of lolitrem B per g of dry grass material) and total amount of lolitrem B (Fig. 4.30) (µg of lolitrem B per tissue) in different components of vegetative and reproductive tillers from 'reproductive' ryegrass plants sampled on 14/1/92. Values are a mean of eight different A. lolii-infected plants.

Figure 4.31

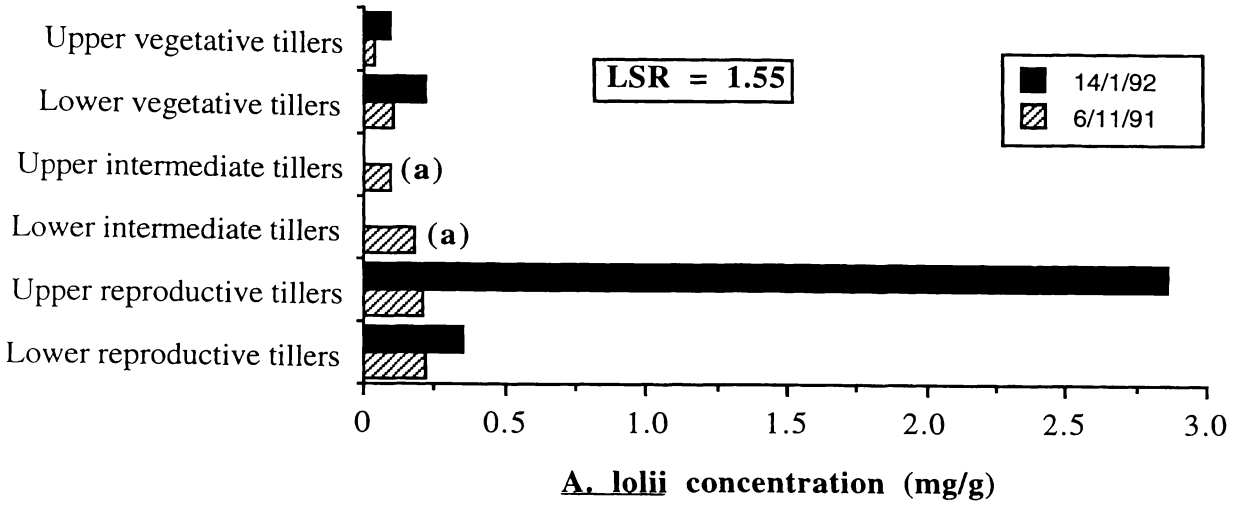
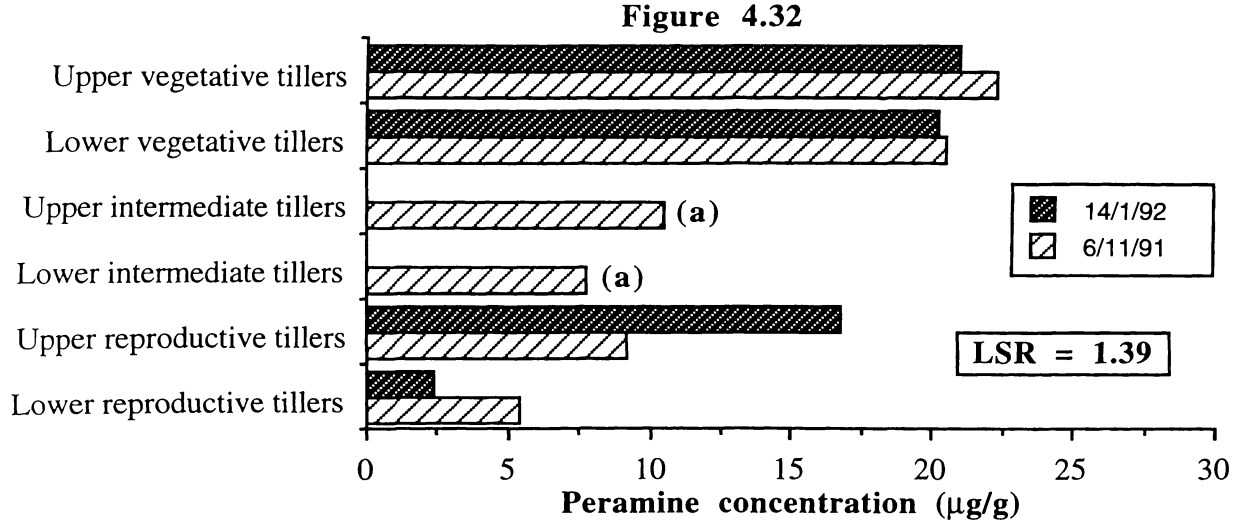


Figure 4.32



Figures 4.31 and 4.32 A. lolii concentration (µg of freeze-dried mycelium per g of dry grass material) (Fig. 4.31) and peramine concentration (µg of peramine per g of dry grass) (Fig. 4.32) in lower (from ground level to 8cm) and upper (above 8cm) components of vegetative, intermediate (for 6/11/91) and reproductive tillers from 'reproductive' ryegrass plants sampled on 6/11/91 and 14/1/92. Values are a mean of eight different A. lolii-infected plants. (a) denotes that no intermediate tillers were present on 14/1/92).

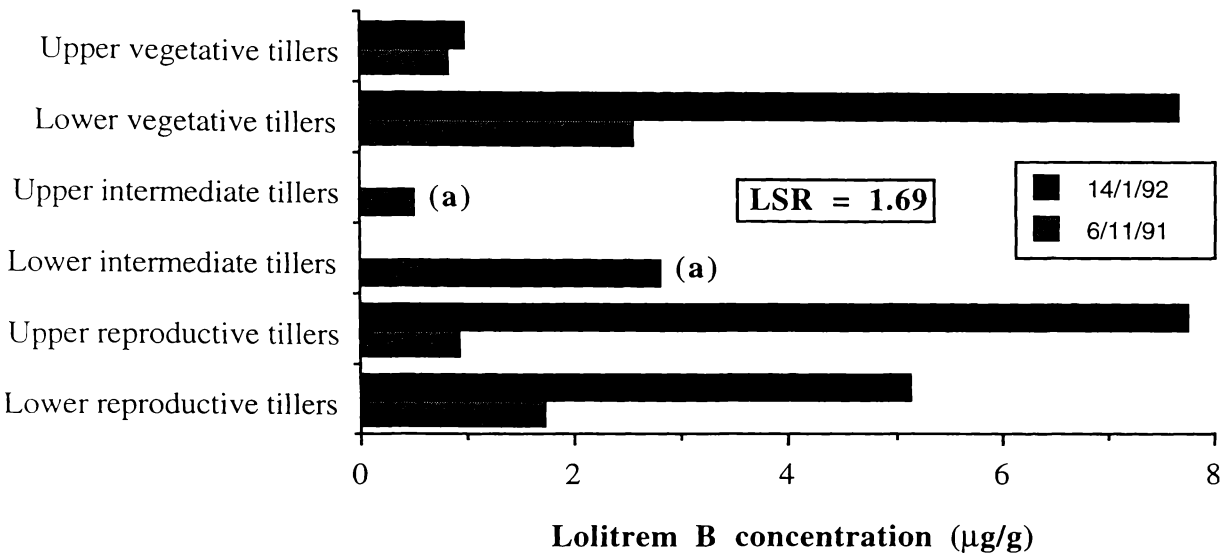


Figure 4.33 Lolitrem B concentration (µg of lolitrem B per g of dry grass material) in lower (from ground level to 8cm) and upper (above 8cm) components of vegetative, intermediate (for 6/11/91) and reproductive tillers from 'reproductive' ryegrass plants sampled on 6/11/91 and 14/1/92. Values are a mean of eight different A. lolii-infected plants. (a) denotes that no intermediate tillers were present on 14/1/92).

vegetative tillers than crown tissue from reproductive tillers. Also, peramine and lolitrem B concentrations were significantly higher ($P< 0.001$) in senescent leaf material from vegetative tillers compared to senescent leaf material from reproductive tillers.

The plant component containing the greatest quantities of *A. lolii*, peramine and lolitrem B was the seed (Figs. 4.26, 4.28 and 4.30 respectively). In comparison with the seed, the quantity of *A. lolii*, peramine and lolitrem B in all other plant parts was small. The seed component alone accounted for 92.9%, 76.5% and 58.4% of the total *A. lolii*, peramine and lolitrem B content of the plant respectively.

4.3.4 Total quantities and mean concentrations of *A. lolii*, peramine and lolitrem B in 'vegetative' and 'reproductive' plants.

6/11/91 harvest: The total quantity of *A. lolii*, peramine and lolitrem B was significantly ($P< 0.01$) higher in the 'reproductive' plants than in the 'vegetative' plants (Tables 4.2, 4.3 and 4.4 respectively). The mean *A. lolii* concentration (excluding as always the contribution from the roots) was also significantly higher ($P< 0.05$) in the 'reproductive' plants compared to the 'vegetative' plants (Table 4.2). However, the 'reproductive' plants produced significantly ($P< 0.001$) more biomass than the 'vegetative' plants (Table 4.1). This resulted in a significantly higher ($P< 0.01$) mean concentration of peramine in 'vegetative' compared to 'reproductive' plants (Tables 4.3). The mean lolitrem B concentration in the 'vegetative' plants was also higher compared to the 'reproductive' plants, but this difference was not significant (Table 4.4).

14/1/92 harvest: As mentioned previously, sample shortages of some plant components in both 'vegetative' and 'reproductive' plants meant that it was not possible to analyze all of the 14/1/92 samples for *A. lolii* content. In the 'reproductive' plants an average of 0.54% of the total biomass of the plant (minus the root) was not analyzed for *A. lolii* content for this reason. In the 'vegetative' plants this figure was 1.01%. As

Table 4.1 **Total amounts (mg) of dry matter (excluding roots), and total numbers of tillers produced in vegetative and reproductive perennial ryegrass plants harvested on 6/11/91 (when reproductively mature inflorescences were emerging) and 14/1/92 (when mature seeds were present in the inflorescences). The dry matter removed from the vegetative plants during the trimming regime (accumulated regrowth) is included in the totals.**

	6/11/91				14/1/92			
	Total dry weight (mg)		Total number of tillers		Total dry weight (mg)		Total number of tillers	
Plant Number	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants
1	11075	30600	115	171	16071	34396	130	195
2	9475	25311	160	222	28613	136988	230	496
3	11917	37626	151	291	30748	39788	294	195
4	9166	36733	128	257	11654	38801	145	164
5	16373	30894	191	259	25033	83613	181	450
6	12610	39353	126	180	16144	51778	89	173
7	10261	25573	107	144	17419	25802	126	95
8	11952	40203	132	287	18205	78947	135	330
Mean	11603.6	33286.6	139	226	20486	61264	166	262

Table 4.2 **Total quantities (µg) of *A. lolii* and average *A. lolii* concentrations (µg/g) of vegetative and reproductive perennial ryegrass plants harvested on 6/11/91 and 14/1/92. Quantities of *A. lolii* in the root component have been excluded. The *A. lolii* content present in the accumulated regrowth of the vegetative plants is included (* P<0.05, ** P<0.01, *** P<0.001).**

	6/11/91				14/1/92			
	Total *A. lolii* content (µg)		Mean *A. lolii* concentration (µg/g)		Total *A. lolii* content (µg)		Mean *A. lolii* concentration (µg/g)	
Plant Number	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants
1	1649	10472	148.9	342.2	2299	70088	143.1	2037.7
2	498	2022	52.6	79.9	2408	253879	84.2	1853.3
3	684	4331	57.4	115.1	2196	93914	71.4	2360.4
4	506	2905	55.2	79.1	801	44625	68.7	1150.1
5	479	3805	29.2	123.2	1447	151215	57.8	1808.5
6	770	3917	61.1	99.5	2222	183513	137.6	3544.2
7	518	5783	50.5	226.1	1817	93810	104.3	3635.8
8	1367	4565	114.4	113.5	1392	128990	76.5	1633.9
Mean	809	4725	71.2	147.3	1823	127504	92.9	2253.0
	**		*		**		***	

Table 4.3 **Total quantities (µg) of peramine and average peramine concentrations (µg/g) of vegetative and reproductive perennial ryegrass plants harvested on 6/11/91 and 14/1/92. Quantities of peramine in the root component have been excluded. The peramine content present in the accumulated regrowth of the vegetative plants is included (** P<001).**

	6/11/91				14/1/92			
	Total peramine content (µg)		Mean peramine concentration (µg/g)		Total peramine content (µg)		Mean peramine concentration (µg/g)	
Plant Number	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants
1	366.0	688.0	33.1	22.5	417.0	872.2	25.9	25.4
2	219.8	251.3	23.2	9.9	471.7	1343.1	16.5	9.8
3	184.2	364.7	15.5	9.7	590.3	757.0	19.2	19.0
4	177.0	310.4	19.3	8.5	222.8	487.4	19.1	12.6
5	127.0	190.4	7.8	6.2	140.0	661.1	5.6	7.9
6	275.0	432.5	21.8	11.0	308.9	1109.3	19.1	21.4
7	144.8	262.8	14.1	10.3	234.5	234.8	13.5	9.1
8	149.2	378.7	12.5	9.4	206.4	780.4	11.4	9.9
Mean	205.4	359.9	18.40	10.9	323.9	780.7	16.7	14.4
	**		**		**			

Table 4.4 **Total quantities (µg) of lolitrem B and average lolitrem B concentrations (µg/g) of vegetative and reproductive perennial ryegrass plants harvested on 6/11/91 and 14/1/92. Quantities of lolitrem B in the root component have been excluded. The lolitrem B content present in the accumulated regrowth of the vegetative plants is included (** P<001, *** P<0.001).**

	6/11/91				14/1/92			
	Total lolitrem B content (µg)		Mean lolitrem B concentration (µg/g)		Total lolitrem B content (µg)		Mean lolitrem B concentration (µg/g)	
Plant Number	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants	Vegetative Plants	Reproductive Plants
1	16.7	66.7	1.51	2.18	69.9	415.9	4.35	12.1
2	18.2	20.7	1.92	0.82	40.9	407.5	1.43	3.0
3	15.0	39.5	1.26	1.05	84.6	320.6	2.75	8.1
4	16.4	47.9	1.79	1.30	37.5	279.8	3.22	7.2
5	9.9	25.2	0.60	0.82	28.3	292.0	1.13	3.5
6	27.6	81.4	2.19	2.07	45.9	491.6	2.85	9.5
7	21.0	31.6	2.05	1.24	28.5	132.3	1.64	5.1
8	10.2	28.6	0.85	0.71	25.8	388.3	1.42	4.9
Mean	16.9	42.7	1.52	1.27	45.2	341.0	2.25	6.7
	**				**		***	

these percentages were so small, the contribution of *A. lolii* in the unanalyzed samples to the total *A. lolii* content of the plants was likely to be negligible, particularly since *A. lolii* concentrations in the missing samples were relatively low in the plants in which they were measured. Therefore it was concluded that the total quantity of *A. lolii* in each plant could still be calculated with a high degree of accuracy.

The total quantity of *A. lolii*, peramine and lolitrem B was significantly greater ($P< 0.01$) in 'reproductive' compared to 'vegetative' plants (Tables 4.2, 4.3 and 4.4 respectively). Mean *A. lolii* and lolitrem B concentrations were also significantly ($P< 0.001$) higher in 'reproductive' plants compared to 'vegetative' plants (Tables 4.2 and 4.4 respectively). As the dry matter production in 'reproductive' plants was significantly higher ($P< 0.05$) than in 'vegetative' plants (Table 4.1), average peramine concentrations were slightly (though not significantly) higher in the 'vegetative' plants compared to the 'reproductive' plants (Table 4.3).

4.4 DISCUSSION

***A. lolii*:** Results of the analyses of the *A. lolii*-free clones using ELISA suggest that in most cases, the *A. lolii* concentrations quoted for the *A. lolii*-infected samples are probably close to being a true reflection of the actual amount of mycelium present in them, as absorbances of most of the *A. lolii*-free samples were low. However, absorbances for the senescent leaf and root samples from the *A. lolii*-free line were consistently higher than average, indicating that non-specific binding was more prevalent in these two plant components. Thus, although absorbances of the root and senescent leaf samples from *A. lolii*-free clones were lower than those from *A. lolii*-infected clones, it is probable that compared to the other plant tissues, *A. lolii* concentrations in the roots and senescent leaf tissues of *A. lolii*-infected clones have

been slightly overestimated.

Compared to previous studies (Musgrave 1984, Musgrave and Fletcher 1984, Keogh and Tapper 1993), *A. lolii* concentrations recorded in the present study were low. There could be several reasons for this difference. The time of year in which the harvests were taken, and the geographical location of the plants could have influenced the results. Also the strain of endophyte used in the *A. lolii* dilution series could have affected the results. The fact that the antibodies used were produced using the soluble *A. lolii* antigen fraction instead of the usual mycelial fraction may also have been an important factor. Alternatively, sample deterioration could have reduced the final absorbances. After the present study had been completed, fresh grass samples were taken to test this last possibility. The results indicated that no, or only a very minor amount of sample deterioration had occurred during storage. Irrespective of the reasons for the lower *A. lolii* concentrations observed in the present study, the important point is that consistent results were obtained. Although quoting *A. lolii* concentrations in μg/g probably gives a much more accurate impression of the amount of mycelium present in grass samples, it should also be remembered that the reactivity of certain concentrations of *A. lolii* may be highly dependant upon whether the fungus is in its natural state (ie within the plant) or produced under artificial conditions (ie cultured).

Results from 'vegetative' and 'reproductive' plants on all three harvest dates indicate that, irrespective of leaf age, *A. lolii* concentrations were significantly higher in the leaf sheath of vegetative tillers, than they were in the leaf blade (Figs. 4.1, 4.7, 4.13, 4.19 and 4.25). These results are in complete agreement with results from other studies. Using a simple staining technique, Niell (1940) was the first to observe that the leaf sheath contained a greater abundance of hyphae of what was almost certainly *A. lolii*, than the leaf blade. Using ELISA, Musgrave (1984) and Musgrave and Fletcher (1984) found that *A. lolii* concentrations were high in basal sections of vegetative tillers,

and more specifically were highest in leaf sheaths from vegetative tillers.

The results of the present study are also in close agreement to those of Keogh and Tapper (1993) who also reported that concentrations of *A. lolii*, in all but the oldest leaf, progressively increased from the tip of the leaf blade to the lower part of the sheath. Results from the present study indicate that in vegetative tillers (whether from 'vegetative' or 'reproductive' plants), the *A. lolii* concentration gradient may extend into the crown (Figs. 4.1, 4.7, 4.13, 4.19) as mean *A. lolii* concentrations were higher in the crown than in any other vegetative tissue. Musgrave and Fletcher (1984) also found that *A. lolii* concentrations in the crown of vegetative tillers were high although they were not as high as in the leaf sheath. Differences in time of harvest, method of sample preparation and sample extraction technique may account for the differences between the results of the present study and those of Musgrave and Fletcher (1984).

The fact that in the present study *A. lolii* concentrations were consistently at their highest in the crown component of vegetative tillers, indicates that the crown tissue acts as a concentrated reservoire of *A. lolii* mycelium. *A. lolii* hyphae may then grow up into the leaf sheath and undergo vegetative propagation. Alternatively, much of the vegetative propagation of *A. lolii* may occur in the crown rather than the leaf sheath. In the latter scenario, the endophyte would literally be dragged up the blade and sheath as the tiller develops.

The higher *A. lolii* concentration in the leaf sheath compared to the leaf blade recorded in vegetative tillers was also observed in reproductive tillers, irrespective of leaf age (Fig. 4.19). A similar phenomenon was reported by Musgrave and Fletcher (1984) who also found that *A. lolii* concentrations were considerably higher in leaf sheaths from vegetative tillers compared to those from reproductive tillers. In the present study, *A. lolii* concentrations were also generally slightly higher in leaf sheaths and blades from vegetative tillers than reproductive tillers. This may reflect the greater

biomass of each leaf sheath and blade component in reproductive tillers compared to vegetative tillers. As was seen in vegetative tillers, *A. lolii* concentrations in the crown of reproductive tillers were higher than in any leaf sheath or blade component. This was also reported by Musgrave and Fletcher (1984).

The effect of leaf age on *A. lolii* concentration in vegetative tillers appears to be dependant upon time of harvest. In plants harvested on 1/10/91, *A. lolii* concentrations were very low in the newest emerging leaf (1st leaf) compared to sheaths of the older leaves (Fig. 4.1) (interestingly, Neill (1940) found that hyphae were difficult to detect in very young tissues). Also, in the 2nd to 4th leaves, *A. lolii* concentrations increased as leaf sheath age decreased. Similar *A. lolii* concentration distributions to this were observed by Keogh and Tapper (1993) who concluded that the large difference in *A. lolii* concentration between the first and second leaves was probably due to a short period of hyphal growth which occurred concurrently with or immediately after the leaf elongation phase. Data from the 1/10/91 sample support this hypothesis. The decline in *A. lolii* concentrations between the 2nd and the 4th sheath was probably due to a reduction, or a complete stoppage, in the growth of the mycelium at a time when the leaf sheath biomass was still increasing. From this, the time period during which *A. lolii* was actively growing in the sheath appears to have been very short. A similar pattern as this was observed in the vegetative tillers derived from 'reproductive' plants sampled on 6/11/91 (Fig. 4.19). However, in the 6/11/91 harvest of the 'vegetative' plants, *A. lolii* concentrations in leaves 2-4 increased, rather than decreased, with increasing leaf age (Fig. 4.7). Also, *A. lolii* concentrations in the 1st leaf were as high as those in the 2nd sheath. This indicates that either *A. lolii* hyphae continued to grow in the sheath for longer, or that leaf sheath growth declined more quickly compared to the earlier harvest (1/10/91). The observation that *A. lolii* concentrations on 6/11/91 in the leaf blades were

much closer to those in the leaf sheaths in 'vegetative' compared to 'reproductive' plants, may be due to the trimming regime. During trimming, much of the upper parts of the leaf blades, which only contain very small amounts of *A. lolii* (Keogh and Tapper 1993), were removed. Thus the leaf blade components from the 'vegetative' plants were in reality, the lower leaf blade components which would be expected to contain higher concentrations of *A. lolii* than the entire blade. There was no effect of leaf age on *A. lolii* concentration in vegetative tillers from 'reproductive' or 'vegetative' plants sampled on 14/1/92 (Figs. 4.25 and 4.13), or in reproductive tillers from 'reproductive' plants sampled on 6/11/91 (Fig. 4.19).

A. lolii concentrations were generally at their lowest in leaf blades, roots (from both 'vegetative' and 'reproductive' plants), and regrowth material (from 'vegetative' plants) (Figs. 4.1, 4.7, 4.13, 4.19 and 4.25). Musgrave (1984) and Musgrave and Fletcher (1984) also recorded low levels of *A. lolii* in the roots and leaf blades. These tissues represent the extremities of the vegetative parts of the ryegrass plant. Also, roots and leaf blade tips are a comparatively long distance from where the *A. lolii* hyphae are concentrated (crown and sheath), hence plant biomass production in these tissues may dilute the small quantity of fungus which manages to reach them. It is also probable that the environment in these tissues is not conducive for the proliferation *A. lolii*. *A. lolii* hyphae have yet to be observed visually (ie: by staining) in the roots, leading to the possibility that only translocated antigens of *A. lolii*, rather than the actual mycelium, are being recorded by the ELISA. The non-specific binding which appears to be associated with the root samples mentioned earlier complicates the picture still further.

It is interesting to note that on 1/10/91, the plant component with the highest concentration of *A. lolii* was the stem tissue (Fig. 4.1). This is significant as the stem tissue was the only reproductive tissue present in the plants sampled on 1/10/91. *A. lolii* concentrations were also high in the stem tissue component on 6/11/91 in vegetative

tillers from both 'reproductive' and 'vegetative' plants (Figs. 4.7 and 4.19 respectively). In 'vegetative' plants sampled on 1/10/91 and 6/11/91, the plant component containing the largest quantity of *A. lolii* was the stem (Figs. 4.2 and 4.8 respectively). Also, barring the leaves (alive and dead) and roots, all structures associated with reproductive tillers from 'reproductive' plants, contained particularly high concentrations of *A. lolii* (Figs. 4.19 and 4.25). These structures included the upper and lower reproductive stem (Figs. 4.19 and 4.25), inflorescence (Fig. 4.19), seed head (minus seed) (Fig. 4.25) and seed (Fig 4.25). The seed component in particular contained large quantities of *A. lolii*. Musgrave (1984) also found large amounts (up to 20% w/w) of *A. lolii* in seeds compared to mature vegetative plants, although the seeds did not originate from the plants analyzed. Relatively high concentrations of *A. lolii* were also measured in flowering stem tissues by Musgrave and Fletcher (1984). However, in their study, slightly higher *A. lolii* concentrations were recorded in the crown and leaf sheaths from reproductive tillers compared to the flowering stem. In the current experiment, *A. lolii* concentrations in the crown and leaf sheaths from reproductive tillers were considerably lower than most parts of the flowering stem (Fig. 4.19). Also, Musgrave and Fletcher (1984) recorded higher *A. lolii* concentrations in crowns, pseudostems and leaf sheaths from vegetative tillers than in any component from reproductive tillers. This contrasts with the results obtained in the present experiment. However, it is difficult to compare the results of Musgrave and Fletcher (1984) with those of the present study, partly for the reasons outlined earlier in this section, but also because Musgrave and Fletcher (1984) did not report on the precise composition of the flowering stem.

The proliferation of *A. lolii* in the plant reproductive structures observed in the present study is almost certainly linked to the fact that the endophyte is seed disseminated, viz proliferation of *A. lolii* in these tissues all but ensures the successful transmission of the endophyte to the next plant generation. This hypothesis is supported

by the results of chapter 3 which indicated that *A. lolii* proliferation occurred in response to the plant becoming reproductive, rather than as a result of direct environmental (ie external to the plant) influences. The pattern of *A. lolii* concentrations from whole undissected tillers (Fig. 4.31) and the mean *A. lolii* concentrations from 'vegetative' and 'reproductive' plants (Table 4.2) also support this hypothesis. During the early part of the ryegrass reproductive season (6/11/91), *A. lolii* concentrations increased as tillers became reproductively more mature (Fig. 4.31). By 14/1/92, by far the greatest concentration of *A. lolii* was recorded in the upper portion of reproductive tillers. Much of the *A. lolii* present in this portion was already located in the target structure, the seed (Figs. 4.25 and 4.26). Also, the significantly higher mean *A. lolii* concentrations in the 'reproductive' compared to the 'vegetative' plants (on both 6/11/91 and 14/1/92) (Table 4.2) indicates that the endophyte was in a more actively growing state in reproductive compared to vegetative tissues. The higher *A. lolii* concentrations in the 'reproductive' plants were achieved even in the face of large biomass increases in 'reproductive' compared to 'vegetative' plants (Table 4.1). Even if the amount of *A. lolii* contributed by the seed component is subtracted from the total *A. lolii* content of the 'reproductive' plants, mean *A. lolii* concentrations are still higher in 'reproductive' compared to 'vegetative' plants. The factors which enable *A. lolii* proliferation to occur in reproductive structures are not known, but plant hormone balance and/or the nutrient status of the plant tissues could be involved. Also, the structural nature of the plant reproductive tissues may provide an environment which is more conducive for the growth of *A. lolii*.

In the only similar experiment performed with *A. coenophialum*-infected tall fescue, Siegel *et al.* (1984a) found that concentrations of mycelium were highest (in descending order) in the leaf sheath (from vegetative tillers presumably), seed (not harvested from the same plants), crown, and stem (presumably pseudostem). *A.*

coenophialum concentrations were lowest in the roots and leaf blades. With the exception of the result for the seed component (which was lower than would be expected), this is generally what was observed in the present study and what was observed by Musgrave (1984) and Musgrave and Fletcher (1984) for *A. lolii* in perennial ryegrass.

Peramine: Results from the 1/10/91 harvest indicate that, at this time of year, peramine concentrations were higher in the leaf sheath than in the leaf blade irrespective of leaf age (Fig. 4.3). However, in none of the other harvests was peramine concentration consistently higher in the leaf sheath than the leaf blade of vegetative tillers. This was true for vegetative tillers in both 'reproductive' and 'vegetative' plants (Figs. 4.9, 4.15, 4.21 and 4.27). Thus, compared to *A. lolii*, peramine appears to be more uniformly distributed throughout the vegetative leaf. In leaves from reproductive tillers, peramine concentrations were significantly higher in sheaths compared to the blades (Fig. 4.21). This was similar to the vertical distribution of *A. lolii* (Fig. 4.19). Thus, at certain times of the year (ie: 6/11/91), it appears that the vertical distribution of peramine in leaves from reproductive tillers is different to that in leaves from vegetative tillers (whether they are from 'reproductive' or 'vegetative' plants or not), with peramine concentrations being higher in the sheath compared to the blade in the former, but not in the latter. Also peramine concentrations in the sheaths and blades from reproductive tillers were significantly lower than in the same tissues from vegetative tillers (Fig. 4.21). A possible explanation for this is that *A. lolii* concentrations were also generally lower in the sheaths and blades from reproductive tillers compared to those from vegetative tillers.

Since relative peramine concentrations in the leaf blades of vegetative tillers were high (except for the 1/10/91 harvest) compared to relative *A. lolii* concentrations, some peramine may be translocated from areas of high *A. lolii* concentration (crown and leaf

sheath) to the leaf blade. Translocation of peramine throughout the plant is probably accomplished quite easily as this alkaloid is hydrophillic (Rowan and Gaynor 1986). However, it is also possible that instead of being translocated, peramine synthesis by the small amount of *A. lolii* in the blade, may have been enhanced. Results discussed later give more credence to this possibility.

Similar results to those above were obtained by Davies *et al.* (1993), who concluded that the vertical distribution of peramine throughout all but the oldest leaves from vegetative tillers was relatively even. Davies *et al.* (1993) harvested their plant samples in late-March and mid-April indicating that in leaves from vegetative tillers, peramine concentration differences in late spring and summer are probably similar to those in the autumn. Similar concentrations of peramine in leaf sheath and leaf blade tissues from vegetative tillers were also reported by Keogh and Tapper (1993). However, Keogh and Tapper (1993) also observed that peramine concentrations increased progressively down the blade.

Although there was no consistent vertical peramine concentration gradient in leaves from vegetative tillers with time, there was a consistent horizonal one. In all harvests of 'reproductive' and 'vegetative' plants, peramine concentrations in leaves (from vegetative tillers) which were mature enough to be dissected into sheath and blade, increased as leaf age decreased (Figs. 4.3, 4.9, 4.15, 4.21, 4.27). In most cases, this horizontal concentration gradient was maintained through to the youngest (first) leaf (which was not sectioned). Also, the senescent leaf material (the oldest of the leaf tissues) consistently had the lowest concentrations of peramine of any of the components derived from leafy tissues. Apart from the 1/10/91 harvest (Fig. 4.3), this peramine concentration gradient was not related to *A. lolii* concentrations. In fact, in the 6/11/91 'vegetative' plant sample, the horizontal peramine and *A. lolii* concentration gradients were negatively correlated (Fig. 4.9). There was no effect of leaf age on

peramine concentrations in leaf sheaths from reproductive tillers, although peramine concentrations in the 1st blade were significantly higher ($P< 0.05$) than those in the 3rd blade (Fig. 4.21). A similar horizontal concentration gradient as that seen for peramine in the vegetative tillers was reported for loline alkaloids in *A. coenophialum*-infected tall fescue (Hardy *et al.* 1986).

The same effect of leaf age on peramine concentrations in vegetative tillers was observed by Keogh and Tapper (1993) and Davies *et al.* (1993) who harvested their plant material in late-March and mid-April respectively.

The results of Keogh and Tapper (1993) as well as those of the present experiment indicate that peramine concentrations in the different leaf tissues of vegetative tillers are not necessarily determined by the *A. lolii* concentrations of those tissues. Keogh and Tapper (1993) suggested that as well as a net movement of peramine from sheath to blade, there may also be some translocation occurring from older to younger leaves. Results from the current experiment lend support to this hypothesis, particularly since *A. lolii* concentrations in the youngest leaf tended to be low while peramine concentrations were high. However, there is also the possibility that peramine could have been translocated from the crown, which according to the results of the present study, usually contains higher concentrations of *A. lolii* than the leaf sheath. As discussed earlier in relation to the distribution of peramine in the leaf sheaths and blades, a third possibility is that peramine synthesis was enhanced in areas where *A. lolii* was scarce. If peramine was translocated from the crown (second option) or was synthesised in the younger tissues (third option), a breakdown of peramine in the senescing leaves would be implied. Which ever mechanism is responsible, the peramine concentration gradient presumably ensures that the young developing leaves receive greater protection from peramine sensitive invertebrate herbivores, although there is evidence that in *A. coenophialum*-infected tall fescue, alkaloid levels are not necessarily correlated with the

degree of insect resistance (Hardy *et al.* 1986). Protection of the developing and younger leaves from invertebrate herbivore attack is more important than the protection of the older leaves, as it is more likely to ensure the continued growth and persistence of the plant.

Comparing all plant parts together, peramine concentrations in vegetative tillers from both 'reproductive' and 'vegetative' plants were usually at their highest in the 2nd and 3rd leaf sheaths and blades and 1st leaves (Figs. 4.3, 4.9, 4.15, 4.21 and 4.27). Except for the 1/10/91 harvest (Fig. 4.3), peramine concentrations in the regrowth category of 'vegetative' plants were also relatively high (Figs. 4.9 and 4.15), while both *A. lolii* and lolitrem B were scarce (Figs. 4.1, 4.7, 4.5, 4.11 and 4.17).

The lowest peramine concentrations were recorded in the roots (where it was often not detectable at all), crown (from vegetative and reproductive tillers) and senescent leaf tissue (from vegetative and reproductive tillers) (Figs. 4.3, 4.9, 4.15, 4.21 and 4.27). Low peramine concentrations in the root and senescent leaf tissue have also been reported respectively by Fannin *et al.* (1990) and Davies *et al.* (1993). The scarcity of peramine in the roots could have major implications on the vulnerability of this tissue in regard to attack from soil-borne invertebrate herbivores such as grass grub (*Costelytra zealandica*) and black beetle (*Heteronychus arator*) larvae. As discussed earlier, it appears that much of the peramine in the senescing leaf tissue could be translocated to the younger leaves, so that by the time the leaf dies, very little peramine remains. It is slightly surprising therefore that peramine concentrations in the roots are so low, given that peramine appears to be such a translocatable compound. The fact that the roots contain appreciable (though not high) amounts of lolitrem B, a compound supposedly translocated with difficulty, makes this even more surprising.

The dynamics of peramine production and how this varies according to plant component are not made clear from the results of the 'vegetative' plants. However,

regression analyses show that for the 6/11/91 and 14/1/92 harvests, peramine concentrations of all plant components (excluding roots) were significantly negatively correlated with *A. lolii* concentrations. Thus, if peramine production is directly dependent on *A. lolii* concentrations, it is evident that peramine was translocated from areas of high *A. lolii* concentrations to areas of low *A. lolii* concentrations. However, the results could be interpreted differently. Peramine production may also be dependent upon the growth phase of the endophyte. Thus, peramine production may be enhanced while *A. lolii* is in the early stages of growth, and would be inhibited during proliferation of *A. lolii*. Such an hypothesis would also explain the inverse relationship between *A. lolii* and peramine concentrations. Results from the 'reproductive' plants discussed below indicate that several factors, including the two just discussed (ie: translocation and *A. lolii* growth phase) may be important in determining the peramine concentrations of plant tissues.

In complete contrast to what was observed with *A. lolii*, peramine concentrations in 'reproductive' plants sampled on 6/11/91 and 14/1/92 were higher in the leaf sheaths and blades from vegetative tillers than in almost all of the structures associated with reproductive tillers (ie: upper and lower reproductive stems and inflorescence) (Fig. 4.21). Thus, as was seen in the crown component, the proliferation of *A. lolii* in most parts of the reproductive tillers did not result in an increase in the peramine concentration. This effect can also be clearly seen in whole undissected tillers, where peramine concentrations on both 6/11/91 and 14/1/92 decreased as tillers became reproductively more mature (Fig. 4.32), while *A. lolii* concentrations increased (Fig. 4.31). The rise in peramine concentration seen in the upper reproductive tillers on 14/1/92 compared to 6/11/91 (Fig. 4.32), was almost completely due to the contribution from the seed component which was extremely high in peramine (Figs. 4.27 and 4.28). The inhibitory effect of plant reproductive activity on peramine production, at least in the

6/11/91 harvest, is also clearly evident from the results presented in Table 4.3. The mean peramine concentration of the 'reproductive' clones sampled on 6/11/91, was significantly lower than the mean peramine concentration of the 'vegetative' clones. Due to the huge amounts of peramine produced in the seed component of the 'reproductive' clones, the mean peramine concentrations in 'vegetative' and 'reproductive' plants on 14/1/92 were similar, although they were still slightly higher in the 'vegetative' clones.

The cause of the inhibitory effect on peramine production in reproductive tissues, excluding the seed, is unclear. The fact that peramine concentrations were considerably higher in the vegetative leaves of 'vegetative' plants compared to vegetative leaves from 'reproductive' plants sampled on 6/11/91, strongly suggests that peramine was not diverted from reproductive to vegetative tillers. By calling it an inhibitory effect, it is being assumed that peramine concentrations are driven by *A. lolii* concentrations. It has been confirmed that this is generally the case for the whole plant (chapter 3), but this relationship may be different for the different plant components. In other words, a particular concentration of *A. lolii* in different plant tissues, may not give rise to the same peramine concentration in those tissues. It is more likely that peramine production and the resulting peramine concentrations in the different tissues of the plant, are determined by several, rather than just one or two, factors.

As discussed for the 'vegetative' plants, there is evidence that the growth phase of *A. lolii*, coupled sometimes with changes in plant biomass production, may influence peramine concentrations in plant tissues. In the reproductive tissues (with the exception of the seed) where *A. lolii* growth appears to be in the prolific stage (ie: past the stage where concentrations are still low), and where plant biomass increases are large, peramine concentrations are low, while in the vegetative herbage where *A. lolii* concentration and plant biomass increases are relatively low, peramine concentrations are at their peak. The possibility that a lag effect, whereby peramine production

increases during and/or after *A. lolii* proliferation, could be responsible for the low peramine concentration in the reproductive tissues is unlikely. Evidence from the upper and lower reproductive stems (Figs. 4.21 and 4.27) and the lower reproductive tillers (Fig. 4.32) suggest that there was virtually no lag at all, as peramine concentrations were higher in lower and upper stems from reproductive tillers on 6/11/91 than they were on 14/1/92 (Figs. 4.21 and 4.27). Also peramine concentrations on 6/11/91 were more than double those on 14/1/92 in the lower reproductive stems (Fig. 4.32). As with the vegetative plants, these results suggest that peramine maybe produced at the very start of the *A. lolii* growing phase (which is usually when concentrations of *A. lolii* are low), shortly after which, peramine production may slow down or stop. These results also support the idea that peramine concentrations in the different tissues are determined by a complex of several factors. These could include the concentration of mycelium in the tissue or in other parts of the plant, the growth pattern of the mycelium and the length of time that the mycelium is in an enhanced peramine producing stage, the rate of plant biomass production for each tissue and how much peramine has been translocated to, or away from, the tissue. Mycelium concentrations and growth rates could themselves be influenced by factors such as the nutrient status, structural nature or hormone balance of the tissue.

The arguments outlined above could also be applied to the seed component which contained very high concentrations of both *A. lolii* and peramine (Figs. 4.25 and 4.27). The physical nature of the seed may be of particular relevance in determining peramine concentrations there. Being a small compact entity whose biomass does not keep increasing, the seed may be able to accumulate relatively large quantities of peramine in a short space of time. However, being at an extremity, an alternative possibility to all of this is that the seed may act as a sink for the peramine. This peramine may have been produced by *A. lolii* in other parts of the reproductive tiller, and then

translocated to the seed. This hypothesis would explain the reduction in peramine concentrations observed between 6/11/91 and 14/1/92 in the upper and lower stem tissues from reproductive tillers (Figs. 4.21 and 4.27) and in the lower portion of undissected reproductive tillers (Fig. 4.32). Irrespective of how they are brought about, high peramine concentrations in the seed have almost certainly arisen to protect the seed and/or developing seedling (see chapter 5) against attack from herbivores. Although they did not measure alkaloid concentrations, Cheplick and Clay (1988) have previously suggested that seed predators may be adversely affected by the presence of endophyte (*A. lolii* and *A. coenophialum*) in seed (perennial ryegrass and tall fescue respectively).

With the revelation that peramine may be an important factor in leading to faecal contamination in grazing stock (Fletcher and Sutherland 1993a, Pownall *et al.* 1993), the figures for the total quantity of peramine produced in each plant component are of considerable interest. On 1/10/91, the peramine concentration in the regrowth component was moderate (Fig. 4.3). However, since the accumulated dry weight of this component compared to other plant parts was considerable, the total amount of peramine present in the accumulated regrowth was higher than in any other part of the plant (Fig. 4.4). By 6/11/91, the total quantity of peramine which had been recovered from the regrowth component of 'vegetative' plants during the clipping regime (these plants were trimmed on 2/10/91, 14/10/91, 24/10/91, 30/10/91 and 6/11/91), comprised over 55% of the total peramine content of the plant, and was far greater than the amount of peramine recovered from any other tissue (Fig. 4.10). By 14/1/92, the total quantity of peramine which was removed from the plant as regrowth since the start of the clipping regime (2/10/91), accounted for 75% of the total amount of peramine in the whole plant (Fig. 4.16). The quantity of peramine in the regrowth component was over 16 times higher than the quantity of peramine in any other plant part. Thus, during this period of time, grazing animals would ingest a considerable amount of peramine, as much of the

herbage ingested by stock is often regrowth. This lends a considerable amount of support to the idea that peramine may be linked with faecal contamination. Interestingly, if peramine is a primary cause of faecal contamination, stock feeding on reproductive or partly reproductive material may also be at risk. Ingestion of mature inflorescences (Fig. 4.22) and in particular fully developed seeds (Fig. 4.28) (both of which may be present in hay) could result in large uptakes of peramine.

Lolitrem B: Lolitrem B concentrations in older leaves (ie 3rd and, where present, 4th) from vegetative tillers were consistently higher in leaf sheaths compared to leaf blades, irrespective of whether the tillers were derived from 'reproductive' or 'vegetative' plants (Figs. 4.5, 4.11, 4.17, 4.23 and 4.29). Higher concentrations of lolitrem B in sheaths compared to blades from plants in a vegetative state, have been reported on a number of occasions (Gallagher *et al.* 1982a, 1985, 1987, di Menna *et al.* 1992, Davies *et al.* 1993 and Keogh and Tapper 1993). This difference was not seen in the youngest dissected leaf (2nd leaf), where lolitrem B concentrations were often slightly higher in the blade component compared to the sheath. However, in leaf sheaths from reproductive tillers, lolitrem B concentrations were consistently higher than in the blade, irrespective of leaf age (Fig. 4.23). Also, lolitrem B concentrations in the outer sheaths were substantially lower in leaves from reproductive tillers compared to leaves from vegetative tillers. Interestingly, higher ergot alkaloid concentrations in leaf sheaths compared to leaf blades have been recorded in *A. coenophialum*-infected tall fescue (Lyons *et al.* 1986).

Also, there was an effect of leaf age on lolitrem B concentration in the sheaths, viz lolitrem B concentrations increased with increasing sheath age, irrespective of whether the sheaths were derived from vegetative or reproductive tillers (Figs. 4.5, 4.11, 4.17, 4.23 and 4.29). This pattern of lolitrem B distribution was also reported by

Gallagher *et al.* (1987) and Keogh and Tapper (1993) for vegetative tillers, and is the opposite to what was observed for peramine. Lolitrem B concentrations in the senescent leaf category (the oldest leaves) from reproductive tillers were higher than in any other reproductive tiller category (Fig. 4.23). Senescent leaf material from vegetative tillers also contained high concentrations of lolitrem B, though usually not as high as those in the outer sheath (Figs. 4.5, 4.11, 4.17, 4.23 and 4.29). The fact that the senescent leaf sample from vegetative tillers contained material from both the leaf sheath (which was high in lolitrem B) and the leaf blade (which was low in lolitrem B) may have accounted for the slight reduction in the lolitrem B concentration in the senescent leaf tissue compared to that in the outer sheath(s). It is a little mystifying why this was not also the case for the senescent tissue from reproductive tillers. Nevertheless, the results indicate that it is unlikely that much, if any, lolitrem B was translocated from the senescing leaf material (either from vegetative or reproductive tillers) back into the living plant, as may have been the case with peramine. As it is a lipophilic compound, the ability of the plant to translocate lolitrem B would be expected to be low.

Davies *et al.* (1993) also found high concentrations of lolitrem B in senescent leaf tissues from vegetative tillers. They found that the concentrations of lolitrem B were approximately double those in the lower pseudostem. It is not possible to directly compare the results of the present study with those of Davies *et al.* (1993) as the latter took their samples during March and April when lolitrem B concentrations may have been higher in the senescent leaf component compared to any other. Also, the pseudostem samples of Davies *et al.* (1993) consisted of leaf sheaths of all ages. Thus the high concentrations in the outer sheaths would probably have been diluted by the low concentrations present in the inner sheaths.

The effect of leaf age on lolitrem B concentration was not seen in the leaf blade tissues, the lolitrem B concentrations of which remained relatively constant with leaf age

(Figs. 4.5, 4.11, 4.17, 4.23 and 4.29).

In relation to the whole plant, lolitrem B concentrations in 'vegetative' plants were consistently at their highest in the 3rd, and where present, 4th leaf sheaths and the senescent leaf tissue (Figs. 4.5, 4.11, 4.17). The crown, which, with the exception of the stem, contained the highest *A. lolii* concentration, generally contained variable, but intermediate amounts of lolitrem B.

The lowest concentrations of lolitrem B in 'vegetative' plants were usually registered in the roots, 1st leaves, leaf blades and regrowth (part of the leaf blade material). With the exception of the 1st leaf, these categories are at the vertical extremities of the plant, providing more evidence for the low translocatability of lolitrem B.

Compared to the situation with peramine, the roots of 'vegetative' plants contained appreciable amounts of lolitrem B. A mean lolitrem B concentration of nearly 1μg/g was registered in the root component on 14/1/92. Either the lolitrem B in the root was translocated there from areas such as the crown or leaf sheaths, or it was produced by the endophyte which, according to the ELISA results, appears to reside there. Since most of the evidence suggests that lolitrem B is a poorly translocated molecule, the second option is perhaps the more likely.

Results from the leaf sheaths of tillers (both vegetative and reproductive) from 'vegetative' and 'reproductive' plants indicate that lolitrem B production increases just before leaf senescence occurs. At this stage, results from the ELISA test indicate that the growth of the *A. lolii* present has slowed considerably, or actually stopped. Therefore, it appears that, like peramine, lolitrem B production may be partly dictated by the growth phase that the endophyte is in, viz *A. lolii* which is growing rapidly produces little lolitrem B, while lolitrem B production in *A. lolii* which is not actively growing, but

which has gone through an actively growing stage, is enhanced. This would explain the consistently low levels of lolitrem B recorded in the 1st leaf. Thus, peramine production by *A. lolii* appears to be enhanced at the start of the growth cycle of *A. lolii*, while lolitrem B production appears to occur mainly at the end of the *A. lolii* growth cycle. However, like peramine, other factors such as the rate of plant biomass increase, the *A. lolii* concentration, the growth pattern of the mycelium and the length of time the mycelium is in an enhanced lolitrem B producing stage and the rate of translocation of lolitrem B to, or away from tissues (although for reasons discussed earlier, translocation is probably of little consequence), are also probably involved in determining lolitrem B concentrations.

The highest concentrations of lolitrem B in the 'reproductive' plants sampled on 6/11/91 occurred in the 4th sheath and senescent leaf of vegetative tillers (Fig. 4.23). This was similar to the situation recorded in the 'vegetative' plants sampled at the same time (Fig. 4.11). Similarly, the roots and the 1st leaf and 2nd leaf sheath from vegetative tillers, contained low concentrations of lolitrem B (Fig. 4.23). Also, lolitrem B concentrations were low in all of the leaf blades from reproductive tillers. Despite the relatively high *A. lolii* concentrations, the inflorescence and stem tissues from both vegetative and reproductive tillers sampled on 6/11/91 contained only moderate concentrations of lolitrem B. However, results from the 14/1/92 harvest of the 'reproductive' plants show that lolitrem B concentrations rose in the plant reproductive structures relative to the vegetative structures (Fig. 4.29). At this time, the lolitrem B concentration in the upper stem tissue from reproductive tillers was as high as that in the senescent leaf material from vegetative tillers (6.3μg/g) and lolitrem B concentrations in the seed (20.0μg/g) were more than three times higher than in any other tissue, vegetative or reproductive.

Like those for the 'vegetative' plants, these results indicate that there may be a lag between firstly, the proliferation of *A. lolii* in the plant, and secondly, the production of lolitrem B once *A. lolii* proliferation has ceased. This notion is supported by the results presented in Table 4.4. In the early phase of *A. lolii* proliferation (6/11/91), the mean lolitrem B concentration was slightly higher (though not significantly) in the 'vegetative', compared to the 'reproductive' clones. This was probably due to the greater increase in plant biomass relative to the increase in lolitrem B production in 'reproductive' plants. Lolitrem B production may also have been inhibited in the 'reproductive' plants relative to the 'vegetative' plants, as lolitrem B concentrations in all components (except the root) on 6/11/91 were substantially higher in 'vegetative' plants compared to the same structures on the 'reproductive' plants (Figs. 4.11 and 4.23). However, by 14/1/92 when endophyte proliferation had probably slowed or ceased (see chapter 3), the mean lolitrem B concentration in the 'reproductive' plants had risen by a factor of more than five since 6/11/91, and was nearly three times higher than that in the 'vegetative' plants sampled at the same time. Although much of this increase was due to the contribution from the seed, subtraction of the dry weight and lolitrem B content of the seed component from the 'reproductive' plants, still resulted in the mean lolitrem B concentration of the 'reproductive' clones being, in most cases, higher than that of the 'vegetative' clones.

There are few reports of lolitrem B concentrations having been monitored in reproductive structures of *A. lolii*-infected plants. Using a mouse bioassay, Gallagher *et al.* (1982b) reported that seeds contained greater concentrations of extractable neurotoxin than the grass itself. However, Gallagher *et al.* (1982b) did not quantify the amount of lolitrem B present in the seed. Lolitrem B concentrations of 3.5-4.3μg/g and 2.5-3.0μg/g have been measured in seed and reproductive stalk tissue respectively by di Menna *et al.* (1992). Compared to results from the present experiment where lolitrem B

concentrations ranged from 12.5 to 29.3μg/g, the seed concentration of 3.5-4.3μg/g measured by di Menna *et al.* (1992) is extremely low. Differences in the accuracy of the assay used in the two experiments are almost certainly not the cause of this unexpected result, as the lolitrem B analyses were performed by the same person on the same machine. If composite pasture samples made up of several plants were used in the study by di Menna *et al.* (1992), it is possible that seed and stalk from *A. lolii*-free plants were included in the samples diluting the lolitrem B somewhat. However, this is unlikely as the range of lolitrem B concentrations in their study was small. The large difference in the lolitrem B concentrations of the seed component in the two studies, may be as a result of the time of sampling. Di Menna *et al.* (1992) sampled the seed between 12th to 21st of December, while in the current study, the seed was harvested on 14th of January. Using conclusions drawn from the present experiment, it is possible that the earlier harvest time of di Menna *et al.* (1992) incorporated little or none of the lag phase, which is when lolitrem B production appears to increase. Whatever the reason, the very high lolitrem B concentrations recorded in the seed component in the present study could, like peramine, be an adaptation to protect the seed from destruction by seed eating herbivores (Cheplick and Clay 1988).

The distribution of lolitrem B in the plant, and the grazing patterns of the stock which feed on it, have a considerable impact on the development of ryegrass staggers. The higher concentration of lolitrem B in leaf sheaths compared to leaf blades from vegetative tillers observed in the present study, and recorded previously by Gallagher *et al.* (1982a, 1985, 1987), di Menna *et al.* (1992), Davies *et al.* (1993) and Keogh and Tapper (1993), support the observations of Keogh (1973) who reported that ryegrass staggers was usually only prevalent when stock were allowed to graze pastures too closely. The higher lolitrem B concentrations in the outer compared to the inner sheaths reported by Gallagher *et al.* (1987) and Keogh and Tapper (1993) and observed in the

current study, also has major implications on pasture management. Keogh and Tapper (1993) concluded that ryegrass staggers outbreaks could be minimized if the ryegrass component of pastures was maintained at a young leafy stage. According to Keogh and Tapper (1993), this could be achieved by not allowing pastures to be spelled for too long. In reducing the mature and senescent leaf content of the pasture, shorter spelling periods would probably decrease the levels of lolitrem B. Thus, by shifting stock daily to avoid the higher lolitrem B concentrations present in the basal tissues (Keogh 1973,1978) and by ensuring that pastures are spelled for short periods, it appears that simple grazing management practises could, in some situations, do much to control the ryegrass staggers problem.

Although the time of year that the present study was undertaken did not include much of the period during which ryegrass staggers outbreaks tend to be most prevalent (February-April), and although the plants used for the experiment were not in a pasture situation, results from the present study may still be of use when examining the ryegrass staggers problem. Concentration and production data from 'vegetative' plants sampled on 1/10/91 show that much of the lolitrem B contained in the plant was present in the outer two sheaths (Figs. 4.5 and 4.6). The same was also true for the 'vegetative' plants harvested on 6/11/91, except that the component containing the largest quantity of lolitrem B was the senescent leaf (Figs. 4.11 and 4.12). Also, the total quantity of lolitrem B in the regrowth components sampled since 2/10/91 was higher than in the third sheath (Fig. 4.12). By 14/1/92, the two plant components containing most of the lolitrem B were the senescent leaf and the accumulated regrowth (Fig. 4.18). Even though the outer sheath (3rd sheath) contained the highest concentrations of lolitrem B (Fig. 4.17), the absolute quantity it contained was small (Fig. 4.18). The fact that the lolitrem B accounted for in the accumulated regrowth is present in such a large quantity of herbage (ie: is very diluted), and that it is produced over a long period of time,

indicates that the regrowth component is probably of little importance in ryegrass staggers. Thus, within the limitations of the experiment, the indication from the present study is that the senescent leaf component, rather than mature sheaths, becomes more important as the "ryegrass staggers season" approaches. Results from the present experiment therefore support the view of Keogh and Tapper (1993) that senescent leaves are important when considering the principal causes of ryegrass staggers, but the role of mature sheaths requires further study. However, as Keogh and Clements (1993) have also pointed out, hard grazing before neurotoxin levels reach their peak could remove much of the mature leaf and leaf litter components from pastures.

The observations that serious outbreaks of ryegrass staggers occurred in animals grazing ryegrass allowed to bolt with seed head formation (Mortimer and di Menna 1985) and that the incidence of ryegrass staggers was significantly higher on untopped (flowering stems present) compared to topped (flowering stems removed) subplots (di Menna *et al.* 1992), are also interesting in the context of the present study. Prestidge and Gallagher (1988a) and di Menna *et al.* (1992) suggested that a lolitrem B concentration of more than 2.0-2.5μg/g was required for ryegrass staggers symptoms to develop in lambs. The mean lolitrem B concentration of 'reproductive' plants (equivalent to the untopped herbage) sampled on 14/1/92 (6.7μg/g) was considerably higher compared to that in the 'vegetative' plants (2.4μg/g) (equivalent to the topped herbage) (Table 4.4). The important contribution of the reproductive tillers as a source of lolitrem B can also be seen from the results of the undissected tillers which show that lolitrem B concentrations well in excess of 2.5μg/g were present in the upper, as well as the lower part of reproductive tillers (Fig. 4.32). From the results of the current study, much of the increase in the incidence of ryegrass staggers observed by di Menna *et al.* (1992) could have been due to the seed component which contained massive amounts (20.0μg/g) of lolitrem B (Figs. 4.29 and 4.30). However, the seed head and upper and

lower reproductive stem components also contained over 2.5μg/g of lolitrem B and would probably be important in contributing to ryegrass staggers. Thus, the reproductive state of the ryegrass plants in the pasture may affect the amount of lolitrem B ingested by stock and hence the incidence of ryegrass staggers. Regular trimming or grazing would help to maintain the ryegrass plants in a vegetative state. Compared to plants in an advanced reproductive state, lolitrem B production in plants in a vegetative state does not appear to be enhanced. In order to avoid the situation of plants becoming "too reproductive" (ie: setting seed), short spelling periods, also recommended to reduce the amount of mature and senescent sheath material, would seem to be desirable, as the lolitrem B increase associated with stalk and seed development could be largely avoided.

Comparing the distributions of peramine and lolitrem B observed in the present study with alkaloid distributions in endophyte-infected grasses from other studies, is often difficult due to the variable nature (ie: the environment in which the plants are grown, the time of year that the plants are sampled and the composition of the samples) of the different experiments. However, some comparisons and observations with these limitations in mind, may be useful. As mentioned in the preceding discussion, leaf sheaths of *A. coenophialum*-infected tall fescue contained higher concentrations of ergot alkaloids than leaf blades (Lyons *et al.* 1986). This was similar to what was observed in the present study with lolitrem B. Investigating the distribution of ergovaline in *A. coenophialum*-infected tall fescue, Azevedo *et al.* (1993) found that the crown component contained the highest concentrations, followed by the pseudostem, leaf blades and finally the root, which contained only low levels of ergovaline. Rottinghaus *et al.* (1991) who also investigated the distribution of ergovaline in tall fescue, found that concentrations were low in the leaf blades, intermediate in the stem with leaf sheath and highest in the seed head. This was similar to the distribution of both peramine and lolitrem B in the reproductive structures in the present experiment. It is also interesting

to note that in the study by Rottinghaus *et al.* (1991), ergovaline concentrations in the seed head component increased with time (ie: as the seeds matured). This is almost certainly similar to what would have been observed in the current study with peramine and lolitrem B if the methodology of Rottinghaus *et al.* (1991) had been used to sample the plants, as peramine and lolitrem B concentrations in the seed were very high. High concentrations of N-acetyl and N-formyl loline have also been recorded in seeds of *A. coenophialum*-infected tall fescue (Bush *et al.* 1982). Thus reports to date indicate that seed from endophyte-infected grasses invariably contain high concentrations of alkaloids. As mentioned in the preceding discussion, this is probably a defence mechanism which increases the likelihood that the seed and/or the resulting seedling will not succumb to effects of herbivory.

4.5 CONCLUSIONS

Although a small number of previous studies (reviewed in the discussion) have examined some aspects of *A. lolii* and alkaloid production in endophyte-infected perennial ryegrass, some of the results from the present experiment have further helped to define the relationship between *A. lolii* and alkaloid production. This is the first report of *A. lolii*, peramine and lolitrem B concentrations having been monitored in separate components from all parts of individual plants. It is also the first detailed report of peramine and lolitrem B concentrations in reproductive structures, and how they compare with peramine and lolitrem B concentrations in vegetative structures, whether on the same plant, or on a clonal plant maintained in a vegetative state. Since the experiment was conducted during the time of year in which plant reproductive development occurs, this is also the first report of how the reproductive state of the plant, and how plant management effects production of *A. lolii*, peramine and lolitrem B production. Results from this experiment have also been useful in elucidating the

potential significance that each part of the plant has on animal disorders such as ryegrass staggers and faecal contamination.

Several patterns concerning concentrations of *A. lolii*, peramine and lolitrem B in leaves from vegetative and reproductive tillers were apparent. *A. lolii* and lolitrem B concentrations were consistently higher in leaf sheaths (except for the 2nd leaf in the case of lolitrem B) compared to leaf blades, irrespective of whether the tillers or plants were vegetative or reproductive. It was concluded that the hydrophobic nature and therefore probable low translocatability of lolitrem B within the plant (suggested in previous studies) was the likely cause of the low concentrations of lolitrem B in the leaf blades. For the most part, peramine concentrations were more evenly distributed between leaf sheaths and leaf blades. However, there were two exceptions to this rule. Firstly, higher peramine concentrations were recorded in leaf sheaths compared to leaf blades of vegetative tillers on 1/10/91, indicating that the relative distribution of peramine between sheath and blade may vary according to season; and secondly, leaf sheaths from reproductive tillers also contained higher peramine concentrations than the blade. As suggested from previous studies, it was concluded that the relatively high concentrations of peramine in the leaf blades was due to the hydrophilic, and therefore probable highly translocatable nature of the compound. As well as differences between leaf sheaths and blades, leaf age was also often significant. *A. lolii* concentrations were nearly always low in the 1st (youngest) leaf of vegetative tillers compared to the older leaves. The results usually indicated that most of the *A. lolii* growth in the leaf had taken place by the time it had reached the 2nd leaf stage. However, there was no consistent effect of leaf age on *A. lolii* concentration in leaves 2 to 4. Peramine concentrations consistently decreased with increasing leaf age; this effect persisted into the senescent leaf material which was very low in peramine. This decrease was attributed either to the breakdown

of the peramine in the senescing tissues, or to the translocation of the peramine elsewhere (ie: the younger leaves). In contrast to peramine, lolitrem B concentrations rose as leaf age increased; this pattern was noticeable in leaves from both vegetative tillers (from 'vegetative' or 'reproductive' plants) and reproductive tillers. There were high concentrations of lolitrem B in the senescent leafy tissue. It was hypothesised that there was a lag between *A. lolii* growth and lolitrem B production.

In vegetative tillers, the crown and the small amount of stem tissue contained the highest concentrations of *A. lolii*.These tissues only contained moderate concentrations of peramine and lolitrem B. Low concentrations of *A. lolii*, lolitrem B and particularly peramine were recorded in the root component. Relatively speaking, lolitrem B concentrations were higher in the roots than peramine concentrations. It was suggested that the lolitrem B in the roots was synthesised there by the small amount of *A. lolii* present in the tissue. However, the generally low levels of *A. lolii*, peramine and lolitrem B in the root indicated that the endophyte probably confers little resistance against root feeding invertebrates. The regrowth component from 'vegetative' plants contained low concentrations of *A. lolii* and lolitrem B but relatively high levels of peramine. The total amount of peramine in the regrowth component which was removed from the 'vegetative' plants during the trimming regime was considerable. It was suggested that the regrowth component was probably the primary source of peramine acquisition by grazing stock. This may be a significant observation as peramine has been implicated as a cause of faecal contamination in livestock. Calculations from the 'vegetative' plants also showed that as the season progressed, the senescent leaf tissue contribution to the total pool of lolitrem B increased. By 14/1/92, nearly 50% of the total quantity of lolitrem B present in the 'vegetative' plants or removed during the trimming regime, was contained in the senescent leaf component. Although the conditions of the experiment were far removed from those in pastures, it was suggested that the senescent

leaf component could be more important in contributing to ryegrass staggers than the outer leaf sheaths even though the latter contained higher concentrations of lolitrem B.

Plant reproductive development had a considerable impact on *A. lolii*, peramine and lolitrem B concentrations. *A. lolii* concentrations were higher in most reproductive tissues compared to vegetative tissues. Also, mean *A. lolii* concentrations were higher in 'reproductive' compared to 'vegetative' plants; this was noticeable by 6/11/91. Thus, relative to 'vegetative' plants, *A. lolii* production was stimulated in 'reproductive' plants. The seed component in particular contained massive concentrations of *A. lolii*, although it did not account for all of the increase in *A. lolii*. Several factors, including plant hormone balance, plant nutrient status and the structural nature of the plant reproductive tissues, may have been responsible for the proliferation of *A. lolii* in the 'reproductive' clones, and in particular, in the reproductive structures. The stimulation of *A. lolii* growth in the 'reproductive' plants was almost certainly related to the fact that the endophyte is seed disseminated, and proliferation of *A. lolii* would increase the likelihood of successful transmission.

Peramine concentrations in the 'reproductive' plants did not increase in response to the increased *A. lolii* concentrations. On 6/11/91, mean peramine concentrations were in fact lower in 'reproductive' compared to 'vegetative' clones. By 14/1/92, mean peramine concentrations in 'reproductive' clones were almost as high as those in 'vegetative' clones. However, if the contribution of the seed, which contained extremely high concentrations of peramine, is subtracted, mean peramine concentrations were again substantially higher in 'vegetative' compared to 'reproductive' clones. Thus, with the exception of the seed component, peramine production appeared to be inhibited with reproductive development of the plant. This inhibition also appeared to apply to vegetative tillers from 'reproductive' plants as peramine concentrations in tillers from 'vegetative' plants sampled on 6/11/91 contained substantially higher concentrations of

peramine than vegetative tillers from 'reproductive' plants sampled at the same time. However, it is also possible that peramine produced in the plant reproductive structures could have been translocated to the seed. This would explain the low levels of peramine recorded in the reproductive structures and the high peramine concentrations in the seed. The high peramine concentrations in the seed have probably arisen to protect the seed and seedling from herbivore attack. Results from 'vegetative' and 'reproductive' plants over the whole period of the trial indicated that peramine was probably produced at a very early stage in the growth of *A. lolii* and that concentrations of peramine in each tissue may be determined by several factors. These could include the concentration of mycelium in the tissue or in other parts of the plant, the growth pattern of the mycelium and the length of time that the mycelium is in an enhanced peramine producing phase, the rate of dry matter production of the plant tissue and how much peramine has been translocated to, or away from, the tissue.

Lolitrem B concentrations reacted slightly differently than peramine to plant reproductive development. Initially (ie: on 6/11/91), there was a slight reduction in the mean lolitrem B concentration in 'reproductive' compared to 'vegetative' clones. Like peramine, this was also apparent in the vegetative tillers which contained higher concentrations of lolitrem B when derived from 'vegetative' compared to 'reproductive' clones. However, by 14/1/92 the mean lolitrem B concentration in the 'reproductive' clones was substantially higher than that of the 'vegetative' clones. The seed component contained extremely high concentrations of lolitrem B. Like peramine, this may have evolved as a defense against herbivore attack. Although the seed component accounted for much of the increase in the 'reproductive' clones, these results, as well as those for the vegetative structures and clones, indicated that there was probably a lag between the proliferation of *A. lolii* and the production of lolitrem B. Thus, the results from the present study suggest that peramine is produced early in the growth phase of *A. lolii*,

and lolitrem B is produced late in the growth phase of *A. lolii*. In this regard, it would be interesting to monitor peramine and lolitrem B concentrations during seed development to see whether peramine is produced before lolitrem B. The relatively high lolitrem B concentrations in the more mature reproductive structures, particularly the seed but also the stalk, indicated that plant reproductive development could be associated with an increased risk of ryegrass staggers in livestock. Lolitrem B concentrations in the different plant tissues are probably determined by a number of factors. These factors are likely to be similar to those discussed in relation to peramine.

A number of questions concerning the production and distribution of *A. lolii*, peramine and lolitrem B in perennial ryegrass plants and how this could be affected by the reproductive status of the grass have been clarified in this experiment. However, many of the results from the present study could be interpreted in several ways indicating that more research into the dynamics of *A. lolii* and alkaloid production is required before a full understanding of the ryegrass-*A. lolii* relationship is gained.

CHAPTER 5

EFFECT OF PLANT AGE AND ENDOPHYTE VIABILITY ON PERAMINE AND LOLITREM B CONCENTRATION IN PERENNIAL RYEGRASS SEEDLINGS.

5.1 INTRODUCTION

Little is known about the accumulation and distribution of lolitrem B and peramine in seedling ryegrass. Before the role of *A. lolii* in insect resistance was discovered, Kain *et al.* (1982a) and Goldson (1982) found that seedling resistance to Argentine stem weevil was a poor indicator of mature plant resistance. Stewart (1985) later explained these phenomena when he found that infected seed in which the endophyte was no longer viable, developed into seedlings resistant to Argentine stem weevil. It was assumed that this resistance was due to the translocation of peramine from the seed into the developing seedling. Although the duration of this seedling resistance has not been determined, Stewart (1985) showed that mature plants were susceptible to Argentine stem weevil. The relative importance of seed-stored peramine and that produced by the developing endophyte in *A. lolii*-infected seedlings, is unknown. This is also the case with lolitrem B.

The following study was therefore undertaken to examine the dynamics and significance of seed-stored peramine and lolitrem B and that produced by viable *A. lolii* in developing perennial ryegrass plants.

5.2 MATERIALS AND METHODS

In June 1991, 18 polystyrene seed trays (500mm x 300mm x 85mm) were filled to within 2cm of the top with Horotiu sandy loam soil. Six trays were sown with *A. lolii* -infected perennial ryegrass (cv. Yatsyn 1, 1991 harvest) seed. Seeds were sown at a depth of 0.5-1.0cm, and were spaced 2-3cm apart so that there were approximately 300 seeds per tray. Three of the six trays were marked so that they could be identified on each sampling occasion. Material from the three marked trays would be bulked at each harvest, constituting one replicate, while bulked material from the remaining three trays would constitute another replicate. This was necessary as the mass of each young seedling was such that one tray contained an insufficient number of plants for one replicate, particularly during the early harvests. A further six trays were similarly sown with *A. lolii* -infected (cv. Ellett, 1991 harvest) perennial ryegrass. The final six trays were sown with aged perennial ryegrass seed (cv. Ellett, 1986 harvest) which was infected with *A. lolii* but which was no longer viable.

Twenty mature perennial ryegrass plants grown from the three seedlines used, were tested for the presence of *A. lolii* histochemically. Leaf sheaths were placed individually into test tubes containing aniline blue/lactophenol stain (100ml lactic acid, 100ml glycerol, 500ml water, 100g phenol, 0.4g aniline blue). The test tubes were heated to 100^{0}C for ten minutes, after which the leaf sheaths were removed with tweezers, mounted on a microscope slide (with the outer side of the sheath against the slide) with a drop of glycerol and examined at a magnification of x200 under a compound microscope. Also, ten seeds from the aged cv. Ellett seedline were examined for *A. lolii* infection. Seeds were sectioned (20µm thickness) longitudinally using a freeze-microtome. With a pair of fine tweezers, the seed sections were carefully transferred to a microscope slide onto which a drop of aniline blue/lactophenol stain had

been placed. A coverslip was placed over the sections and the slide was gently heated for 5-10 seconds over a Bunsen flame. The stained seed sections were examined under the compound microscope as described above.

All of the trays were placed randomly in a constant environment room at 20^{0}C in a 16 hour/8 hour light/dark regime. During the experiment, the trays were reorganized randomly twice a week to negate any possible effects of environmental gradients within the room. Each tray was also top watered twice a week with measured volumes of water.

Subsamples of seedlings in each tray were destructively harvested 12, 24, 36, 48 and 124 days after sowing. On day 12, approximately half of the contents of the three marked trays of cv. Yatsyn 1 perennial ryegrass were removed and bulked. The plant material was separated from much of the soil by sieving. The seedlings were then washed under cold tap water to remove the remaining soil debris, counted and stored at 4^{0}C until dissection. This procedure was followed for the unmarked trays of cv. Yatsyn 1, and for the marked and unmarked trays of the 1991 and 1986 lines of cv. Ellett. Thus, for the harvest on day 12 (and all other subsequent harvests), there were six replicates in total, two of cv. Yatsyn 1 (1991), two of cv. Ellett (1991) and two of cv. Ellett (1986).

Using a scalpel, each seedling from a bulked and washed replicate, was dissected into four components. These were: seed residue (the remnants of the seed which was sown), roots (including crown), shoot from ground level to 3cm (lower shoot), and shoot above 3cm (upper shoot). Bulked material of each plant component was placed in a 25ml glass vial, dried at 60^{0}C for 40 hours, weighed and finely ground in an Ika model MFC culatti micro hammer mill. Dried ground samples were stored at -20^{0}C until peramine and lolitrem B analysis.

On each of the subsequent harvests half of the remaining soil and plant material left in each tray was removed and treated as described for the day 12 harvest. Thus approximately 50%, 25%, 12.5%, 6.25% and 6.25% of the seedlings in each tray were used in the 12, 24, 36, 48 and 124 day harvests respectively.

The plants remaining in the trays on day 70 were trimmed to 15cm. For material harvested on days 48 and 124, chemical analyses were not performed on the seed residue as insufficient quantities of this component were recoverable.

Lolitrem B and peramine analyses:

Grass samples were analyzed by HPLC for peramine using the method described in section 3.2.2, and lolitrem B by the method of Gallagher *et al.* (1985).

Statistical analyses and calculations:

Mean peramine and lolitrem B concentrations within each tissue were calculated for both seedlings grown from seed infected with viable endophyte, and for seedlings grown from seed infected with non-viable endophyte. Paired difference *t* tests were used to determine whether changes in mean alkaloid concentration within particular tissues with time were statistically significant in seedlings grown from seed infected with viable endophyte. Statistical tests were not performed on seedlings grown from seed infected with non-viable endophyte as the number of replicates used was insufficient. The quantity of alkaloid in each plant component was calculated by the multiplying the alkaloid concentration by the dry weight of the plant component. The quantity of alkaloid per seedling was then calculated by dividing the total alkaloid content of the bulked samples by the number of seedlings in the samples.

5.3 RESULTS

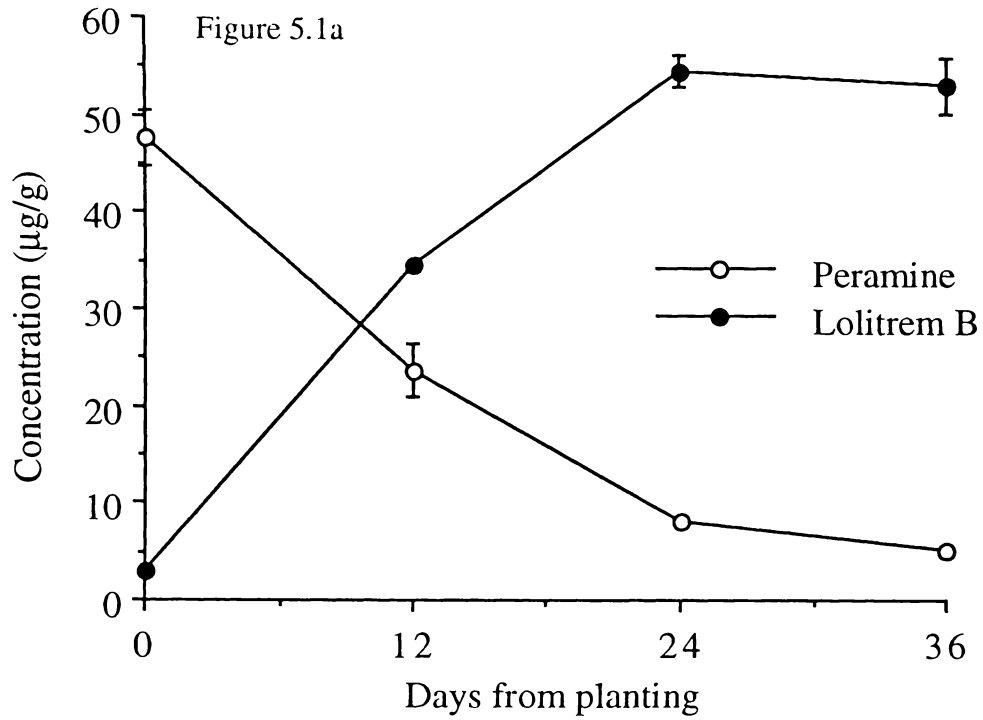
Seed infected with viable endophyte:

Microscopic examination of stained sheath material showed that 95% and 90% of the cv. Yatsyn 1 (1991) and cv. Ellett (1991) ryegrass plants respectively, were infected with *A. lolii*.

From an initial concentration of 47.6μg/g in the seed, the mean peramine concentration declined in the seed residue to 23.7μg/g by day 12 ($P<0.05$) and 5.1μg/g by day 36 ($P<0.01$) (Fig. 5.1a). Lolitrem B concentrations rose sharply from 2.92μg/g in the seed, to 34.52μg/g in the seed residue on day 12 ($P<0.001$) and 54.45μg/g on day 36 ($P<0.001$) (Fig. 5.1a).

Compared to levels within the lower and upper leaf categories, the mean peramine concentration in the root/crown component was low (Fig. 5.2a). The highest value was recorded in the day 12 sample, after which peramine levels decreased and fell to below the level of detection (0.5μg/g) by day 36 ($P<0.05$). Mean lolitrem B concentrations in the root/crown were also at their highest on the day 12 harvest (Fig. 5.3a). Concentrations fluctuated up to the day 36 harvest, then decreased to their lowest levels on the day 48 harvest ($P<0.05$). A slight increase was recorded in the harvest taken on day 124.

At days 12 and 24, the mean peramine concentration in the lower leaf was higher than in the upper leaf (Fig. 5.2a). From day 12, levels in the lower leaf declined sharply with the lowest value being recorded on day 48 ($P<0.01$). After day 36, levels in the upper leaf also fell, and were also at their lowest on day 48 ($P<0.05$). By day 124, peramine levels in the lower leaf were higher once again than those in the upper leaf. Also concentrations in the lower leaf were similar to day 12 levels while concentrations in the upper leaf were higher ($P<0.05$).



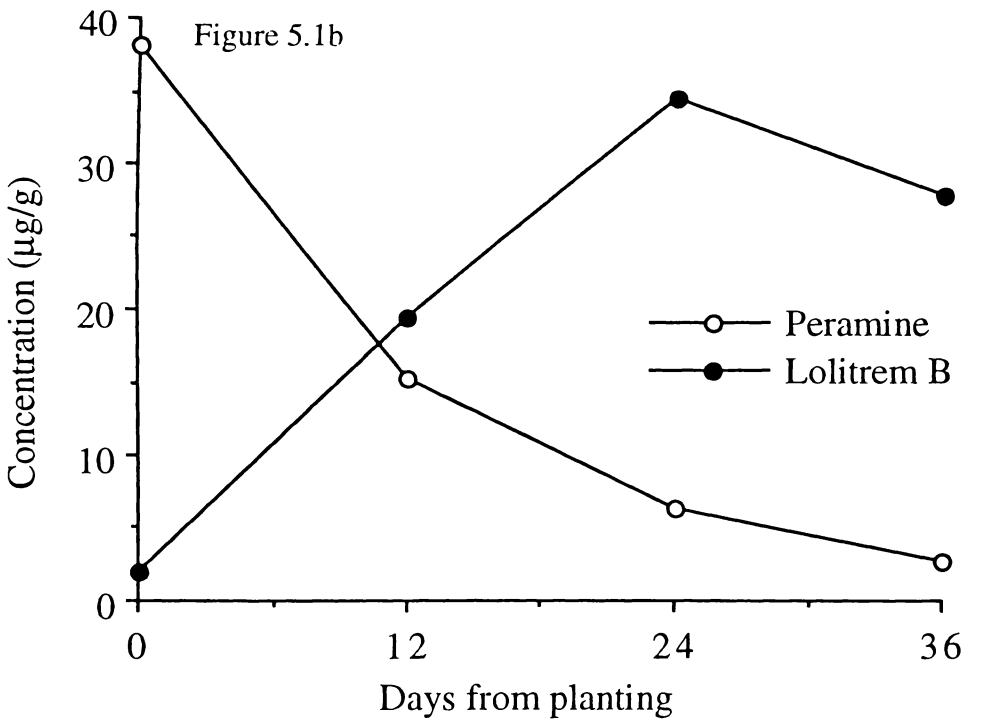
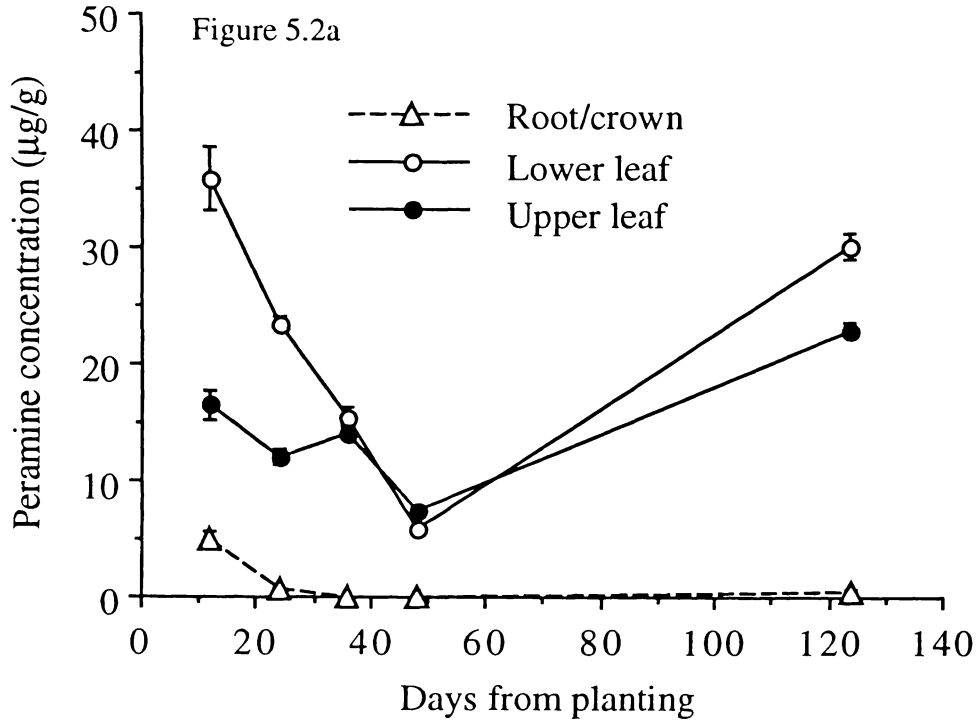


Figure 5.1 **Changes in mean peramine and lolitrem B concentrations in seed (day 0) and seed residue (day 12-36) components of (a) *A. lolii*-infected seedlings (error bars = SEM), and (b) seedlings grown from seed infected with non-viable *A. lolii*.**



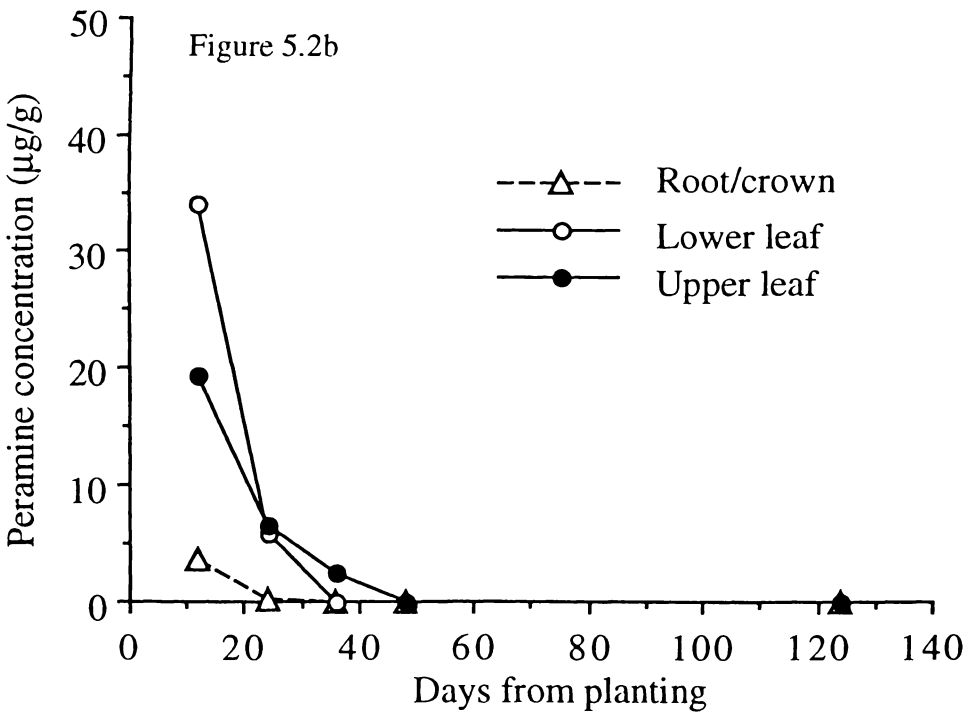


Figure 5.2 **Changes in mean peramine concentration in root/crown, lower leaf and upper leaf components of (a) *A. lolii*-infected seedlings (error bars = SEM), and (b) seedlings grown from seed infected with non-viable *A. lolii*.**

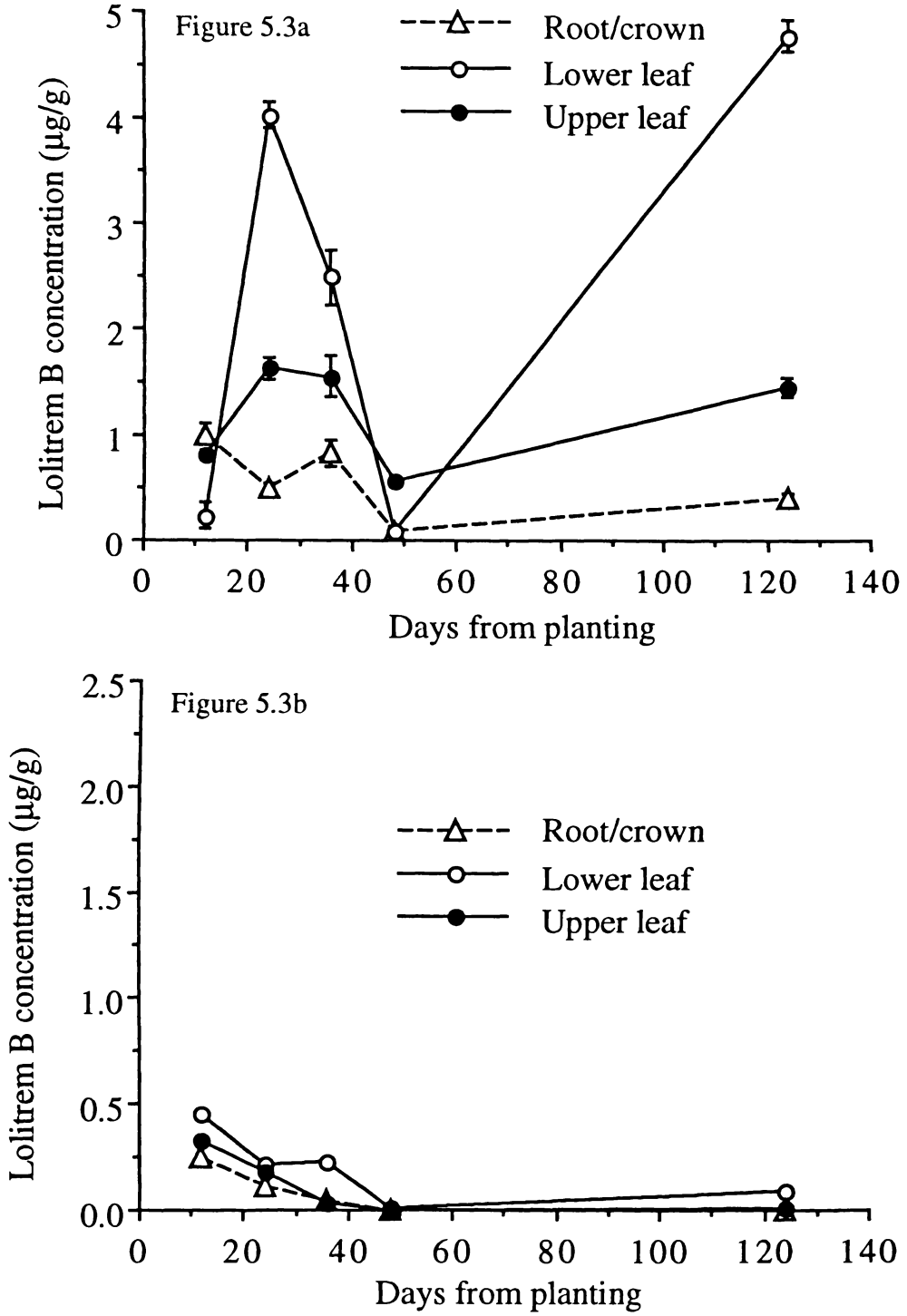


Figure 5.3 **Changes in mean lolitrem B concentration in root/crown, lower leaf and upper leaf components of (a) *A. lolii*-infected seedlings (error bars = SEM), and (b) seedlings grown from seed infected with non-viable *A. lolii*.**

Between days 12 and 24, mean lolitrem B concentrations increased in both lower ($P<0.01$) and upper ($P<0.05$) leaf material (Fig. 5.3a). At this point, lolitrem B concentrations in the lower leaf were over twice as high as those in the upper leaf. Lolitrem B concentrations in both tissues then decreased, and reached their lowest levels on the day 48 harvest ($P<0.05$). On the day 124 harvest, concentrations in the lower leaf increased to the highest point recorded ($P<0.001$). Upper leaf lolitrem B concentrations also rose on the day 124 harvest ($P<0.05$), but were not as high as those in the lower leaf.

Seedling dry matter production in the root/crown and lower leaf components followed a typical sigmoidal curve (Fig. 5.4a), with the highest rate of production recorded between days 36 and 48. Since all of the remaining plants were trimmed on day 70, no day 124 value for the upper leaf is included.

Seed infected with non-viable endophyte:

Of the seeds and mature plants examined for endophyte, 100% and 0% respectively contained *A. lolii.*

Changes in mean peramine and lolitrem B concentrations in the seed residue were similar to those observed in seed infected with viable endophyte (Fig. 5.1b).

Mean peramine concentration changes in the root/crown and shoot tissue were initially similar to those recorded for endophyte-infected seedlings (Fig. 5.2b). However, the concentrations declined to a non-detectable level by day 48 and did not exhibit an increase at day 124. Lolitrem B concentrations were lower in seedlings grown from seed containing non-viable endophyte than for seedlings grown from seed containing viable endophyte (Fig. 5.3b). Furthermore, the lolitrem B concentrations did not exhibit a rise and fall during early seedling growth, or show a rise as the seedlings matured.

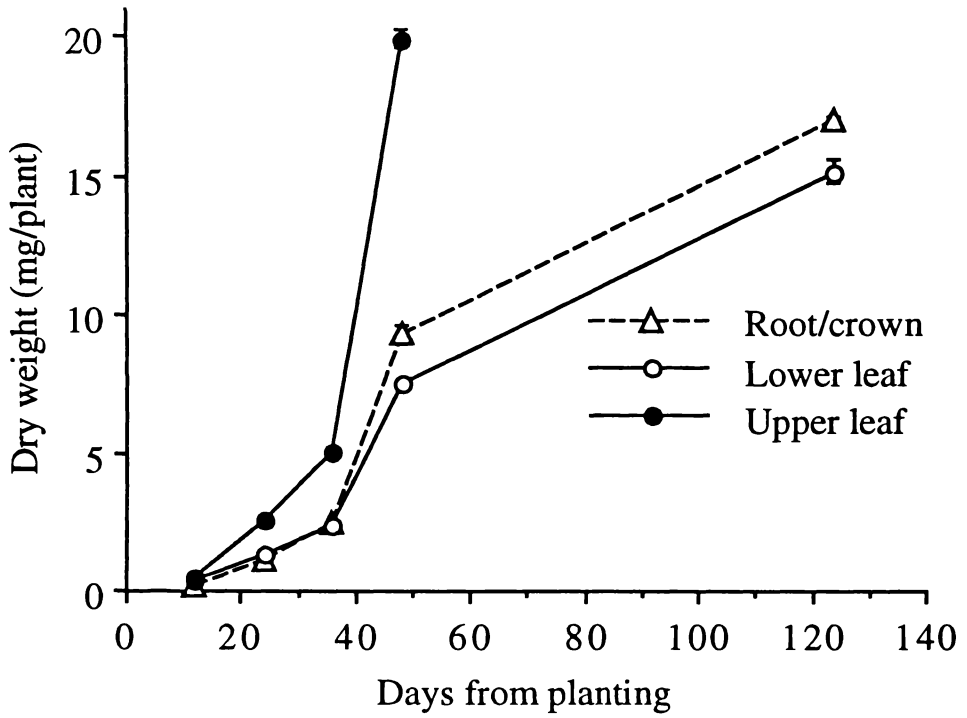


Figure 5.4a **Changes in dry weight production per plant in root/crown, lower leaf and upper leaf components of *A. lolii*-infected seedlings (error bars = SEM).**

Translocation of peramine and lolitrem B from the seed to the seedling:

On day 12, the total amount of peramine present in each seedling grown from a seed infected with non-viable endophyte contained 28.7% of the total amount of peramine initially present in each seed (Table 5.1). This percentage decreased with time such that by day 48, zero percent of the peramine which was initially in the seed, was in the seedling. On day 12, each seedling grown from a seed infected with viable endophyte contained 23.0% of the total amount of peramine present in the original seed (Table 5.1). Thereafter, the total amount of peramine present in the seedlings expressed as a percentage of the total amount of peramine in the original seed, increased sharply with time.

On day 12, each seedling grown from a seed infected with non-viable endophyte contained 9.2% of the total amount of lolitrem B present in the original seed (Table 5.1). The total amount of lolitrem B present in the seedlings expressed as a percentage of the total amount of lolitrem B in the original seed, increased to 17.1% by day 24, but decreased sharply to 2.3% by day 48. On day 12, the total amount of lolitrem B present in each seedling grown from a seed infected with viable endophyte contained 17.3% of the total amount of lolitrem B initially present in each seed (Table 5.1). This percentage increased sharply by day 24 and reached a maximum of 233.2% on day 36.

5.4 DISCUSSION

Although the results from the seed line infected with non-viable endophyte indicate that some lolitrem B was translocated from the seed into the developing seedling (Fig. 5.3b and Table 5.1), the large increase in lolitrem B concentration in the seed residue during seedling establishment and growth compared to initial levels in the seed

Table 5.1 **Mean quantities of peramine and lolitrem B per seedling after 12, 24, 36 and 48 days, expressed as percentages of the quantity of each alkaloid in the original seed.**

	Harvest day			
	12	24	36	48
Lolitrem B				
A.lolii-free seedlings (old seed)	9.2	17.1	17.3	2.3
A.lolii-infected seedlings	17.3	161.7	233.2	201.4
Peramine				
A.lolii-free seedlings (old seed)	28.1	26.1	13.7	0.0
A.lolii-infected seedlings	22.6	60.4	101.6	187.2

(Figs. 5.1a and 5.1b), indicates that much of the lolitrem B present in the seed was not translocated. This suggests that either lolitrem B is not easily translocatable, or that the bulk of the lolitrem B is stored in areas of the seed from which translocation into the developing seedling is difficult. Evidence indicates that both of these suggestions are possible. The hydrophobic nature and the dynamics of lolitrem B production in the plant (chapters 3 and 4, this thesis), suggest that lolitrem B is not an easily translocatable compound. However, it is also possible that lolitrem B is located principally in the testa of the seed. In mature seed, *A. lolii* hyphae are located within the embryo, between the scutellum and endosperm, and in and around the aleurone layer just beneath the testa (Philipson and Christey 1986). By day 24, seed residue samples were composed almost entirely of testa indicating that lolitrem B concentrations may be high in this region. Analysis of different parts of *A. lolii*-infected perennial ryegrass seeds for lolitrem B would indicate whether this was correct.

Contrary to what was observed with lolitrem B, peramine concentrations in the seed residue compared with initial seed levels, declined during seedling development (Figs. 5.1a and 5.1b), indicating that peramine was either translocated from the seed into the developing seedling, or metabolised. Results from Figure 5.2b show that in the seedline infected with non-viable *A. lolii*, some peramine was translocated from the seed into the developing seedling. It is likely that this was also the case in the seedlines infected with viable *A. lolii*. However, on the days in which the harvests were taken, translocated peramine only accounted for a maximum of 28.1% (on day 12) of the total amount of peramine initially present in the seed of the ryegrass line infected with non-viable endophyte (Table 5.1) (it is possible that the time at which the largest amount of seed-stored peramine was present in the developing seedling, occurred earlier than day 12). The amount of peramine remaining in the seed residue of the aged seed line on day 12 brought the total amount of peramine accounted for to only 41.9% of that initially

present in the seed, indicating that much of the peramine had already been metabolised.

The fact that much of the lolitrem B was retained by the seed instead of being translocated into the developing seedling, indicates that this alkaloid may be more useful in protecting the seed, rather than the seedling. The depletion of peramine from the seed and seed residue indicates that the opposite may be true of peramine.

The relatively low concentrations of peramine and lolitrem B recorded in the root/crown component of *A. lolii*-infected seedlings (Figs. 5.2a and 5.3a respectively) is in close agreement with the low levels recorded in chapter 4 of this thesis. The results from seedlings grown from seed containing non-viable endophyte (Fig. 5.2b and 5.3b) show that both peramine and lolitrem B can be translocated from the seed to the root/crown tissues of developing seedlings. Translocation from the seed was the only means by which the two alkaloids could have occurred in the seedling, as the endophyte, being non-viable, could not have grown into the seedling and produced the compounds there. The similarity of the curves for peramine in the root/crown component in Figures 5.2a and 5.2b indicate that much of the peramine present in the root/crown component of *A. lolii*-infected seedlings on day 12 (Fig. 5.2a) was probably derived from that stored in the seed. As the *A. lolii*-infected plants matured, there was only a hint of a recovery in the peramine concentration of the root/crown component (Fig. 5.2a). Results from Figures 5.3a and 5.3b indicate that, like peramine, a large proportion of the lolitrem B present in the rootcrown component of 12 to 36 day old *A. lolii*-infected seedlings may have been derived from that stored in the seed. However, unlike peramine, there was an appreciable increase in the lolitrem B concentration in the root/crown component of 124 day old *A. lolii*-infected plants. These results indicate that compared to what is observed with peramine, roots and crowns of mature *A. lolii*-infected plants contain relatively high concentrations of lolitrem B.

The low concentrations of peramine and lolitrem B in the root/crown component

of *A. lolii*-infected plants may be to the advantage of root feeding insects, and therefore to the disadvantage of the ryegrass. However, the "artificially" high (ie due to translocation from the seed) levels of peramine and lolitrem B recorded in the root/crown tissue on day 12 and on days 12, 24 and 36 respectively, may lead to enhanced resistance against soil-borne pests so benefiting the seedling at this crucial phase of its existence.

Peramine and lolitrem B can be translocated from the seed to the shoot tissues of the developing seedling (Figs. 5.2b and 5.3b), indicating (particularly in the case of peramine) that much of the lolitrem B and peramine recorded in the early harvests in *A. lolii*-infected seedlings (Figs. 5.2a and 5.3a) was derived from that stored in the seed. The decline in peramine and lolitrem B content in the shoot (and root) components of seedlings grown from seeds infected with both viable and non-viable *A. lolii* after day 12, suggests that after the initial translocation from the seed, there was a period of alkaloid metabolism and/or dilution (ie due to plant dry matter production), the effects of which were greatest on the day 48 harvest. In the case of *A. lolii*-infected seedlings, the increases in peramine and lolitrem B concentration recorded in the shoot components on day 124, indicate that by this time, alkaloid production by the endophyte in the plant was the dominant process, and that the metabolism or dilution of both translocated and newly synthesised alkaloid was secondary.

The vertical distribution of peramine in mature ryegrass plants appears to be dependent upon several factors making consistent patterns difficult to detect. Some studies have shown that peramine concentrations in upper and lower parts of the vegetative leaf are similar (Keogh and Tapper 1993, chapter 4 this thesis), while others have indicated that peramine concentrations are higher in the upper part of the leaf compared to the lower part of the leaf (Fannin *et al.* 1990, chapter 3 this thesis). Results from Tapper *et al.* (1989) indicated that the opposite was true, viz the lower part of the

plant contained higher concentrations of peramine compared to the upper part of the plant. It is difficult to compare results from all of these studies with each other and with those of the present study, as so many factors are different (ie how the plants were dissected, the conditions under which they were grown). In the present study, the pattern of alkaloid distribution within the different components of *A. lolii*-infected seedlings changed considerably with time. Initially (day 12), peramine concentrations in the lower leaf component were approximately double those in the upper leaf (Fig. 5.2a). This distribution probably largely reflects the ease with which peramine was translocated from the seed into the different parts of the shoot as a similar pattern was observed in seedlings grown from seed containing non-viable endophyte (Fig. 5.2b). In the more mature *A. lolii*-infected plant, peramine was distributed more evenly between upper and lower leaf portions (Fig. 5.2a).

A number of studies have shown that lolitrem B concentrations are consistently higher in basal parts (ie leaf sheath) of vegetative tillers compared to upper portions such as the leaf blade (Gallagher *et al.* 1982a, 1985, 1987, di Menna *et al.* 1992, Davies *et al.* 1993 and Keogh and Tapper 1993). Except for the day 12 and day 48 harvests, lolitrem B concentrations in the lower leaf of *A. lolii*-infected seedlings were considerably higher than those in the upper leaf (Fig. 5.3a). The low lolitrem B concentration in the lower leaf of *A. lolii*-infected seedlings on day 12 is a little mystifying as lolitrem B concentrations on day 12 in seedlings grown from seed infected with non-viable *A. lolii* were higher in the lower leaf than they were in the upper leaf (Fig. 5.3b).

Lolitrem B concentrations in the root/crown, lower leaf and upper leaf components were at their lowest level on day 48. This result differs from that of Gallagher *et al.* (1987) who found that lolitrem B concentrations had reached a high level plateau at this time. Although practical aspects of the two trials differ making direct comparisons difficult, the differences between the two sets of results are striking and

unexplainable. In the present study, seedling dry matter production followed a typical sigmoid curve (Fig. 5.4a) and the results show that the lowest concentrations of both peramine and lolitrem B in *A. lolii*-infected seedlings corresponded with the highest rate of dry matter production by the grass (Figs. 5.2a, 5.3a and 5.4a) adding weight to the plant dilution effect discussed previously.

From the limited number of harvests taken in this study, seedlings were probably at their most vulnerable to Argentine stem weevil attack around day 48, when peramine concentrations were at the lowest recorded levels in all seedling components. Using artificial diets, Dymock *et al.* (1988), Popay *et al.* (1990) and Rowan *et al.* (1990a) found that a peramine concentration of less than 1.0μg/g was sufficient to deter adult Argentine stem weevil from feeding, although Popay *et al.* (1990) found that 10μg/g was required in a no-choice test. Peramine levels recorded in seedlings in the present study were therefore probably sufficient to deter adult feeding throughout the seedling growth phase. It is interesting to speculate on how long the Argentine stem weevil resistance observed by Stewart (1985) in seedlings grown from seed infected with non-viable endophyte could last. Using the results of the current study, the Argentine stem weevil resistance observed by Stewart (1985) may be expected to last for up to 36 days.

The alkaloid budget calculated on a per seedling basis indicated that a larger proportion of peramine was translocated, and more rapidly, from the seed to the developing seedling, than lolitrem B (Table 5.1). It is possible that due to the sampling regime, the peak of the translocation waves of peramine and lolitrem B could have been missed. However, the above hypothesis is supported by the results for the seed residue component which was high in lolitrem B but low in peramine (Fig. 5.1a). Being hydrophilic, peramine is probably more easily translocatable within the plant than lolitrem B which is a hydrophobic compound.

Comparisons of alkaloid budgets for the seedlings grown from endophyte-infected seed and seed infected with non-viable endophyte, indicate that up to day 12, no extra peramine was produced by *A. lolii*-infected plants (Table 5.1). The initial burst of peramine from the seed is therefore probably crucial to the ability of the young seedling to resist attack from insect pests such as Argentine stem weevil. Lolitrem B production by endophyte-infected seedlings appears to begin within the first 12 days after sowing, as concentrations were approximately twice those expected if the lolitrem B had been derived solely from that translocated from the seed (Table 5.1). Assuming that a high level of lolitrem B confers an advantage to the plant, the early synthesis of lolitrem B in the seedling may be necessary to counter the poor translocation of this alkaloid from the seed.

CHAPTER 6

THE EFFECTS OF *ACREMONIUM LOLII* INFECTION, *A. LOLII*-DERIVED ALKALOIDS AND *ACREMONIUM* ENDOPHYTE STRAIN ON FEEDING BY ADULT BLACK BEETLE, *HETERONYCHUS ARATOR* (COLEOPTERA: SCARABAEIDAE).

6.1 INTRODUCTION

In New Zealand, perennial ryegrass infected with *A. lolii* has been shown to be resistant to Argentine stem weevil (*Listronotus bonariensis* Kuschel) (Prestidge *et al.* 1982), cutworm (*Graphania mutans* Walker) (Dymock *et al.* 1988) and pasture mealy bug (*Balanococcus poae* Maskell) (Pearson 1988). With an understanding of the mechanisms involved in the resistance against each pest, endophytes possessing the most desirable characteristics for each pasture pest or group of pests, and therefore for each region of New Zealand, could be selected. The aim of chapter 6 was to investigate the effect of *A. lolii* in perennial ryegrass on black beetle (*Heteronychus arator* F.) and to identify the mechanism of the resistance. Although there were no published reports, it was already suspected that *A. lolii* conferred resistance to perennial ryegrass against black beetle (R. Watson pers. comm.). Before discussing the experiments performed in this investigation, a brief description of the biology of the black beetle in New Zealand will be given.

Accidentally introduced from South Africa in ca. 1937, black beetle is a sporadic but significant pest of northern New Zealand pastures. Black beetle is found only in the warmer northern areas of the North Island of New Zealand, its southern limit corresponding closely with the isotherm of 12.8^{0}C mean annual surface temperature

(Watson 1979). Mature adult black beetle are glossy black, females being approximately 1.4cm in length while males are usually slightly smaller. The sexes are easily distinguished by the shape of the fore tarsi. In the female, the segments are long and narrow, while in the male they are short and broad (Cumpston 1940, Todd 1959).

In New Zealand, black beetle is univoltine. Oviposition occurs during spring and early summer. Eggs (whitish in colour and approximately 2mm in length) are deposited singly in the soil from September to December and larvae emerge from November to January. Larvae pass through three instars and pupate from February to April. Adults emerge from March to May, at which time dispersal flights may occur. Dispersal flights may take place on warm autumn evenings providing temperatures exceed the threshold of 17-18^{0}C (Watson 1979). Autumn flights are relatively uncommon in New Zealand as the threshold temperature is only rarely exceeded. During the winter months, adults become less active. Feeding resumes in the spring when temperatures increase, at which time mating occurs. Although they may occur, spring flights are very uncommon.

The dependence on relatively high temperatures to complete their life cycle within one year is the factor most responsible for the inability of black beetle to colonise areas further south. Even in its current distribution, cooler than normal temperatures may delay adult oviposition and reduce larval development rates. This occasionally results in overwintering "out of phase" larvae which usually fail to survive through to the following spring.

Both the larval and adult stages of black beetle are soil dwelling and both are potentially damaging to plants. In the pasture situation however, the larval stage (the third instar in particular) is responsible for most of the damage (Watson and Webber 1976).

First instar black beetle larvae feed predominantly on organic matter. However, as development progresses, the larvae switch to feeding on the roots of pasture plants.

During heavy infestations (30-50 black beetle larvae/m^2 in perennial ryegrass and 80-100 larvae/m^2 in paspalum (*Paspalum dilatatum* Poir.) (Watson and Wrenn 1980), the effect of this root pruning may be such that the turf may be rolled back as is seen during severe grass grub (*Costelytra zealandica*) outbreaks. All grass species tested, with the exception of *Phalaris aquatica* L., are favourable to third instar black beetle larvae, while legumes such as white clover (*Trifolium repens*) are unfavourable (King 1979a).

Although not as damaging as the larval stage, adult black beetle play an important role in determining subsequent levels of larval damage. Graminaceous plants (with the exception of *P. aquatica*) rather than legumes are favoured by black beetle adults. In particular, adult black beetle performance (ie fat accumulation, fecundity and longevity) was markedly better on paspalum compared to ryegrass (King 1979b). Watson and Wrenn (1980) and King *et al.* (1981a) also found that perennial ryegrass pastures were a poor host for black beetle adults over the autumn/winter period as mortality and/or dispersal rates were high. "Winter disappearance" in paspalum pastures however was relatively slight resulting in higher larval populations the following summer (Watson and Wrenn 1980, King *et al.* 1981a). Watson and Wrenn (1980) also reported that black beetle numbers were generally positively correlated to paspalum levels and that the occurrence of damaging numbers of larvae in ryegrass based pastures was dependant upon adult immigration in spring from surrounding areas of paspalum. The abundance of paspalum appears therefore to be an important factor in determining damage levels in both paspalum and surrounding ryegrass pastures. Perennial ryegrass is less tolerant to larval black beetle attack than paspalum as fewer larvae are required for the damage threshold to be reached (Watson and Marsden 1981).

Until 1967, a degree of black beetle control was achieved with organochlorine insecticides such as dieldrin (Todd 1967). However, due to residue problems, organochlorine insecticides were withdrawn and replaced by the more transient

organophosphorus and carbamate based compounds, of which isofenphos (Watson *et al.* 1981), isazophos and fensulfothion (Blank *et al.* 1982) were effective against black beetle. Economically and ecologically, insecticide use has become prohibitive. Management control measures in black beetle infested areas include cropping, pasture renovation to incorporate resistant species such as white clover, lucerne (*Medicago sativa* L.) or kikuyu (*Pennisetum clandestinum* Hochst.), and lax summer grazing to decrease pulling. Attempts to introduce predators and parasites to control black beetle and pasture scarabs in general, have been unsuccessful (Cameron *et al.* 1979, Valentine 1979). Pathogens found to be naturally associated with black beetle include a small isometric RNA virus, a rickettsia, a neoplectanid nematode and a coccidian protozoan parasite (*Adelina* sp.) (Longworth and Archibald 1975). The long term effect of these pathogens on black beetle populations is uncertain, although King and Mercer (1979) concluded that *Adelina* sp. may have contributed to population declines.

As there are no reliable biological control mechanisms to manage black beetle in New Zealand pastures, the following series of experiments were undertaken to determine whether *A. lolii* has potential as a biocontrol agent against black beetle attack on perennial ryegrass.

6.2 MATERIALS AND METHODS

6.2.1 The effect of *Acremonium lolii* infection on adult black beetle feeding (experiment 1).

Bioassays testing the effect of *A. lolii* infection in perennial ryegrass on adult black beetle feeding were performed on two separate occasions, the first in the spring of 1991, the second in the autumn of 1992. Two experiments were considered necessary for the following reasons: 1). To test whether there were any differences between *A.*

lolii-infected ryegrass cultivars 2). To determine whether time of year and therefore black beetle age had any bearing on the effect of *A. lolii* on adult black beetle feeding 3). It was felt that the experimental technique could be improved so that plant assessments could be made on a regular basis making the data easier to interpret and analyze. Unless specified, the following methodology applies to both of the bioassays.

Adult black beetle collection and maintenance:

Adult black beetle were collected using pitfall traps between April and June 1991 for the spring 1991 bioassay, and between March and April 1992 for the autumn 1992 bioassay, from pastures at the Ruakura Agricultural Research Centre, Hamilton. Each pitfall trap consisted of a honey pot (90mm x 80mm diameter) buried such that the lip of the pot was at or just below ground level. Two small holes had been drilled into the base of each pot to allow them to drain. Two hundred such pitfall traps, spaced approximately 5 yards apart, were used to collect the black beetle. The traps were checked weekly. Black beetle were sexed according to the criteria outlined earlier and maintained in groups (sexes separate) of approximately 20-30 in liverpails (170mm x 170mm x 190mm) of sieved Horotiu sandy loam. Black beetle were fed on slices of fresh carrot which were changed weekly, and were not starved prior to experimentation.

Preparation of grass plants:

Mature perennial ryegrass plants were used in both bioassays, cv. Ellett in the 1991 trial, and cv. Yatsyn 1 in the autumn 1992 trial. The endophyte status of all plants used was assessed using the sheath staining method described in appendix 1.

Spring 1991 trials: Choice and no choice feeding tests with respect to *A. lolii* infection were performed.

Choice test: In June 1991, ten liverpails (170mm x 170mm x 190mm) were filled to within 50mm of the top with sieved Horotiu sandy loam soil. Four small holes were drilled into the base of each pail to aid in soil drainage. One *A. lolii*-infected and one *A.*

lolii-free cv. Ellett perennial ryegrass plant, each with between 2-10 tillers, were planted into each pail. The plants were maintained in a greenhouse over the winter, and trimmed and watered as required.

No choice test: In June 1991, 16 liverpails were filled with sieved Horotiu sandy loam soil as in the choice experiment. Half of the liverpails were planted with two *A. lolii*-infected cv. Ellett perennial ryegrass plants, and the remainder with two *A. lolii*-free cv. Ellett plants, each with between 2-13 tillers. Plants were maintained as described in the choice test.

As a source of extra plants, two seedling trays of mature *A. lolii*-infected and two trays of mature *A. lolii*-free perennial ryegrass plants were also maintained in the glasshouse.

Autumn 1992 trial: A no choice test was the only bioassay performed at this time. Four days before the trial commenced, 20 three inch diameter soil cores were taken from a local pasture containing mature *A. lolii*-infected cv. Yatsyn 1 perennial ryegrass, from which sufficient numbers of complete plants were recovered. Each plant was confirmed as being infected with *A. lolii* by microscopic examination of stained leaf sheath material. Two *A. lolii*-infected cv. Yatsyn 1 perennial ryegrass plants were planted into each of ten liverpails as described in the previous no choice test. Two *A. lolii*-free cv. Yatsyn 1 plants (which had been maintained outdoors in seedling trays of Horotiu sandy loam soil) were similarly planted into a further ten liverpails. Each plant, whose endophyte-free status had been visually confirmed, consisted of between 2-9 tillers.

Adult black beetle feeding bioassays:

Spring 1991 trial: Choice test: In September 1991, three male black beetle were introduced into half of the liverpails and three females were introduced into each of the remainder. Each liverpail was covered with nylon mesh supported by a wire frame to prevent the black beetle from escaping, while allowing plant growth. The nylon mesh

was secured to the upper rim of the liverpail with a rubber band. The trial lasted for 22 days and water was added to each liverpail as required. The number of healthy, black beetle-damaged, and black beetle-killed tillers on each plant was assessed on days 0, 7, 11, 15, 19 and 22. The criteria for deciding whether a tiller was healthy, black beetle-damaged, or black beetle-killed, were as follows: tillers with shredding so extensive at their base that they had collapsed, and in the view of two observers, were moribund, or those that had become detached from the crown completely, were classed as black beetle-killed. Tillers with signs of black beetle feeding activity at their bases (ie: slight shredding), but which were upright and healthy and not considered moribund, were classed as black beetle-damaged. The actual level of damage in tillers classed in this category was highly variable. Some tillers were extensively shredded at their base but not considered moribund, while others sustained only slight damage. A healthy tiller was classed as such if no sign of black beetle feeding activity was detected. Tillers which showed signs of "test bites" (ie where the outer most leaf sheath had been disturbed but not damaged by black beetle) were classed in the healthy category.

No choice test: In September 1991, two male and two female adult black beetle were introduced into each of the 16 liverpails which were then covered with mesh as described for the choice test. The number of healthy, black beetle-damaged and black beetle-killed tillers on each plant was recorded every 4-8 days using the criteria described in the choice test. The experiment was terminated 27 days after introducing the black beetle to the liverpails. Black beetle survival in each liverpail was recorded after the final assessment.

Autumn 1992 trial: No choice test: On April 29th 1992, two male and two female adult black beetle were introduced into each of the 20 liverpails and were confined using the nylon mesh. The experiment was terminated on 26th May 1992 as this coincided with the approximate time of year that adult black beetle enter diapause and largely cease

to feed in pastures. The number of black beetle-damaged or killed tillers were counted, removed and replaced with healthy tillers every nine days during the trial. Three such assessments were made after which all liverpails were dismantled and assessed for black beetle mortality.

Statistical analyses:

Data from the spring 1991 choice and no choice trials were analyzed using paired difference *t* tests. A pooled standard deviation was calculated for the spring 1991 no choice test. Data from the three day 9 assessments carried out in autumn 1992, were combined, rank transformed and subjected to analysis of variance.

6.2.2 Development and use of an artificial diet to test the effect of four *A. lolii*-associated alkaloids on feeding by adult black beetle (experiment 2).

Preparation of alkaloid solutions:

It was considered important to have only one control diet which could be used for all of the treatments. This was firstly to reduce the amount of material and time needed to make up the diets, and secondly to ensure that it would be possible to directly compare results for all treatments.

Each alkaloid was dissolved in ethanol as this was the least toxic universal solvent available. Lolitrem B, paxilline (both obtained from Dr C. Miles, AgResearch, Ruakura), ergotamine tartrate (Sigma) and peramine hydrochloride (obtained from D. Rowan, Hort + Research, Palmerston North) were each dissolved in absolute ethanol (Analar) to make final concentrations of 0.25, 0.25, 0.5 and 0.7mg/ml respectively. Solutions were prepared on the same day that the diets were made up so as to minimise deterioration of the compounds. The alkaloid solutions were stored at 4^0C until use.

Diet preparation:

For each diet, 400g of washed carrot and 200ml of distilled water, plus 85mg of penicillin-G (Sigma) and 85mg of streptomycin sulphate (Serva), were blended to a fine paste using a Waring commercial blendor. 5ml of absolute ethanol (Analar) for control diets, or 5ml of absolute ethanol containing dissolved alkaloid at the appropriate concentration for treatment diets, was then added dropwise to the carrot mix with the blendor running to ensure maximum uniformity in the distribution of the ethanol or ethanol/alkaloid mix.

20g of agar was added to 250ml of distilled water in a 1500ml glass beaker and stirred with a glass rod. The contents of the beaker were microwaved for two minutes, stirred, microwaved for 60 seconds, stirred again, and microwaved once more for 30 seconds. 300mg of p-hydroxy benzoic acid (Sigma) and 250mg of sorbic acid (BDH) (mould inhibitors) were added to the hot agar which was thoroughly stirred. While the agar was left to cool slightly, the carrot mix was poured into a metal bowl maintained at 50^{0}C in a constant temperature water bath. Once the agar had cooled to approximately 60^{0}C, it was added to the carrot mix in the metal bowl. The diet was thoroughly mixed, poured into two plastic trays (230mm x 150mm x 50mm) and left to set at room temperature. Each tray of diet was covered and stored at 5^{0}C until use.

Alkaloid treatments and calculations:

Alkaloid concentrations used in this experiment were based upon the range (from low to high) of concentrations commonly found in *A. lolii* -infected perennial ryegrass plants (Gallagher *et al.* 1987, Rowan and Shaw 1987, Rowan *et al.* 1990b, Davies *et al.* 1993, Keogh and Tapper 1993, Ball this thesis).

To calculate the amount of alkaloid required for each treatment, the percent dry weight composition of the diet was determined. Eight discs of newly made diet were

weighed, dried at 90^{0}C for 48 hours, and reweighed. The mean percent dry matter composition of the diet was calculated at 6.57%. From the ingredients, the wet weight of each diet was 870.72g. Since 6.57% of this was dry matter, the amount of dry matter in each diet was 57.2g. After the volume of stock solution required for each treatment diet had been determined, the final volume of ethanol was brought up to 5ml to standardize all diets.

Lolitrem B and paxilline diets: 1ml of a 0.25mg/ml stock solution added to the diet was equivalent to a concentration of 250µg/57.2g or 4.37µg/g. The quantity of stock solution required to make each treatment diet was then calculated.

Peramine diets: 1ml of a 0.7mg/ml stock solution added to the diet was equivalent to a concentration of 700µg/57.2g or 12.24µg/g. Quantities of stock solution used to make each treatment diet were then calculated.

Ergotamine diets: 1ml of a 0.5mg/ml stock solution of ergotamine tartrate added to the diet was equivalent to a 500µg/57.2g or 8.74µg/g diet. The amount of stock solution used to make each treatment diet was then calculated.

A diet containing 5.0µg/g of lolitrem B and paxilline, 10.0µg/g of peramine, and 1.0µg/g of ergotamine was also made up to test for any synergistic effects. These concentrations were used as they closely reflect the levels found in *A. lolii* -infected ryegrass. The alkaloid stock solutions used in this diet were the same as those used for the previous diets.

Adult black beetle choice feeding tests:

In November 1992, numbered honey pots (90mm x 80mm diameter) were filled to within 2cm of the top with moist sieved Horotiu sandy loam. One adult black beetle was then introduced to each honey pot (black beetle were collected as described in section 6.2.1). After approximately 15 minutes, all pots were checked to ensure that the black beetle had burrowed into the soil. Insects which had failed to burrow were

removed and replaced with another of the same sex. Fourteen black beetle, seven male and seven female, were used in each treatment. Once the black beetle had burrowed into the soil, a 3cm diameter plug of control diet and similar sized plug of alkaloid treated diet, were placed on the soil surface. These were lightly pressed into the soil so that the black beetle could feed on them from underneath. The pots were covered with the lid or base of petri dish and arranged randomly on a laboratory bench top (18-24^{0}C).

After 96 hours, the extent of adult black beetle feeding was assessed by giving each disc a feeding score. The percentage of each disc eaten was estimated visually and given a score between 1-10 corresponding to 10-100% feeding. A score of zero was given when no feeding was visible. Black beetle mortality was also assessed at the end of the trial.

Statistical analyses:

Feeding scores from control and alkaloid treated discs were compared using a paired difference *t* test.

6.2.3 Effect of selected isolates of *Acremonium* endophytes on adult black beetle feeding (experiment 3).

Source of plant material:

Bioassays individually testing the effects of five different *Acremonium* endophyte isolates (Tf13, Tf16, Lp5, Lp14 and Lp17) in Nui perennial ryegrass on adult black beetle feeding were performed between 17/11/93 and 12/12/93. Limited alkaloid analyses (lolitrem B, peramine, ergovaline, total ergot alkaloid and loline alkaloid) using HPLC and ELISA techniques had shown that the chemical profile of Nui ryegrass plants infected with these *Acremonium* endophytes was strain dependant (Table 6.1). Isolates Tf13 and Tf16 were derived from tall fescue, the endophyte of which is usually referred to as *A. coenophialum*. However, Tf13 and Tf16 are not classified as

Table 6.1 ***Acremonium*** **endophyte strains tested for resistance against adult black beetle, and their alkaloid profiles when in associations with "Nui" perennial ryegrass plants.**

Strain	Taxon[1]	lolitrem B	peramine	ergopeptines	ergovaline	lolines
Lp5	*A. lolii*	+	+	+	+	-
Lp14	*A. lolii*	-	-	-(?)	-	-
Lp17	*A. lolii*	-	-	-(?)	-	-
Tf13	FaTG-2[2]	-	-	+	+	-
Tf16	FaTG-3[3]	-	+	+(?)	-	-(?)

[1] Christensen *et al.* 1993.
[2] *Acremonium* taxon, "*Festuca arundinacea* taxonomic grouping 2"
[3] *Acremonium* taxon, "*Festuca arundinacea* taxonomic grouping 3"
(?) Not consistently detected or present only in trace amounts.

A. coenophialum, but are recognised as belonging to the *F. arundinacea* taxonomic groupings (FaTG) two and three respectively (Christensen *et al.* 1993). Endophyte strains Lp5, Lp14 and Lp17 were derived from perennial ryegrass and are all classified as *A. lolii*. The plants used in the study were obtained from M. Christensen of AgResearch Grasslands, Palmerston North. Cultured isolates of each of the *Acremonium* strains had been inoculated by M Christensen into Nui perennial ryegrass seedlings.

Preparation of grass plants:

Mature perennial ryegrass plants, the endophyte status of which were verified prior to each trial, were used in all feeding tests. All feeding tests were of the choice type and were performed in the laboratory. Four tillers from each of the two test plants to be compared were planted into a honey pot (90mm x 80mm diameter) which was filled to within 2cm of the top with sieved Horotiu sandy loam soil.

Adult black beetle choice feeding bioassays:

Adult black beetle were collected and maintained as described in section 6.2.1. Six choice feeding tests comparing different plant combinations were performed.

Test 1: Ten Nui perennial ryegrass plants infected (five genotypes) infected with strain Lp5 were compared with ten endophyte-free Nui plants (ten genotypes).

Test 2: Seven Nui plants (seven genotypes) infected with strain Tf13 were compared with seven endophyte-free Nui plants (seven genotypes).

Test 3: Ten Nui plants (five genotypes) infected with strain Lp5 were compared with ten Nui plants (five genotypes) infected with strain Tf13.

Test 4: Nine Nui plants (three genotypes) infected with strain Tf16 were compared with nine endophyte-free Nui plants (six genotypes).

Test 5: Eight Nui plants (four genotypes) infected with strain Lp14 were compared with eight endophyte-free Nui plants (eight genotypes).

Test 6: Eight Nui plants (three genotypes) infected with strain Lp17 were compared with eight endophyte-free Nui plants (six genotypes).

After planting, each honey pot was seeded with one adult black beetle. Where possible, equal numbers of male and female adult black beetle were used for each test. Each honey pot was covered with an inverted clear plastic container with ventilation holes to prevent the black beetle from escaping. The number of healthy, black beetle-damaged, and black beetle-killed tillers on each plant was assessed every day for ten days. Tillers were scored according to the black beetle damage sustained. Tillers which were killed were given a score of 2. Tillers which were damaged were given a score of 1. Healthy tillers were given a score of zero. Daily feeding scores for each plant were calculated by adding together the feeding scores for the four tillers of each plant. Thus the highest feeding score possible for each plant was 8. Daily mean feeding scores per plant for all replicate plants in each test were calculated.

Statistical analyses:

Mean feeding scores in each experiment were analyzed using paired difference *t* tests.

6.3 RESULTS

6.3.1 Experiment 1.

Choice test (1991): The greatest percentage of *A. lolii*-free tillers in the "damaged" category (46.3%) was recorded on the first assessment day (day 7) at which time a significantly ($P<0.001$) higher percentage of *A. lolii*-free tillers were damaged compared to *A. lolii*-infected tillers (Fig. 6.1). A significantly ($P<0.01$) higher percentage of *A. lolii*-free tillers were also in the damaged category on day 11 of the trial. Until day 15 of the trial, no *A. lolii*-infected tillers were recorded as being

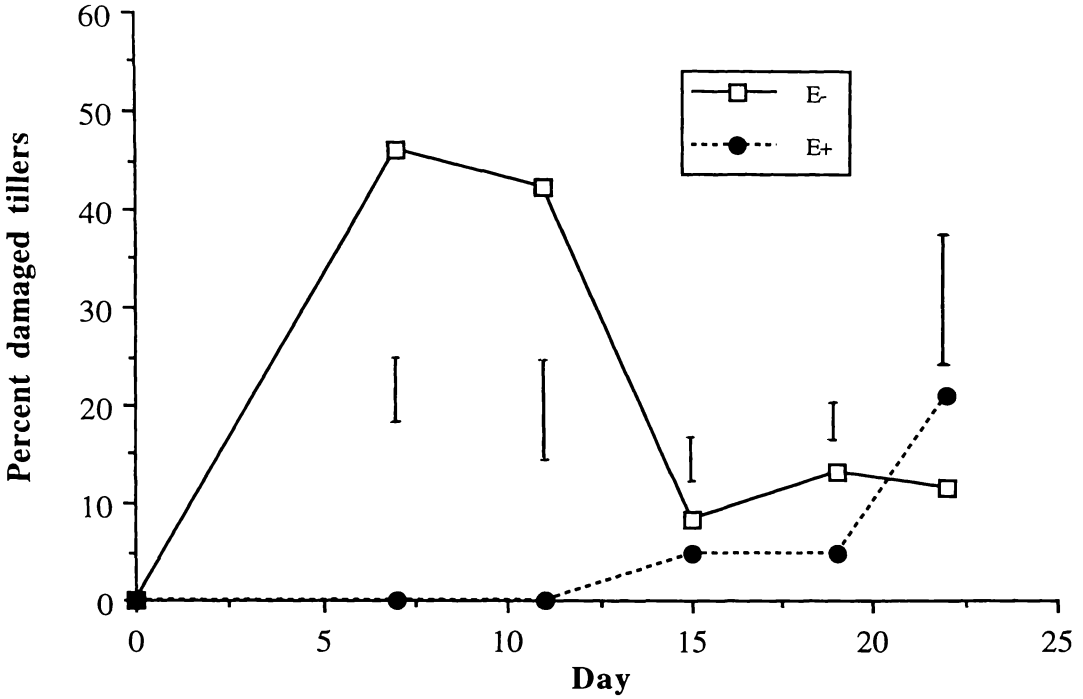


Figure 6.1 Damage to A. lolii-free (E-) and A. lolii-infected (E+) perennial ryegrass due to adult black beetle feeding in a choice test. (Bars represent S.E.D.).

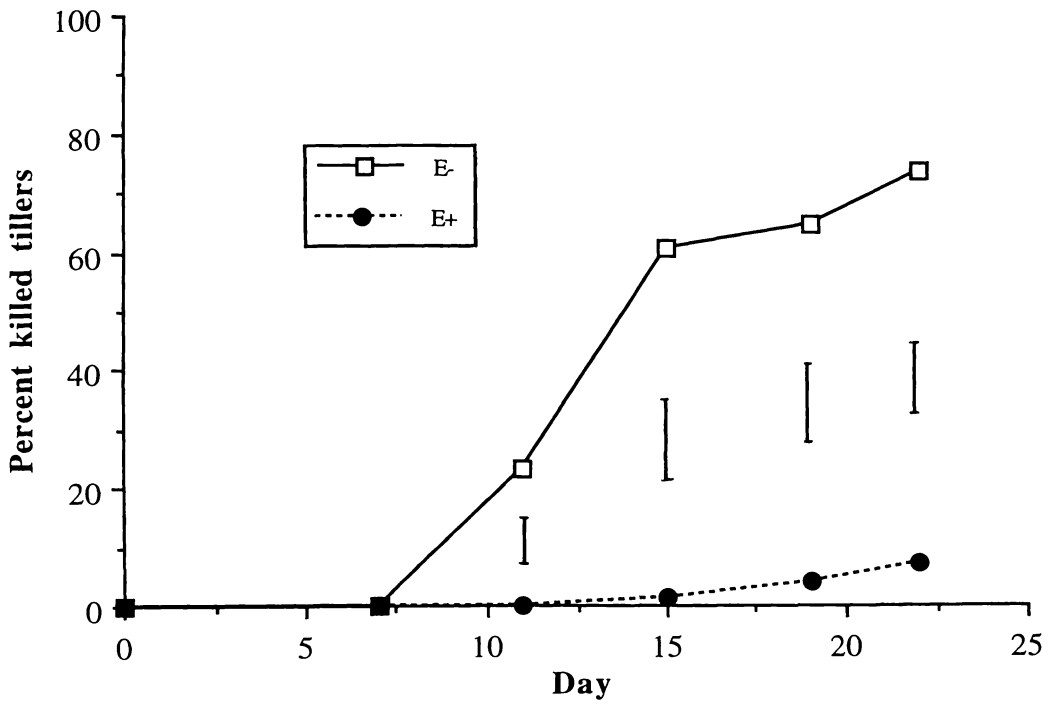


Figure 6.2 Mortality of A. lolii-free (E-) and A. lolii-infected (E+) perennial ryegrass due to adult black beetle feeding in a choice test. (Bars represent S.E.D.).

damaged. The percentages of *A. lolii*-free and *A. lolii*-infected tillers in the damaged category on day 15 were similar. The highest percentage of *A. lolii*-infected tillers in the damaged category (21.0%) was recorded on day 22. Although the percentage of *A. lolii*-infected tillers that were damaged was higher than the percentage of damaged *A. lolii*-free on day 22 (the only assessment day where this was the case), the difference was not significant.

From day 11 onwards, significantly ($P<0.05$ - $P<0.001$) more *A. lolii*-free tillers were killed by black beetle than *A. lolii*-infected tillers (Fig. 6.2). By day 22, 73.8% of the *A. lolii*-free tillers had been killed, compared to 7.3% in the *A. lolii*-infected plants.

The extent of tiller damage and tiller mortality was not influenced by the sex of the black beetle (Tables 6.2 and 6.3).

No choice test (1991): The percentage of *A. lolii*-free tillers damaged increased sharply between days 8 and 15, and reached a maximum of 34.4% on day 19 (Fig. 6.3). On days 15 and 19, the percentages of *A. lolii*-free tillers in the damaged class were significantly ($P<0.05$) higher than the percentages of *A. lolii*-infected tillers in this class which were low for the duration of the trial. Between days 19 and 23, the percentage of *A. lolii*-free tillers in the damaged category declined sharply such that damage levels were similar to those in *A. lolii*-infected plants.

Black beetle killed significantly ($P<0.05$ - $P<0.001$) more *A. lolii*-free tillers than *A. lolii*-infected tillers (Fig. 6.4). Tiller mortality on *A. lolii*-free plants increased steadily from day 8 and by day 27, 87.5% had been killed. Tiller mortality on *A. lolii*-infected plants was very low and by day 27, only 1.4% of the tillers were dead.

At the end of the trial, a black beetle mortality rate of 3.1% was recorded in both the *A. lolii*-infected and the *A. lolii*-free treatments. Mortality rates between the two treatments were therefore not significantly different.

Table 6.2 **Percent tiller damage levels in *A. lolii*-infected and *A. lolii*-free perennial ryegrass by male and female adult black beetle in a choice feeding test.**

	Assessment day									
	7		11		15		19		22	
	-	+	-	+	-	+	-	+	-	+
Female	42.7	0.0	31.3	0.0	12.0	2.5	11.3	0.0	18.0	10.0
Male	50.0	0.0	53.3	0.0	5.0	7.5	15.0	10.0	5.0	32.0
S.E.D.	13.27 (NS)		19.64 (NS)		8.38 (NS)		6.77 (NS)		25.00 (NS)	

NS = Not significant

Table 6.3 **Percent tiller mortality levels in *A. lolii*-infected and *A. lolii*-free perennial ryegrass by male and female adult black beetle in a choice feeding test.**

	Assessment day									
	7		11		15		19		22	
	-	+	-	+	-	+	-	+	-	+
Female	0.0	0.0	19.3	0.0	46.7	2.5	54.7	5.0	62.7	5.0
Male	0.0	0.0	26.7	0.0	75.0	0.0	75.0	2.5	85.0	9.5
S.E.D.	0.0 (NS)		16.09 (NS)		25.88 (NS)		28.20 (NS)		26.63 (NS)	

NS = Not significant

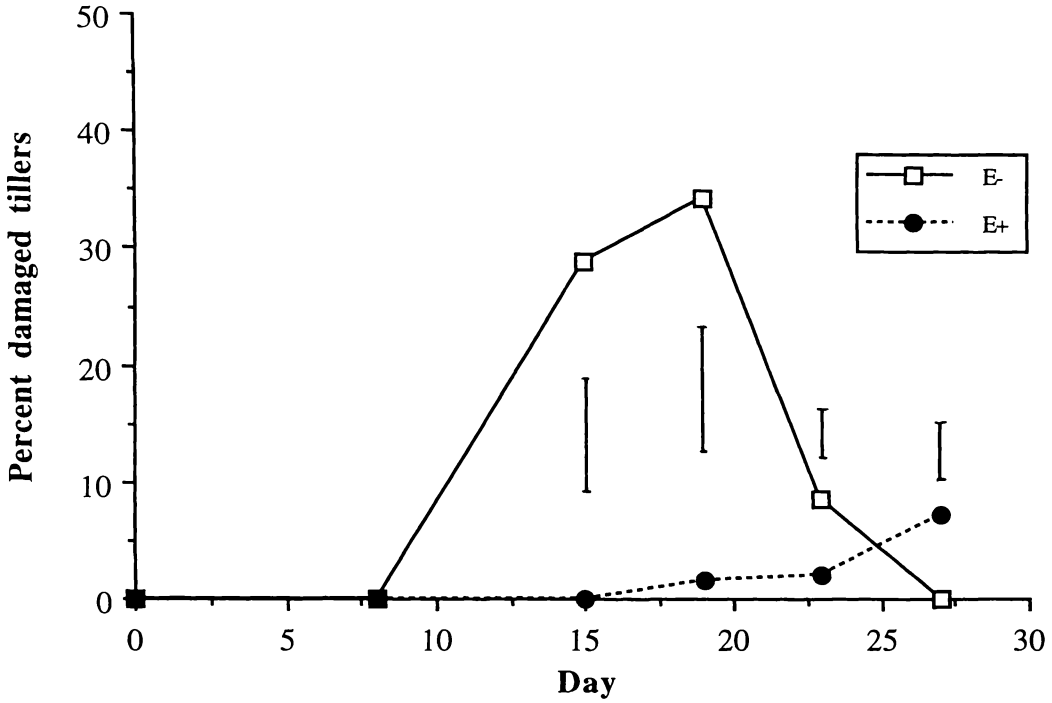


Figure 6.3 Damage to A. lolii-free (E-) and A. lolii-infected (E+) perennial ryegrass due to adult black beetle feeding in a no choice test. (Bars represent S.E.D.).

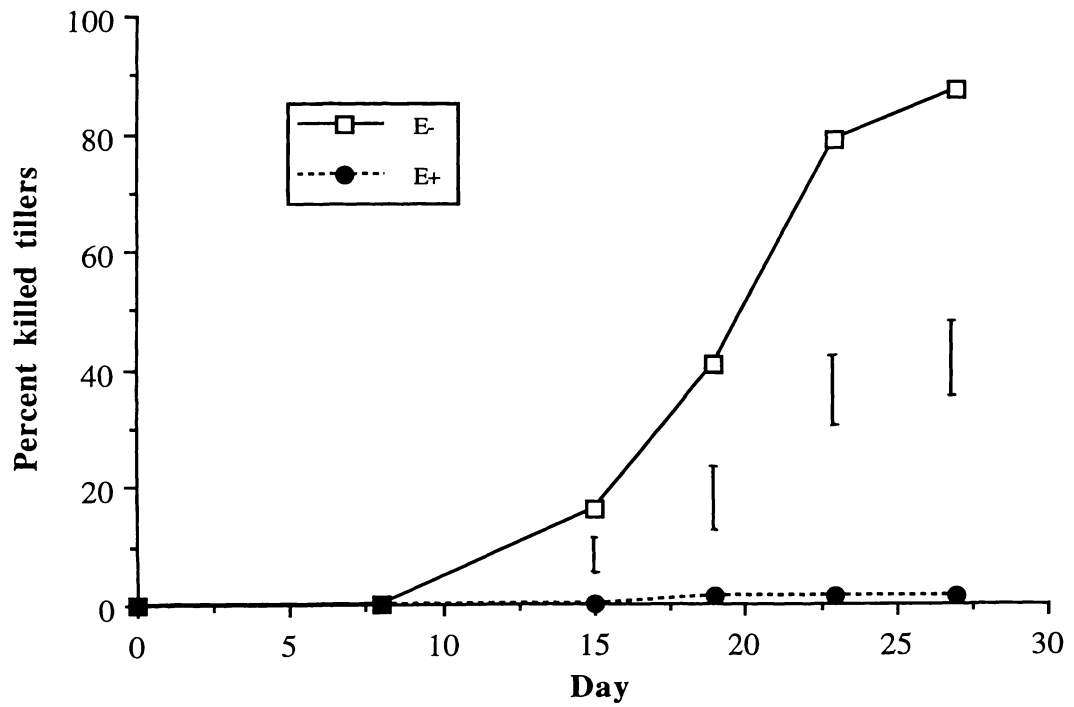


Figure 6.4 Mortality of A. lolii-free (E-) and A. lolii-infected (E+) perennial ryegrass due to adult black beetle feeding in a no choice test. (Bars represent S.E.D.).

No choice test (1992): The mean results of the three day 9 assessments show that there was a significantly ($P<0.001$) higher percentage of healthy *A. lolii*-infected tillers compared to healthy *A. lolii*-free tillers (Table 6.4). Although there was a significantly ($P<0.001$) higher percentage of *A. lolii*-infected tillers in the damaged category compared to *A. lolii*-free tillers, 90% of *A. lolii*-free tillers had been killed compared to only 10% of *A. lolii*-infected tillers.

After 27 days, black beetle mortality was 37.5% in both the *A. lolii*-infected treatment and the *A. lolii*-free treatment. Consequently there was no endophyte effect on black beetle mortality.

6.3.2 Experiment 2.

Adult black beetle mortality was very low (1.9%) during the testing period, and relatively few beetles did not feed at all (Table 6.5). Mean feeding scores for the peramine, lolitrem B and paxilline treatments at all concentrations tested, indicated that black beetle had no preference for either control or alkaloid treated discs (Table 6.6). No long term trends are indicated by the data either (Tables 6.5 and 6.6). Mean feeding scores on control and alkaloid treated diets across the three treatments ranged from 1.07-3.36 and 0.93-2.64 respectively.

Black beetle fed significantly less on discs treated with 5.0μg/g or more of ergotamine. At 10μg/g and 20μg/g of ergotamine, black beetle avoided treated discs almost completely (Table 6.5). Although not statistically significant at the 5% level, black beetle adults consumed more than twice as much control diet as 1.0μg/g ergotamine treated diet (Table 6.6) indicating the start of the trend. As ergotamine concentrations increased, mean feeding scores decreased steadily from the 1.57 recorded for the 0.1μg/g diet, while feeding scores on control discs remained steady.

Mean feeding scores for the mixed alkaloid diet are close to being significant at the 5% level, with feeding on control discs being 2.4 times higher than on treated discs.

Table 6.4 **Damage to, and mortality of *A. lolii*-free and *A. lolii*-infected Yatsyn 1 perennial ryegrass after 9 days, due to adult black beetle feeding in a no choice test. (Figures in brackets represent S.E.M., ***P<0.001).**

	percent healthy tillers	percent damaged tillers	percent killed tillers
A. lolii-free Yatsyn 1	66 (5.3)	5 (2.0)	90 (3.3)
A. lolii-infected Yatsyn 1	5 (2.6)	24 (4.8)	10 (2.5)
	***	***	***

Table 6.5 **Feeding patterns and mortality of adult black beetle in a series of choice tests comparing untreated (Control) and alkaloid treated artificial diet discs.**

compound	concentration (μg/g)	number of dead black beetle	number of black beetle which fed on control discs	number of black beetle which fed on treatment discs	number of black beetle which fed on both discs	number of black beetle which fed on neither
Peramine	0.1	1	2	6	4	2
	1.0	0	2	3	9	0
	10.0	0	4	5	4	1
	30.0	0	3	3	5	3
	60.0	0	5	1	6	2
Lolitrem B	0.1	0	6	2	4	2
	1.0	1	7	4	3	0
	5.0	1	7	3	3	1
	20.0	1	1	0	10	3
Paxilline	0.1	0	4	6	1	3
	1.0	0	3	6	3	2
	5.0	0	6	4	3	1
	20.0	0	3	3	8	0

Table 6.5 Cont.

compound	concentration (μg/g)	number of dead black beetle	number of black beetle which fed on control discs	number of black beetle which fed on treatment discs	number of black beetle which fed on both discs	number of black beetle which fed on neither
Ergotamine	0.1	0	5	5	2	2
	1.0	1	7	1	5	1
	5.0	0	9	0	5	0
	10.0	0	11	1	1	1
	20.0	0	12	1	0	1
Mixed alkaloid diet		0	4	0	7	3

Table 6.6 **Mean adult black beetle feeding scores on control and alkaloid treated artificial diet discs in a series of choice tests. (**P<0.01; ***P<0.001).**

Compound	Concentration (μg/g)	Control	Treated	S.E.D.
Peramine	0.1	1.64	2.21	0.97
	1.0	3.36	2.64	0.87
	10.0	1.86	1.93	1.06
	30.0	2.07	1.14	0.72
	60.0	2.14	1.86	0.62
Lolitrem B	0.1	2.21	1.07	0.72
	1.0	3.14	1.50	1.16
	5.0	1.71	0.93	0.68
	20.0	2.07	1.79	0.54
Paxilline	0.1	1.07	1.71	0.85
	1.0	1.36	1.64	0.78
	5.0	2.21	1.36	0.86
	20.0	1.64	2.21	0.67
Ergotamine	0.1	1.86	1.57	1.02
	1.0	3.07	1.36	1.08
	5.0	3.00	0.93	0.54(**)
	10.0	2.43	0.57	0.68(**)
	20.0	2.57	0.21	0.53(***)
Mixed alkaloid diet:				
Peramine	10.0			
Lolitrem B	5.0	1.93	0.79	0.53
Paxilline	5.0			
Ergotamine	1.0			

Feeding scores for the mixed alkaloid diet show that although very few treatment discs alone were fed upon, a number of black beetle fed on both control and treated discs (Table 6.5). This was also the case with the 1.0μg/g ergotamine diet.

6.3.3 Experiment 3.

No adult black beetle deaths were recorded in five of the six choice tests (tests 2-6). A single adult black beetle, which had already fed extensively, had died by the time choice test 1 (Lp5 x endophyte-free Nui) was dismantled.

Test 1: Two days after black beetle were introduced into the honey pots, mean feeding scores were significantly ($P<0.05$) higher in the endophyte-free Nui plants than the Lp5-infected Nui plants and remained so for the rest of the trial (Fig. 6.5). From day five onwards, the difference in the mean feeding scores was significant at the 0.1% level. Mean feeding scores in the Lp5-infected Nui plants remained low for the duration of the trial. On day ten, the mean feeding score in Lp5-infected plants was 0.5 (equivalent to half a damaged tiller out of four), while the mean feeding score in endophyte-free Nui plants was 6.4 (equivalent to 3.2 killed tillers out of four).

Test 2: From day 7 onwards, tiller damage and tiller mortality due to adult black beetle feeding was significantly ($P<0.001$) higher in endophyte-free Nui than in Tf13-infected Nui (Fig. 6.6). Although mean feeding scores on days 4-6 were considerably higher in endophyte-free Nui compared to Tf13-infected Nui, the differences were not significant at the 5% level. After ten days, the mean feeding score in endophyte-free plants was 6.1 compared to 0.4 in Tf13-infected plants.

Test 3: Mean feeding scores in Lp5-infected Nui and Tf13-infected Nui were low throughout the ten days of the trial and at no point were they significantly different (Fig. 6.7). On day ten, the mean feeding score in both Lp5-infected Nui and Tf13-infected Nui was 0.3.

Test 4: On days one and two of the trial, mean feeding scores in endophyte-free

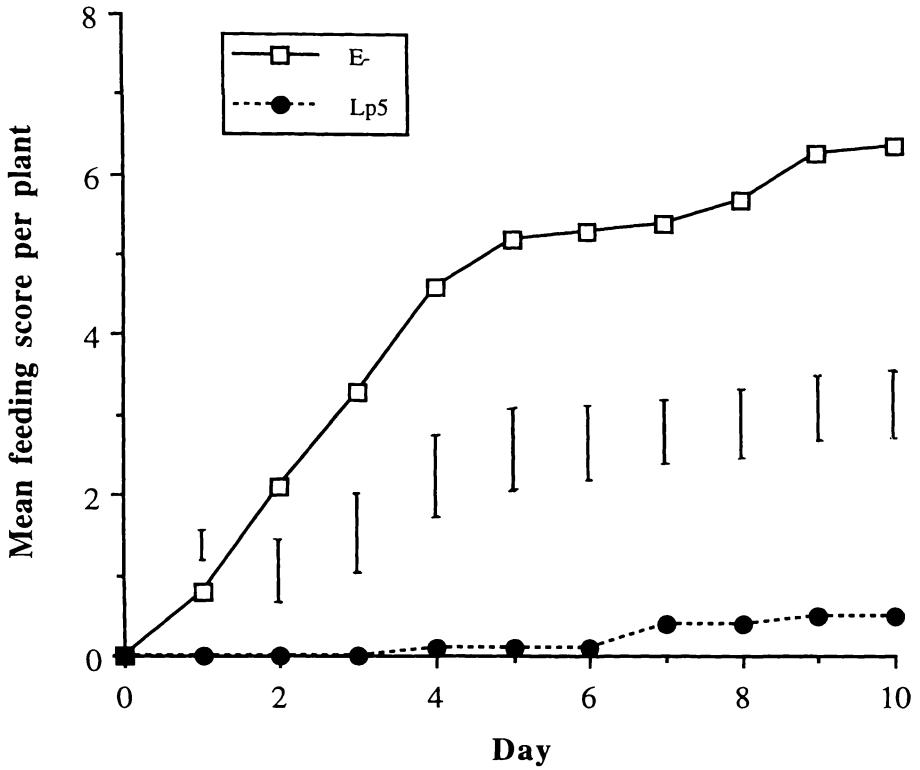


Figure 6.5 Mean feeding scores from an adult black beetle choice feeding test comparing endophyte-free Nui (E-) and Nui infected with Acremonium endophyte strain Lp5. (Bars represent S.E.D.).

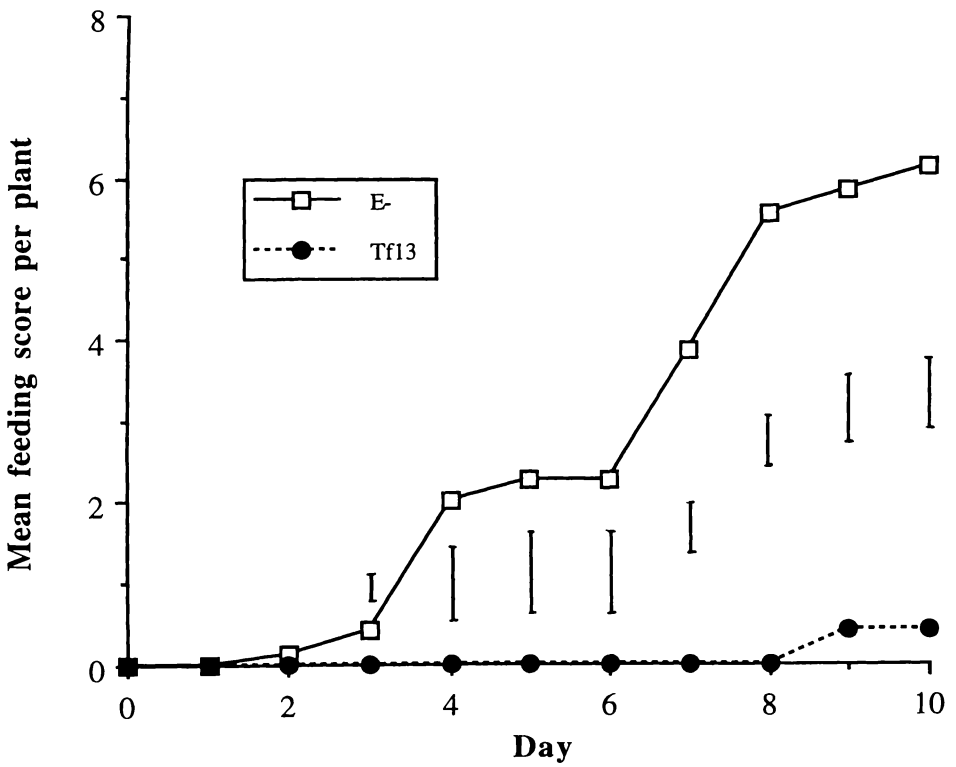


Figure 6.6 Mean feeding scores from an adult black beetle choice feeding test comparing endophyte-free Nui (E-) and Nui infected with Acremonium endophyte strain Tf13. (Bars represent S.E.D.).

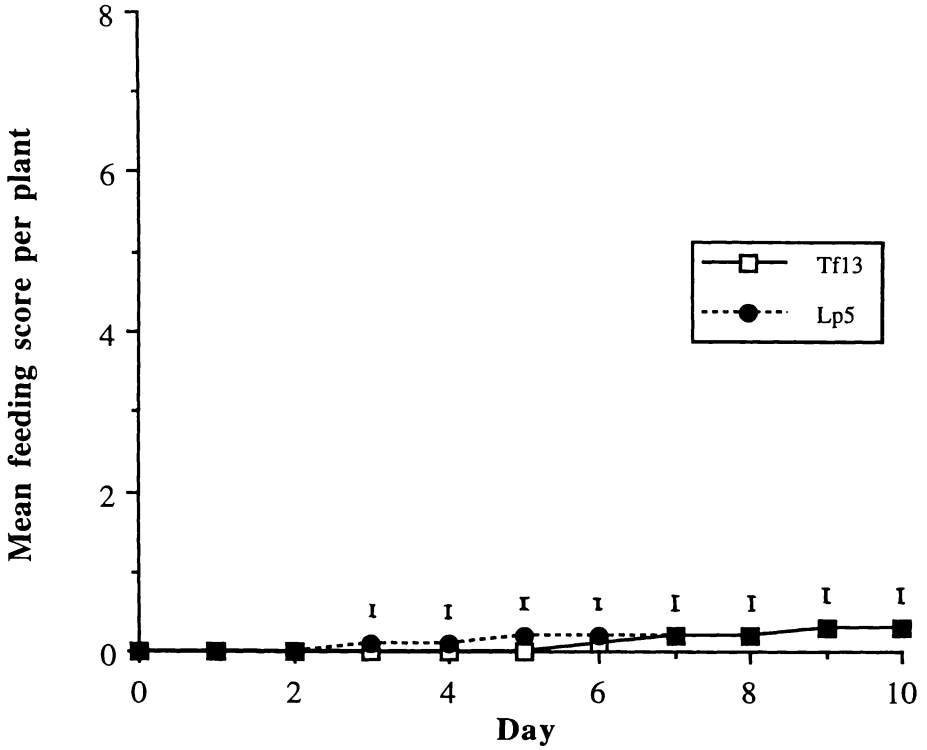


Figure 6.7 Mean feeding scores from an adult black beetle choice feeding test comparing Nui infected with Acremonium endophyte strain Lp5 and Nui infected with Acremonium endophyte strain Tf13. (Bars represent S.E.D.).

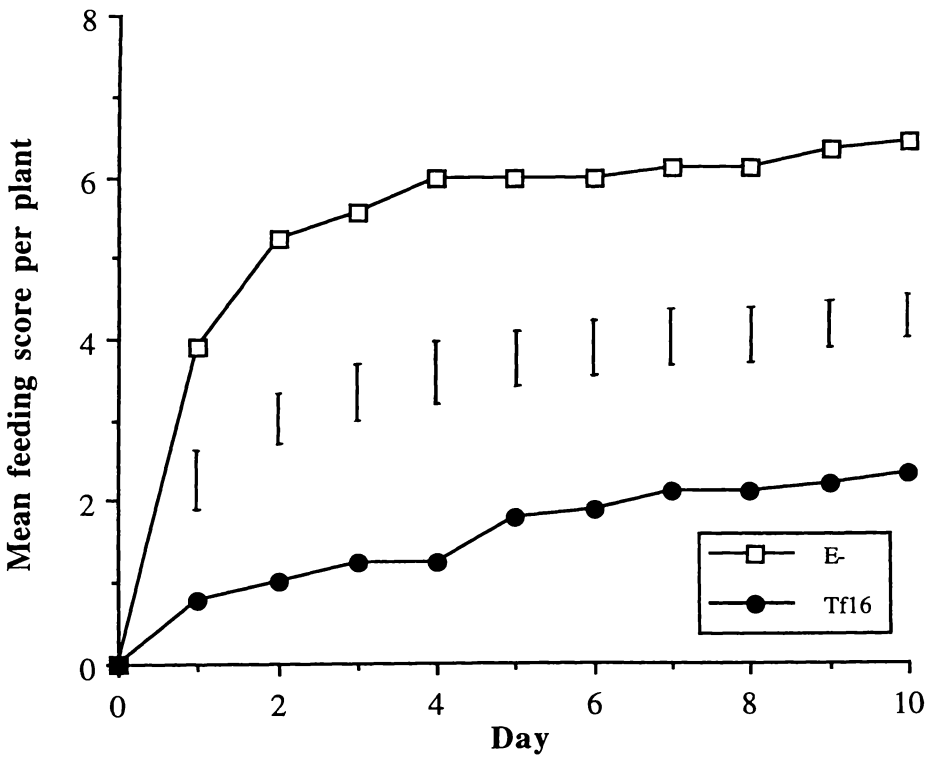


Figure 6.8 Mean feeding scores from an adult black beetle choice feeding test comparing endophyte-free Nui (E-) and Nui infected with Acremonium endophyte strain Tf16. (Bars represent S.E.D.).

Nui plants increased sharply to a high level, while only a minor increase was recorded in Tf16-infected plants (Fig. 6.8). Throughout the entire trial, feeding scores were significantly ($P<0.01$ on day 1, $P<0.001$ from day 2 onwards) higher in endophyte-free plants compared to plants infected with isolate Tf16. On the final day of the trial, feeding scores for the endophyte-free and Tf16-infected plants were 6.4 and 2.3 respectively.

Test 5: Mean feeding scores in endophyte-free plants increased gradually during the trial, and were consistently higher than mean feeding scores in Lp14-infected plants (Fig. 6.9). From day five onwards, mean feeding scores were significantly ($P<0.05$) greater in endophyte-free plants compared to Lp14-infected plants. On day ten, the mean feeding score in endophyte-free Nui was 3.6 compared to 0.9 in Lp14-infected Nui.

Test 6: From day one to day three, mean feeding scores increased steadily in endophyte-free plants, and reached their highest level on day six (Fig. 6.10). Mean feeding scores in Lp17-infected plants increased steadily throughout the trial, although they were consistently lower (significantly ($P<0.05$ - $P<0.001$) so from day two onwards) than feeding scores from the endophyte-free plants. On day ten of the trial, feeding scores for the endophyte-free and Lp17-infected plants were 7.3 and 4.1 respectively.

6.4 DISCUSSION

Results from the initial choice and no choice tests (experiment 1) show that adult black beetle were adversely affected by the presence of *A. lolii* in perennial ryegrass (Figs. 6.1-6.4, Table 6.4) and that males and females are equally adversely affected (Tables 6.2 and 6.3). Specifically, these studies showed that the presence of *A. lolii* significantly reduced perennial ryegrass tiller death due to feeding by adult black beetle. Results from the two no choice pot trials indicate that even when pressured, adult black

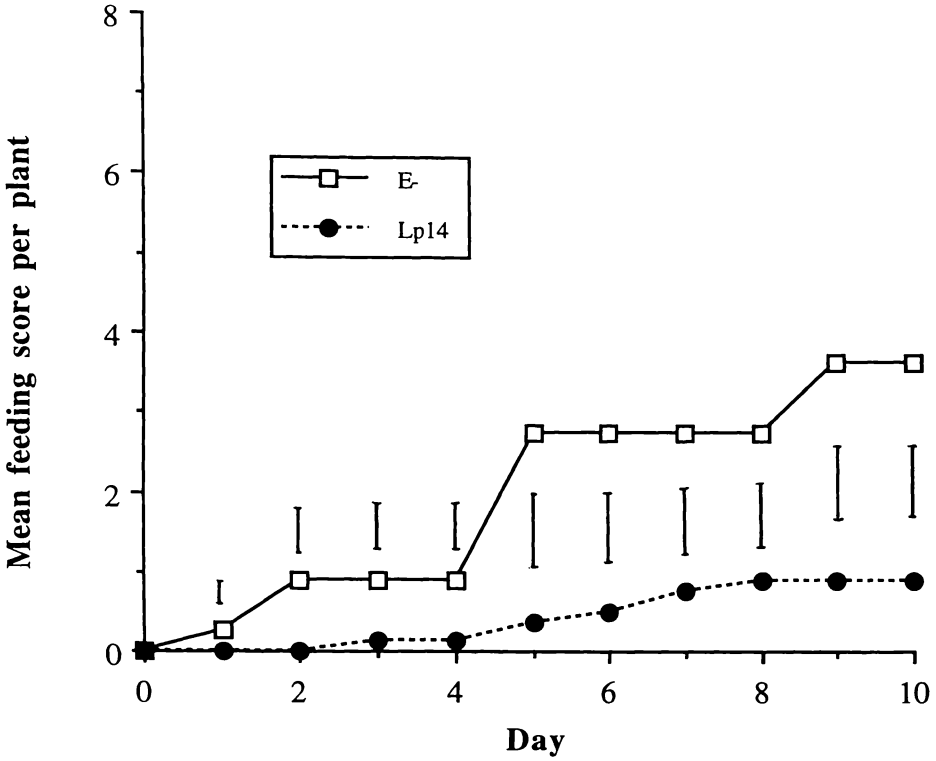


Figure 6.9 Mean feeding scores from an adult black beetle choice feeding test comparing endophyte-free Nui (E-) and Nui infected with Acremonium endophyte strain Lp14. (Bars represent S.E.D.).

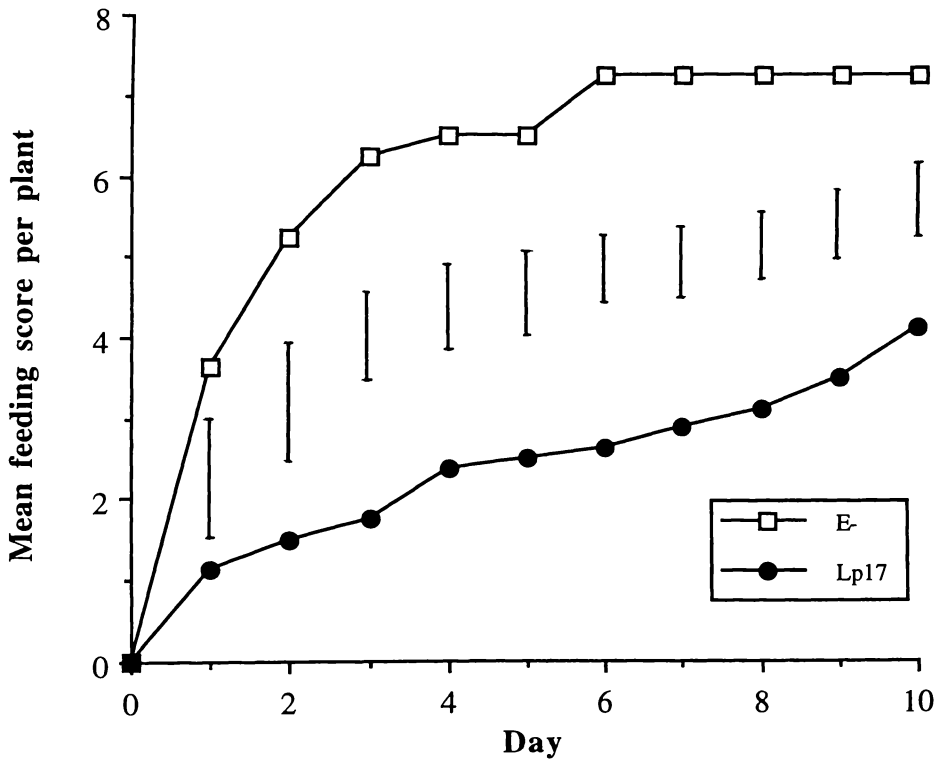


Figure 6.10 Mean feeding scores from an adult black beetle choice feeding test comparing endophyte-free Nui (E-) and Nui infected with Acremonium endophyte strain Lp17. (Bars represent S.E.D.).

beetle avoid feeding on *A. lolii*-infected perennial ryegrass (Figs. 6.3 and 6.4, Table 6.4). The *A. lolii*-mediated feeding deterrency was marked in both the Ellett (Figs. 6.3 and 6.4) and Yatsyn 1 (Table 6.4) lines, indicating that at least some of the commercially available lines of perennial ryegrass are highly resistant to black beetle. The tiller damage patterns were similar in both the choice test and no choice test performed in 1991. Damage to *A. lolii*-free plants peaked early and then declined steeply as many of the tillers which were classed as damaged, were transferred into the killed category (Figs. 6.1-6.4). In comparison to *A. lolii*-free plants, damage to *A. lolii*-infected plants was slight, but generally increased as each trial progressed. The percentage of *A. lolii*-infected tillers which were damaged did not exhibit the decline seen in *A. lolii*-free tillers as the trials progressed. This was probably due to the fact that *A. lolii*-infected tillers were generally only damaged lightly and since they sustained little or no further feeding damage, were not transferred into the killed category. This is also the cause of the higher damage levels recorded in *A lolii*-infected plants compared to *A. lolii*-free plants at the end of all three experiment 1 trials (Figs. 6.1 and 6.3, Table 6.4). Results from these trials show that a small percentage of *A. lolii*-infected tillers may be damaged and killed by adult black beetle.

The black beetle thus joins the list of insects adversely affected by the presence of *A. lolii* in perennial ryegrass and is the first member of the Scarabaeidae to be recorded as such. The effect of *A. lolii* infection on feeding by adult black beetle was surprisingly marked. A possible reason as to why this was so may be due to the fact that black beetle adults feed at the tiller bases just above the crown of the plant where *A. lolii* and several of the toxins associated with its presence are known to be concentrated (Musgrave and Fletcher 1984, Gallagher *et al.* 1987, di Menna *et al.* 1992, Keogh and Tapper 1993). However, in neither no choice test was mortality significantly higher in the black beetle confined to the *A. lolii*-infected plants, even though feeding activity was

considerably reduced. This may have been because the two no choice tests were terminated before the effects of a depleted diet manifested itself as an increase in black beetle mortality. Also, black beetle were well fed (on carrots) prior to each of the two no choice tests. Reduced feeding of black beetle maintained on *A. lolii*-infected ryegrass would be expected to lead to a reduction in fecundity.

Results from experiment 1 may help to explain certain field observations made in previous ecological studies. Watson and Wrenn (1980) showed that black beetle numbers reflected paspalum levels in pasture, and King *et al.* (1981a) reported that perennial ryegrass pastures were an unfavourable host for adult black beetle as "winter disappearance" due to increased levels of adult mortality or dispersal was higher than on paspalum pastures. (King 1979b) also found that fat reserves, longevity and fecundity of female black beetle adults increased on paspalum compared to perennial ryegrass. This may be attributable to the effects of *A. lolii*, although its presence and levels were unknown at that time. Results from the current study indicate that in comparison to *A. lolii*-free grass, *A. lolii*-infected perennial ryegrass will not favour adult black beetle aggregation. Aggregation under favoured plants by black beetle adults leads to more oviposition in those areas (King *et al.* 1981b) resulting in higher larval numbers the following summer. The ability of perennial ryegrass to compete against paspalum in black beetle prone areas may therefore be considerably improved by *A. lolii*.

Results from the artificial diet tests (experiment 2) indicate that unlike Argentine stem weevil, adult black beetle are not deterred from feeding by peramine (Tables 6.5 and 6.6). In choice tests, Dymock *et al.* (1988), Popay *et al.* (1990) and Rowan *et al.* (1990a) found that adult Argentine stem weevil were deterred from feeding on diets containing between 0.1-1.0μg/g of peramine. These concentrations are considerably less than the highest concentration of 60μg/g used in the present study. It is therefore apparent that peramine is not the universal insect feeding deterrent that it was hoped to

be. Lolitrem B which is a toxin and a feeding deterrent to Argentine stem weevil larvae (Prestidge and Gallagher 1985 and 1988b, Dymock *et al.* 1989, Popay *et al.* 1990) was not a deterrent against black beetle. Black beetle adults were also not deterred from feeding on discs treated with paxilline, an adult Argentine stem weevil feeding deterrent (Popay and Latch 1993) and larval argentine stem weevil toxin (Gallagher and Prestidge 1990).

The only alkaloid to significantly deter black beetle was ergotamine (Tables 6.5 and 6.6). The feeding deterrent property of ergotamine observed in this study has also been recorded with other insects. Feeding by adult Argentine stem weevil was significantly reduced by ergotamine and ergocryptine at 0.1μg/g and 1.0μg/g respectively (Dymock *et al.* 1988), and by ergovaline at 0.1μg/g (Popay *et al.* 1990). Pless *et al.* (1993) found that ergotamine and ergocryptine significantly reduced survival of *Drosophila melanogaster* Meigen larvae at 2.9μg/g and 2.45μg/g respectively. Clay and Cheplick (1989) also noted antibiotic and/or antifeedant effects on the fall armyworm (*Spodoptera frugiperda*) with all three classes of ergot alkaloid (clavine, lysergic acid and ergopeptine). Specifically, ergotamine acted as both an antifeedant and an antibiotic. However, Clay and Cheplick (1989) concluded that ergot alkaloids in tall fescue (*Festuca arundinacea* Schreb.) infected with *A. coenophialum*, played only a minor role in observed resistance of endophyte-infected plants to insect herbivores, as the greatest antifeedant effects were seen at 1-1.3g/kg, which is approximately two orders of magnitude higher than concentrations in endophyte-infected tall fescue.

The importance of ergotamine or ergot alkaloids in general in mediating *A. lolii*-infected perennial ryegrass resistance to black beetle is uncertain. There are conflicting reports of whether, and how much ergotamine is found in *A. lolii*-infected perennial ryegrass. Using tandem mass spectrometry, Rowan and Shaw (1987) concluded that ergotamine comprised 30.1% of the total ergopeptine alkaloid content in *A. lolii*-infected

perennial ryegrass. However, except for the seed where concentrations of 2.0μg/g were recorded, ergotamine was found only in trace amounts or was completely absent in herbage using HPLC (Rowan *et al.* 1990b). Tandem mass spectrometry has revealed the presence of eight ergopeptine alkaloids in *A. lolii*-infected perennial ryegrass (Rowan and Shaw 1987), the individual effects of which on black beetle feeding are unknown. Ergovaline, the most abundant ergopeptine in *A. lolii*-infected perennial ryegrass (Rowan and Shaw 1987, Rowan *et al.* 1990b) and the remaining ergopeptine alkaloids are structurally very similar to ergotamine indicating that they may have similar effects.

Results from Figures 6.5-6.10 indicate that all five strains of *Acremonium* endophyte (Lp5, Lp14, Lp17, Tf13 and Tf16) conferred at least some degree of resistance against adult black beetle. Thus, like *A. lolii*, *Acremonium* endophytes derived from tall fescue conferred resistance to perennial ryegrass host plants against adult black beetle attack (Figs. 6.6 and 6.8).

Plants infected with *Acremonium* strains Tf13, Lp14 and Lp17 (Figs. 6.6, 6.9 and 6.10) were, compared to endophyte-free plants, significantly more resistant against adult black beetle. This was despite the fact that Nui plants infected with these three endophyte strains contained no detectable peramine or lolitrem B (Table 6.1). Also a direct comparison indicated that Nui plants infected with *Acremonium* strain Tf13 (which contained ergovaline but no detectable peramine or lolitrem B) were no less resistant to adult black beetle than Nui plants infected with strain Lp5 (which contained peramine, lolitrem B and ergovaline). These results strongly suggest, like the results of the artificial diet work (Tables 6.5 and 6.6), that peramine and lolitrem B are not required for endophyte-mediated adult black beetle resistance in perennial ryegrass.

However, it is also evident that ergovaline was not essential for deterring adult black beetle from feeding, as plants infected with *Acremonium* isolates Tf16, Lp14 and Lp17, which did not contain any detectable ergovaline were still resistant (Figs. 6.8, 6.9

and 6.10). This is a little surprising considering the deterrent effect on black beetle of ergotamine observed in the artificial diet work (Tables 6.5 and 6.6). It is possible that ergopeptine alkaloids other than ergovaline may have been responsible for the resistance observed in these plants. However, total ergot alkaloid analyses indicated that plants infected with *Acremonium* isolates Tf16, Lp14 and Lp17 contained only trace amounts of ergopeptine alkaloids, or no detectable ergopeptines alkaloids at all (Table 6.1). Thus, compounds from a completely different chemical group (or groups) may be involved instead. In the case of Nui infected with *Acremonium* strain Tf16, loline alkaloids (the production of which appears to be inconsistent in perennial ryegrass infected with this endophyte) may also have acted as a black beetle feeding deterrent. However, if this were so, loline alkaloids would, like peramine, lolitrem B and ergovaline, not be essential for deterring adult black beetle as no lolines have been detected in grasses infected with the other four endophyte isolates (Christensen *et al.* 1993).

It should be noted that factors other than the presence or absence of specific alkaloids could control the level of black beetle resistance in these plants. The concentration of endophyte in the plant may influence this. Prestidge *et al.* (1985a) showed that damage to endophyte-infected perennial ryegrass was proportional to the concentration (number of hyphae per mm of leaf sheath) of *A. lolii* in the test grass. Breen (1992, 1993a, 1993b) observed similar effects with greenbug (*schizaphis graminum* Rondani), fall armyworm and bird cherry-oat aphid (*Rhopalosiphum padi* L.). However, Breen (1993b) also found that differences in endophyte concentration did not explain all of the differences between *A. lolii*-infected genotypes. *Acremonium* endophyte concentrations were not determined in the plants used in the present study.

The results of experiment 3 indicate that it may be possible to utilize strains of *Acremonium* endophytes for incorporation into cultivars of perennial ryegrass which do not produce the known mammalian toxins lolitrem B and ergovaline, but which provide

protection from black beetle. However, it is also evident that more research investigating the importance of ergopeptine alkaloids on adult black beetle resistance is required.

6.5 CONCLUDING SUMMARY

This study has shown that adult black beetle prefer to feed on endophyte-free perennial ryegrass compared to *A. lolii*-infected or *A. coenophialum*-infected perennial ryegrass. The extent of the black beetle resistance conferred on the perennial ryegrass cultivars Ellett and Yatsyn 1 as a result of being infected with *A. lolii*, was considerable. Four *A. lolii* associated alkaloids were tested for their effect on adult black beetle feeding using an artificial diet. Peramine, lolitrem B and paxilline had no effect on feeding by adult black beetle. Only ergotamine (at 5.0μg/g and above) had a significant antifeedant effect on adult black beetle. A series of choice feeding tests using *Acremonium* endophyte strains selected on the basis of their alkaloid spectra in host plants, indicated that peramine and lolitrem B were not required for endophyte-mediated adult black beetle resistance. This confirmed the earlier results of the artificial diet work. However, it was found that ergovaline, a compound similar to ergotamine, was also not required for black beetle resistance. It was concluded that either ergopeptine alkaloids other than ergovaline, or compounds from a different chemical group (or groups), may have been responsible for the observed resistance.

GENERAL CONCLUSIONS

Although *Acremonium lolii* is regarded as economically and ecologically important in grassland ecosystems in New Zealand, little attention has been given to the growth of the endophyte and the production of alkaloids inside host plants. Also, research concerning the mechanisms of endophyte-mediated resistance of perennial ryegrass against insect pests, has focussed primarily on the Argentine stem weevil. Few studies have investigated the impact of endophytic fungi on other invertebrates. The results of the studies reported in this thesis extend our knowledge and understanding of the interaction between *A. lolii* and a) the host plant, b) physical factors of the external environment, c) animal health problems, and d) resistance against insect pests. Also, development of an enzyme-linked immunosorbent assay (ELISA) used to detect and quantify *A. lolii* concentrations in plant samples resulted in some modifications to existing techniques. The following general conclusions have been made.

1. Inoculation of rabbits with the soluble antigen fraction purified from *A. lolii* culture fluids, resulted in the production of antibodies which could be used in an ELISA. Previously, only the mycelial fraction had been used to develop an ELISA to *A. lolii*.

2. The use of two different antibodies, derived from two different rabbits, one inoculated with antigens derived from the soluble fraction and the other inoculated with the mycelial antigen fraction, greatly improved detection of *A. lolii* in ryegrass samples.

3. Detection of *A. lolii* was not possible using oven-dried samples. Consistent

absorbance differences between endophyte-free and *A. lolii*-infected samples were only achieved when grass samples were freeze-dried. This was in spite of the fact that oven-drying has been used successfully in previous immunoassays against *Acremonium* endophytes. It was concluded that the majority of antibodies from the antisera used in the present assay were effective against heat sensitive epitopes.

4. Detection of *A. lolii* was enhanced when grass samples were extracted at 25^{0}C compared to 4^{0}C. Previous reports indicated that an extraction temperature of 5^{0}C was preferable to 25^{0}C.

5. Although there was some degree of cross reactivity, higher absorbances meant that *A. lolii* could be easily distinguished from the closely related species *A. coenophialum* and *A. starrii.* The use of soluble *A. lolii* antigens for producing one of the antisera used, may explain the relatively high degree of species specificity achieved in the assay.

6. *A. lolii* concentrations in perennial ryegrass plants maintained in an outdoor trial were lowest in winter, and highest in early summer. As the peak coincided with the time at which reproductive development of the ryegrass host plant occurred, it was suggested that proliferation of *A. lolii* at this time could improve its chances of being successfully disseminated. *A. lolii* concentrations were consistently higher in the basal portion of the plant compared to the regrowth component.

7. Lolitrem B concentrations were lowest in winter and peaked in summer/early autumn. This coincided with the time at which ryegrass staggers outbreaks usually occur. Also, lolitrem B concentrations were markedly higher in the basal part of the

plant compared to the regrowth component, supporting the results of previous studies and explaining the observation that ryegrass staggers outbreaks are more prevalent when stock are forced to close graze pastures.

8. Peramine concentrations, although lowest in winter, were relatively stable over the year compared to lolitrem B. This could be advantageous to the ryegrass/endophyte association as a degree of insect resistance may be maintained throughout the year. In contrast to lolitrem B and *A. lolii*, peramine concentrations were generally consistently higher in the regrowth component compared to the basal part of the ryegrass plant.

9. Changes in the peramine concentration in the basal component, and changes in the peramine and *A. lolii* concentrations in the regrowth component, were closely related to changes in the grass minimum temperature, total monthly rainfall and photoperiod. This was not the case for lolitrem B.

10. Different plants contained different levels of *A. lolii*, peramine and lolitrem B. These differences were generally maintained throughout the year, and in the case of peramine and lolitrem B, from year to year. Mean yearly concentrations of peramine and lolitrem B in different plants were significantly correlated with mean yearly concentrations of *A. lolii* in the same plants. Although, changes in alkaloid concentration were not correlated with changes in *A. lolii* concentration over time (with the exception of peramine in the basal tissue) it was concluded that peramine and lolitrem B concentrations in the plant were largely determined by the *A. lolii* concentration.

11. In leaves from both vegetative and reproductive tillers, *A. lolii* and lolitrem B concentrations were generally higher in the leaf sheaths compared to the leaf blades. Peramine concentrations were also higher in the leaf sheaths compared to the leaf blades of reproductive tillers. This was not the case in leaves from vegetative tillers where peramine was distributed relatively evenly between sheath and blade. It was concluded that peramine was more translocatable than lolitrem B in the plant.

12. *A. lolii* concentrations were usually low in the youngest (innermost) leaf compared to the older leaves, while peramine concentrations consistently decreased with increasing leaf age. Senescent leaves contained very little peramine. This pattern was attributed either to the breakdown in older tissues, or to the translocation from older to younger tissues, of the peramine. In contrast, lolitrem B concentrations increased as leaf age increased, such that senescent issue contained high concentrations of lolitrem B. Either lolitrem B was produced late in the growth cycle of *A. lolii*, or lolitrem B was deposited in the older plant tissue. Calculations showed that in mid January, nearly half of the total lolitrem B content of the 'vegetative' plants was contained in the senescent leaf component. It was suggested that the senescent leaf component may be the most important in contributing to ryegrass staggers.

13. In vegetative tillers, the crown and the small amount of reproductive stem tissue contained the highest concentrations of *A. lolii*. The root component contained low concentrations of *A. lolii*, peramine and lolitrem B, indicating that the endophyte probably confers little resistance against root feeding invertebrates. The regrowth component of 'vegetative' plants contained low levels of *A. lolii* and lolitrem B, but relatively high levels of peramine. Compared to the quantity of peramine elsewhere in the plant, the total amount of peramine in the regrowth component which was removed

from the 'vegetative' plants during the trimming regime was large. If peramine is the main cause of faecal contamination as has been suggested, this finding could have important implications regarding livestock health.

14. *A. lolii* concentrations were higher in most reproductive plant tissues compared to vegetative plant tissues. Also, relative to 'vegetative' plants, *A. lolii* growth was stimulated in 'reproductive' plants. The seed component of the 'reproductive' plants contained particularly high concentrations of *A. lolii*. It was suggested that the observed proliferation of *A. lolii* in the reproductive structures (with the exception of the leaf sheaths and leaf blades from reproductive tillers) may increase the likelihood of successful transmission via the seed.

15. In 'reproductive' plants, peramine concentrations were highest in the seed component. This may be an adaptation, the result of which is increased protection for the seed or resulting seedling from herbivore attack. During the early stages of plant reproductive development (early November), peramine production was inhibited in 'reproductive' plants compared to 'vegetative' plants.

16. The highest concentrations of lolitrem B in the 'reproductive' plants was present in the seed component. Like peramine, this may have arisen as a defense against herbivore attack. In mid January, lolitrem B production was stimulated in the 'reproductive' plants compared to the 'vegetative' plants, although much of the increase was due to the contribution from the seed.

17. Analysis of seedlings grown from seed infected with non-viable endophyte showed that peramine and lolitrem B could be translocated from the seed to the root and

shoots of the developing seedlings explaining the previous observation that such seedlings maybe resistant to attack from Argentine stem weevil.

18. A large increase in the lolitrem B concentration in the seed residue component, compared to that in the original seed, was recorded in young perennial ryegrass seedlings. This indicated that much of the lolitrem B present in the seed was not translocated into the developing seedling. It was concluded that much of the lolitrem B present in the seed was stored in or around the testa. Contrary to what was observed with lolitrem B, peramine concentrations in the seed residue component declined compared to initial levels in the seed. It was suggested that much of the peramine was translocated from the seed to the developing seedling. These results suggested that lolitrem B may be more useful in protecting the seed, while peramine may be more useful in protecting the seedling. The observation that no extra peramine appeared to be produced by viable endophyte in twelve day old seedlings added strength to this hypothesis.

19. The lowest lolitrem B and peramine concentrations were recorded in 48 day old seedlings. This corresponded with the time at which the highest rate of dry matter production by the grass was recorded, indicating a dilution effect.

20. Adult black beetle were shown to be significantly deterred from feeding on perennial ryegrass by the presence of *A. lolii.*

21. Artificial diet studies indicated that peramine, lolitrem B and paxilline had no effect on feeding by adult black beetle. However, ergotamine at a concentration of 5μg/g or higher significantly deterred adult black beetle from feeding on diet discs.

22. Choice feeding tests using selected *Acremonium* isolates selected on the basis of their alkaloid profiles in Nui host plants, indicated that peramine, lolitrem B, ergovaline and the loline alkaloids were not required for endophyte-mediated adult black beetle resistance.

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APPENDIX

1. Compositions of buffers used in ELISA.

Phosphate buffered saline (PBS):
Sodium chloride (NaCl) 8.0g
Potassium dihydrogen orthophosphate (KH_2PO_4) 0.2g
Disodium hydrogen orthophosphate (Na_2HPO_4) 1.4g
Potassium chloride (KCl) 0.2g
Sodium azide (NaN_3) 0.2g
Distilled water 1.0ℓ

Coating buffer:
Sodium carbonate (Na_2CO_3) 1.59g
Sodium hydrogen carbonate ($NaHCO_3$) 2.93g
Sodium azide (NaN_3) 0.2g
Distilled water 1.0ℓ

Sample (extraction) buffer:
Polyvinyl pyrrolidone (PVP) 20.0g
Polyoxyethylene (20) - sorbitan monolaurate (Tween 20) 5.0ml
PBS to1.0ℓ

Conjugate buffer:
Magnesium chloride ($MgCl_2$) 0.203g
Bovine serum albumin (BSA) 2.0g
Tween 20 ..... 0.5ml
PBS to1.0ℓ

Substrate buffer:
Diethanolamine ($[CH_2(OH).CH_2]_2NH$) 97.0ml
Sodium azide (NaN_3) 0.2g
Distilled water ..... to 1.0ℓ
10N Hydrochloric acid (HCl) ..... to pH 9.8

2. Publications of work reported in this thesis.

Ball, O.J-P., Christensen, M.J. and Prestidge, R.A. 1994. Effect of selected isolates of *Acremonium* endophytes on adult black beetle (*Heteronychus arator*) feeding. *Proceedings of the New Zealand Plant Protection Conference 47*: in press).

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