

## ADVANCES IN ENDOPHYTE RESEARCH. PROGRESS AND PRIORITIES IN TEMPERATE AREAS

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### Background

Fungal endophytes infect a large number of temperate grass species, genera and tribes (Leuchtman and Clay, 1997). The majority of these systemic endophytes fall into the genus *Epichloe* or the closely related *Neotyphodium* genus.

The asexual *Neotyphodium* endophytes are asymptomatic, never emerge from between the host grass cells, and are only transmitted vertically, via seed of the host plant. They are believed to have derived from the *Epichloe* endophytes, (Bacon and White, 2000; Schardl and Wilkinson, 2000) which do emerge from their intercellular habitat and form stromata around the emerging seedhead of their host. This is manifested as the choke disease seen in many grass species. Thus reproduction in the *Epichloe* can be sexual and transmission can be horizontal or vertical or a mix of both.

*Epichloe* endophytes tend to form the most antagonistic relationships with their hosts while the *Neotyphodium* endophyte/grass associations are generally mutualistic (Schardl and Wilkinson, 2000). This paper will focus on interactions in temperate grassland ecosystems between *Neotyphodium* endophytes and their host grasses.

While European cultivars of perennial ryegrass (*Lolium perenne* L.) tend to be endophyte-free (Lewis, 1997), both tall fescue in USA (*Festuca arundinacea* Schreb.) and perennial ryegrass in New Zealand were shown to be much less vigorous and persistent in the field if not infected with their respective endophytes (Prestidge *et al.*, 1985; Read and Camp, 1986). Tall fescue research focused for several years on enhanced tolerance of abiotic stress (West and Gwinn, 1993), while research on perennial ryegrass focused on tolerance of invertebrate pests (Prestidge and Ball, 1993; Popay and Rowan, 1994).

These endophytes, which co-evolved with their hosts under a range of biotic and abiotic stresses associated with different environments, produce a wide range of secondary metabolites (Bush *et al.*, 1997; Lane *et al.*, 2000). Most of these enhance the stress tolerance and fitness of the host plant, protecting against vertebrate and invertebrate herbivores, in particular protecting the base of the plant, site of the apical meristems.

The first metabolites identified were loline alkaloids, ergot alkaloids and peramine in tall fescue infected with *N. coenophialum*, and lolitrem B, peramine and ergovaline in perennial ryegrass infected with *N. lolii*. Other bioactive metabolites, both within these original groupings and in new groups, have now been recognised and characterised (Bush *et al.*, 1997; Lane *et al.*, 2000).

Protection provided against herbivory by endophyte infection operates in both natural grasslands and in intensive grazing systems. However, the toxicity toward livestock is an impediment in managed grazing systems and is progressively less tolerable with the increased emphasis on quality of animal product and efficiency of production. Since the discoveries that ryegrass staggers and fescue toxicoses are caused by *Neotyphodium* endophytes in their respective grasses (Bacon *et al.*, 1977; Hoveland *et al.*, 1980; Fletcher and Harvey, 1981) these

disorders have been more precisely described, and other adverse effects on livestock have been uncovered (see below).

Animal toxicoses are not confined to intensively grazed systems. Several toxicoses associated with natural grasslands in the past have now been linked with endophytes, in several grass species (Petroski *et al.*, 1992; Miles *et al.*, 1996).

After the early discovery of a few major bio-active compounds, simple models were derived to account for the tolerance of infected grasses to biotic stresses and their toxicity to livestock.

In New Zealand, *N. lolii* in perennial ryegrass was linked to the neuromuscular disorder of grazing livestock, ryegrass staggers (Fletcher and Harvey, 1981), and the causative agent was shown to be the indole diterpene, lolitrem B (Gallagher *et al.*, 1981). The same ryegrass/endophyte association deterred feeding by the pasture pest Argentine stem weevil (*Listronotus bonariensis*) (Prestidge *et al.*, 1982) through the pyrrolopyrazine alkaloid peramine (Rowan and Gaynor, 1986) which was assumed, from limited evidence, to be non-toxic to grazing livestock. Later, increased body temperatures and respiration rates and decreased serum prolactin levels in sheep were linked with the ergopeptine alkaloid ergovaline, produced by the same ryegrass/endophyte association (Fletcher, 1993a; Fletcher, 1993b). Low livestock growth rates were also associated with infection by *N. lolii* and are probably linked with ergovaline or other ergot alkaloids but this has not been confirmed (Fletcher *et al.*, 1999).

For tall fescue, early work focused on the loline alkaloids as possibly causing the toxicosis syndrome in cattle grazing fescue pastures in USA. However, soon after the involvement of *N. coenophialum* was established, ergot alkaloids were shown to be present in endophyte-infected herbage (Yates *et al.*, 1985) and were recognised as the primary factor in fescue toxicosis (summer syndrome). As with ryegrass, peramine was recognised as a non-toxic insect deterrent. Loline alkaloids may have some activity in mammalian tissues (Oliver *et al.*, 1990) but are now principally recognised as non-toxic broad spectrum invertebrate pest deterrents which may also have a role in drought tolerance (Bush *et al.*, 1997).

While the original 'models' are now recognised as being over simplified, they did focus research into both plant protection mechanisms and livestock toxicosis, and allowed considerable progress toward robust non-toxic pastures (Lane *et al.*, 2000). This progress has further emphasised the complexity of the endophyte-plant-animal-environment interactions in natural and developed grassland and the constraints on some potential opportunities to exploit endophytes for pasture improvement.

The science of grass/endophyte associations has recently advanced on many fronts.

### **Evolution and taxonomy**

There are now consistent and logical hypotheses for the evolutionary development of endophytes, from the sexual *Epichloe* forms to the asymptomatic asexual *Neotyphodium* forms (White, 1997; Wilkinson and Schardl, 1997; Schardl and Wilkinson, 2000; White *et al.*, 2000).

Modern, and more definitive, DNA and molecular-based techniques have refined and confirmed taxonomic groupings developed from earlier studies involving morphology, reproduction, secondary metabolite spectra, host specificity and geographic distribution (Christensen *et al.*, 1993; Bush *et al.*, 1997; Christensen, 1997; White, 1997; Wilkinson and Schardl, 1997; Latch, 1998; Griffiths *et al.*, 1999; Schardl and Wilkinson, 2000). At the same time, the taxonomy of the *Festuca* genus has been critically examined, with the broad-leaved species, *F. arundinacea*, *F. glaucescens*, *F. pratensis* and *F. gigantea*, now known to be more

closely related to *Lolium* than to the fine-leaved fescues (Darbyshire, 1993). It has been proposed that these species be reclassified as *Schedonorus* (Soreng and Terrell, 1997).

Co-speciation and interspecific hybridisation are both believed to have been involved in the co-evolution of natural grass/endophyte associations (Christensen, 1997; Wilkinson and Schardl, 1997; Schardl and Wilkinson, 2000).

Endophyte-infection of temperate (and other) grasses is now recognised as a very widespread phenomenon, and its ecological significance is increasingly clarified (Clay, 1987a; Clay, 1987b).

### **Host-endophyte interaction**

The growth of the endophyte and its host are closely co-ordinated (Schmid and Christensen, 1999; Schmid *et al.*, 2000; Spiering, 2000). The endophyte proliferates in the meristematic zone of the leaf bud. As the bud begins to elongate, the endophyte grows with it, forming a long strand, with very little branching. Further endophyte strands grow into the leaf as elongation proceeds, so that there are more strands near the base of the leaf than at the tip, and usually a discontinuity, with more strands below the leaf ligular zone than above. Growth of all endophyte strands stops at the same time as leaf elongation ceases, so that there is no further proliferation. However, the metabolic activity of the endophyte mycelium is maintained at the same level after elongation ceases. The synchronised elongation of the leaf and the mycelium is all the more remarkable, given that the mycelium elongates at the tip, while the leaf elongates at the base. The degree to which the mycelium grows in the leaf blade, rather than only in the leaf sheath, varies between host endophyte combinations (Christensen, 1997). All the above indicates precise signalling between the host and its endophyte. The nature of this signalling has not yet been elucidated.

### **Biosynthetic pathways**

Model systems have been used to elucidate the ergopeptine and indole-diterpenoid pathways. For the ergopeptine pathway, studies with *Claviceps* have identified enzymes involved in critical steps, and begun to identify gene sequences coding for them (Tsai *et al.*, 1995). Similar progress is being made for the indole-diterpenoid pathway (Miles *et al.*, 1992), leading to lolitrems, through studies of *Penicillium paxilli* (Scott *et al.*, 1999). These pathways and the genes which control them are of great intrinsic scientific interest, and indicate the evolutionary links between apparently dissimilar compounds. More recently-studied compounds can be located within this evolutionary framework (Lane *et al.*, 2000). Knowledge of the pathways may also allow selective elimination or down- or up-regulation of a metabolite, and allow prediction of possible consequences of loss of particular steps in the pathway, so that an end product disappears and is replaced by an intermediate product.

### **Host influence on endophyte activity and biosynthesis**

It has been known for some time that while production of peramine, ergopeptines, lolitrems and lolines were properties of the endophyte, the host plant may influence concentrations accumulated in herbage (Agee and Hill, 1994; Latch, 1994; Ball *et al.*, 1995a; Ball *et al.*, 1995b). Recent results have confirmed earlier observations and provided more precise knowledge. Adcock *et al.*, (1997b) were able to achieve an 86% reduction in ergot alkaloid

concentration in tall fescue herbage by selecting within the host plant population, with a very high realised heritability. Easton *et al* (manuscript submitted for publication) showed with a diallel of ryegrass infected with a common known endophyte strain, that ergovaline concentration was under strong additive control of the host genotype (Table 1). Endophyte concentration in the leaf tissue, determined by quantitative ELISA was also under host control, and accounted for some but not all of the variation in ergovaline.

### **Factors affecting metabolite concentrations**

Seasonal and climatic factors influence the concentration of grass endophyte metabolites (Davies *et al.*, 1993; Justus *et al.*, 1997; Lane *et al.*, 1997a). Seasonal trends have been documented, with all metabolites tending to increase through the spring, sometimes falling somewhat in late spring after the end of reproductive growth, and continue increasing through summer and early autumn, before falling in late autumn and winter (di Menna *et al.*, 1992; Woodburn *et al.*, 1993; Easton *et al.*, 1996). It is unclear how labile grass/endophyte metabolites are, but significant short term (10 day) fluctuations in lolitrem B and ergovaline concentrations have been noted in ryegrass field samples (Fletcher *et al.*, 2001b).

Some of the variation is driven by changes in the pasture profile. True stem and seed head contain more of all the endophyte metabolites than leaf. Leaf sheath contains more ergovaline and (in ryegrass) lolitrem B than the leaf blade, and the crown of the plant may contain very high levels of ergovaline (and other ergopeptine compounds). Older leaf accumulates ergovaline and lolitrem B, but peramine breaks down as the leaf senesces (Keogh *et al.*, 1996). Hence, as stem and seed head develop, then are removed, and as growth slows and falls behind rate of defoliation under summer stress, thus shifting the ratio of leaf blade to sheath and crown, the mean concentrations of endophyte-derived compounds will change. As well as these changes, there are direct effects of ambient conditions.

The greatest overall climatic influence appears to be ambient and/or soil temperature with most metabolite concentrations increasing with temperature (Lane *et al.*, 1997c). However there appears to be an upper threshold above which concentrations of some metabolites can decline rapidly (Adcock *et al.*, 1997a; Fletcher *et al.*, 2001b). Shorter term climatic influences include drought or moisture stress, which tend to increase concentrations of most metabolites (Rottinghaus *et al.*, 1991; Arechavaleta *et al.*, 1992; Lane *et al.*, 1997a). More recently solar radiation has also been proposed as influencing concentrations of some metabolites e.g. lolitrem B (Fletcher *et al.*, 2001b).

Plant nutrition can influence secondary metabolite production. Concentration of several alkaloids increased with increasing levels of soil nitrogen (Arechavaleta *et al.*, 1992; Lane *et al.*, 1997c; Richardson *et al.*, 1999). It has been suggested that endophyte infection increases concentrations of nutrients associated with protein synthesis (Vazquez de Aldana *et al.*, 1999). Soil phosphorus levels may also influence the concentrations of some alkaloids (Azevedo *et al.*, 1993).

None of the endophyte metabolites have been defined as phytoalexins. However there is evidence that concentrations of some increase as a result of damage through either mechanical or biological mechanisms including vertebrate and invertebrate herbivory and possibly disease (Bultman and Murphy, 2000).

### **Biotic stress tolerance of endophyte infected grasses**

Soon after the discoveries that fescue toxicoses and ryegrass staggers were linked with endophyte in their respective grass hosts it became evident that these same endophytes imparted invertebrate pest resistance or tolerance to their host grass (Prestidge *et al.*, 1982). Since then the range of insect pests and nematodes known to be deterred by grass/endophyte associations has grown considerably (Clement *et al.*, 1994; Ball *et al.*, 1997a; Ball *et al.*, 1997b; Ball *et al.*, 1997c; Ball *et al.*, 1997d; Popay *et al.*, 1999; Bacon and White, 2000). A number of species of insect representing five families and a number of nematodes are now known to be affected by endophytes. Although the deterrent endophyte metabolite has not been identified in many cases, the loline alkaloids, peramine, ergot alkaloids and to a lesser degree lolitrems are all known to have insect deterrent or toxic properties (Dymock *et al.*, 1989; Ball *et al.*, 1997c; Ball *et al.*, 1997d; Bush *et al.*, 1997). Some root and soil nematodes and possibly some other root feeding invertebrates are deterred by endophyte (West *et al.*, 1988; Petroski *et al.*, 1990; Eerens *et al.*, 1997; Schmidt and Guy, 1997; Malinowski *et al.*, 1998; Schoberlein *et al.*, 1998; Malinowski *et al.*, 1999b; Pennell and Ball, 1999). Since loline alkaloids appear to be the metabolites most readily shown to be translocated to the roots it is likely that they are the active factors.

There is evidence that endophytes have potential to increase resistance to plant diseases, including pathogenic fungi (Christensen, 1996; Latch, 1998). However, results have been elusive and inconsistent.

### **Increased competitiveness with legumes**

Evidence of enhanced competitiveness of natural grass/endophyte associations in suppressing both competitive and desirable companion species in the same sward continues to grow (Sutherland and Hoglund, 1989; Malinowski *et al.*, 1997b; Hoveland *et al.*, 1999; Malinowski *et al.*, 1999a; Sutherland *et al.*, 1999).

This can reduce the bio-diversity in the sward which may benefit the infected grass but not the total grassland ecosystem (Clay, 1997a; Clay, 1997b). The suppression of legumes, which provide high quality herbage and fix nitrogen, is of concern in developed grazing systems.

While this suppression appears to be largely through enhanced spatial competition for the essential elements of light, water and nutrients, there is considerable evidence of at least some allelopathic effect on competition (Sutherland and Hoglund, 1989; Petroski *et al.*, 1990; Hoveland *et al.*, 1999; Malinowski *et al.*, 1999a; Sutherland *et al.*, 1999). The loline alkaloids in particular have documented allelopathic effects (Petroski *et al.*, 1990; Bush *et al.*, 1997).

These competitive effects appear to be greatest during drought or moisture deficit (Sutherland and Hoglund, 1989; Sutherland *et al.*, 1999). Dilution of toxic effects and improving pasture quality with legumes has been proposed as a management strategy to reduce animal toxicoses, but the greater aggression of endophyte-infected grass may render this option difficult to achieve, especially under dryland conditions.

### **Abiotic stress tolerance**

There is a convincing body of evidence from field and controlled greenhouse trials that infection with their respective endophytes increases drought tolerance of tall fescue and meadow fescue (West and Gwinn, 1993; West *et al.*, 1993; Buck *et al.*, 1997; Malinowski *et al.*, 1997a; Malinowski *et al.*, 1997b; Latch, 1998). However, no effect has been demonstrated in ryegrass (Barker *et al.*, 1997; Eerens *et al.*, 1998). The mechanisms are not known but it is possible that stomatal closure and osmotic adjustment may be affected (Elmi and West, 1995) and that

morphological and/or physiological modification of the plant may play a role (de Battista *et al.*, 1990a; de Battista *et al.*, 1990b; Malinowski *et al.*, 1997a; Malinowski *et al.*, 1997b; Malinowski *et al.*, 1999b). Increased root mass and modification of roots in endophyte infected, compared to non-infected plants, has been reported in some associations.

Endophyte infection in tall fescue has recently been associated with increased uptake of minerals, especially phosphate, in marginal soils but not under high fertility (Malinowski and Belesky, 1999). However, these results did not apply to all of the few clones tested.

Some studies have shown increased efficiency of nitrogen use in endophyte infected grasses but other studies have not confirmed this (Louis and Faeth, 1997).

Enhanced tolerance to salinity and toxic levels of aluminium and copper in soils have also recently been associated with endophyte infection in tall fescue (Liu *et al.*, 1996; Belguendouz *et al.*, 1998; Malinowski and Belesky, 1999). However, (Simpson and Hume, 2001) did not observe any effects of endophyte on salinity response in ryegrass.

Advantages of endophyte infection in grasses have also been demonstrated in other environmental extremes such as high light intensities, high ambient temperatures and high leaf temperatures (Richardson *et al.*, 1993; Marks and Clay, 1996).

In areas where global warming may become a problem, endophyte infection may give a considerable advantage to grasses.

Drought, marginal fertility and soil toxicity are increasing problems in grassland ecosystems in many regions throughout the world. It must therefore be a priority to study and exploit the potential of endophytes to minimise these effects for more productive, sustainable and persistent grasslands.

### **Livestock toxicoses and physiology**

While wide ranging and substantial benefits of endophyte to the production, sustainability and persistence of temperate grasslands has become increasingly evident, so too has toxicity of some grass/endophyte associations to grazing animals (Cross, 1997; Fletcher and Easton, 1997; Oliver, 1997; Fletcher, 1998). Infection of tall fescue with *N. coenophialum* has been linked with the (summer) fescue toxicosis syndrome in cattle, characterised by reduced intake, poor growth rates, lower milk production, thermo-regulatory dysfunction, excessive salivation, shade seeking, reproductive problems and suppression of some hormones including prolactin (Stuedemann and Hoveland, 1988). Disrupted parturition and agalactia have been documented for horses (Cross, 1997).

Ryegrass infected with *N. lolii* causes reduced liveweight gains, ryegrass staggers, increased body temperatures and respiration rates, increased faecal adhesions (sheep) and reduced prolactin levels (Fletcher *et al.*, 1997; Fletcher *et al.*, 1999).

Suppression of immune function and reduction of serum copper levels in cattle has recently been linked with endophyte infection in tall fescue (Dawe *et al.*, 1997; Saker *et al.*, 1998; Schultze *et al.*, 1999).

The ergopeptine alkaloids, including ergovaline, are dopamine agonists as well as powerful vasoconstrictors. Since prolactin secretion is controlled by dopamine, reduction in circulating prolactin is a primary response to ingestion of ergovaline and other ergot alkaloids. Prolactin concentration in plasma or serum is therefore a consistent and reliable indicator of ergovaline or ergot alkaloid intoxication (Cross, 1997). Prolactin is involved in many physiological processes in animals, and reactions such as thermo-regulatory dysfunction and

immuno-suppression may be secondary responses to reduced prolactin levels (Fletcher *et al.*, 1997).

Vaso-constriction appears to be the primary cause of endophyte related thermo-regulatory dysfunction, heat stress and associated symptoms. It is also the major cause of fescue foot. Constriction of blood vessels and capillaries in the peripheries such as skin, ears, tails and feet reduces the animals ability to dissipate excess heat and may cause gangrene in the extremities in cooler weather (Thompson and Stuedemann, 1993). Ambient conditions such as temperature, humidity, wind and solar radiation effect skin vaporisation and heat transfer from the skin to the environment. Reduced intake and consequent reduction in animal weight gain and milk production may be secondary and tertiary responses to heat stress and reduced prolactin levels.

To quantify the environmental stress on the animal, indices such as the temperature/humidity index have been developed, incorporating both ambient temperature and humidity. When the temperature/humidity index rises above 72, a “normal” animal is likely to begin to suffer heat stress. Animals can tolerate higher temperatures in warm dry environments than in humid environments.

Endophyte alkaloids such as ergovaline are likely to have a direct effect on reproductive hormones although some reduction in reproductive performance may be associated with vaso-constriction, heat stress and lower body weights at mating (Watson *et al.*, 1999).

The drug domperidone is a D2 dopamine receptor blocker, which has been shown to alleviate at least some of the symptoms associated with ergovaline and other ergot alkaloids (Cross, 1997). It has proved particularly successful in treating fescue endophyte-related agalactia and delayed parturition of mares. This is an option for treating high value animals, but because of its cost and need for repeated administration, it is currently uneconomic for regular use on a farm scale.

Other prophylactic drugs, mainly dopamine and biogenic amine receptor blockers, have been studied but again they have not been developed for wide scale use (Stuedemann and Thompson, 1993).

Similarly, potential has been demonstrated for vaccines but to date none have been developed for commercial use (Thompson *et al.*, 1993).

The majority of the staggers syndromes seen in animals grazing both developed and natural grasslands are caused by tremorgenic alkaloids produced by grass/endophyte associations including the indole diterpenoids such as lolitrem B in ryegrass. Lolitrem B has also been shown to affect smooth muscle and gut motility (McLeay and Smith, 1999). Although many of the symptoms of the toxicosis syndrome have been linked directly to specific grass/endophyte alkaloids or groups of alkaloids, others may be secondary or even tertiary responses to these primary effects. Immuno-suppression is likely to predispose animals to a range of infections.

The toxicoses in ryegrass and tall fescue result in substantial production losses especially in USA and New Zealand and possibly in other countries where their impact has not been quantified.

### **Milk production effects**

Milk production is impaired in cows grazing endophyte infected tall fescue (Schimdt and Osborn, 1993). The effect of forages on milk production in dairy cows can be difficult to assess in short-term studies with limited areas of pasture when the differences are small. Cows grazing endophyte-infected ryegrass showed impaired production relative to cows on endophyte-free

ryegrass in an Australian trial (Valentine *et al.*, 1993), but results in New Zealand have been inconsistent (Thom *et al.*, 1999).

A recent trial compared perennial ryegrass with its wild-type endophyte and the same ryegrass cultivar infected with a non-toxic endophyte ('AR1' - producing neither lolitrem B nor ergovaline). Milk production in dairy cows grazing the AR1 association had 8% higher milk production than those grazing the wild-type controls (Bluett *et al.*, 2001). Since the endophyte toxin ergovaline causes thermo-regulatory dysfunction in animals it is likely these effects could be greater in warm humid climates.

A closely monitored farm comparison between cows grazing mixed ryegrass/legume pasture naturally infected with wild-type endophyte and cows grazing similar mixed pasture but with the ryegrass either endophyte-free or infected with AR1, showed cumulative milk solids production over 6 months to be 19% greater in the second group (Figure 1) (Blackwell and Keogh, 1999).

Further large scale milk production trials, especially where the temperature humidity index rises above 72, are needed to determine the extent and magnitude of endophyte effects on milk production.

### **Synergistic action of metabolites**

Simple models of ryegrass and tall fescue toxicoses have ascribed symptoms to specific alkaloids or alkaloids with similar bioactivity within the same group.

Recent demonstrations of apparent synergies between grass/endophyte metabolites in animal toxicoses are evidence of the complexity of endophyte/grass/animal interactions. An example of possible synergy is the apparent enhanced toxicity of lolitrem B in the presence of ergovaline or other ergot alkaloids in endophyte infected ryegrass (Fletcher and Easton, 1997; Bluett *et al.*, 1999).

Ergovaline has been considered the major cause of fescue toxicosis (summer syndrome) yet when pure synthetic ergovaline was fed to cattle along with a diet of endophyte-free tall fescue, the symptoms were less severe than in control animals fed endophyte infected tall fescue seed with the same level of ergovaline (Gadberry *et al.*, 1997). It appears that other factors in tall fescue with its wild-type endophyte may enhance the toxicity of ergovaline (Lane *et al.*, 1999).

Synergies among grass/endophyte metabolites are unlikely to be restricted to mammalian toxicity and may also exist among other bioprotective grass/endophyte metabolites.

### **Release of selected endophytes**

The potential to capitalise on the benefits of endophyte for pasture improvement while eliminating or reducing toxic effects in grazing animals has been recognised (Latch, 1989). Specific objectives for pasture improvement vary with climate, region and farm type. However higher and more reliable annual and seasonal herbage production, reduction of anti-quality factors and improved disease and pest resistance or tolerance are universal (Fletcher and Easton, 1997). The endophyte-related benefits of invertebrate pest resistance, drought tolerance and possibly disease resistance fit with at least some of the objectives for pasture improvement.

Opportunities to capitalise on these can be placed in four broad categories:

- Continue to utilise adapted wild-type endophyte/grass associations but eliminate or alleviate toxic responses in grazing animals through vaccines, drugs, feed additives, detoxification,

pasture management or through breeding animals resistant to toxins or combinations of all or some of these.

- Continue to use wild-type endophyte/grass associations but breed and select grass genotypes, which suppress the production or expression of mammalian toxins.
- Genetically modify one or both symbionts to eliminate mammalian toxins and maintain or enhance disease, insect and drought tolerance. Use the endophyte as a vector to introduce desired genes into forage grasses.
- Develop persistent, insect, disease and drought resistant, non-toxic (to mammals) grass/endophyte associations selected from natural populations of both symbionts.

The advantage of utilising wild-type endophyte/grass associations is that they have already adapted, over a long period, to the biotic and abiotic stresses encountered in the environment in which they will continue to be used.

Large areas of pasture naturally infected with endophyte are grazed, and management options have been developed enabling farmers to minimise the adverse effects on livestock. These involve grazing systems that avoid grazing close to the crown of the plant, use of legumes in the sward and the availability of alternative pasture types to be used when endophyte toxins are at their highest concentrations. However, these constraints on grazing management are not always practicable, particularly during a prolonged period of water stress. The claims of feed additives have not been substantiated, and except for the use of domperidone with horses, palliative drugs and vaccines have not progressed. Selection of livestock genetically tolerant of endophyte-related toxicoses is not unimportant (Morris *et al.*, 1999), but minimising exposure to toxins remains an essential objective.

Considerable potential has also been demonstrated for the suppression or reduction of animal toxins through selection and/or breeding within the grass genome (Adcock *et al.*, 1997b), but it is unlikely to eliminate toxin production completely and there will still be the possibility of toxic concentrations occurring in conditions of stress.

While the benefits and disadvantages of natural endophytes in tall fescue and ryegrass are inseparable in many natural associations, considerable diversity in alkaloid profiles and concentrations exists (Latch, 1989; Christensen *et al.*, 1991; Fletcher *et al.*, 1991; Lane *et al.*, 1999; Lane *et al.*, 2000). This has been exploited in the development of selected endophytes in ryegrass and tall fescue which are non-toxic to grazing animals but retain the majority of their bioprotective properties. Endophytes from natural associations which do not produce the known mammalian toxins are isolated in culture then re-inoculated into elite endophyte-free germplasm of the same grass species (Latch and Christensen, 1985).

'MaxQ', (AR542) a tall fescue endophyte strain which does not produce ergovaline, has been available in USA in the northern autumn 2000 in two tall fescue cultivars. These experimental associations, and others like them, have proved successful in herbage production and grazing animal research evaluations, both in New Zealand and USA (Tables 2 & 3) (Bouton, 2001; Fletcher *et al.*, 2001a; Waller *et al.*, 2001).

'AR1' which is a non-toxic endophyte from ryegrass, developed in New Zealand, is also close to release in a number of proprietary ryegrass cultivars. These and other similar experimental associations have also undergone rigorous evaluation for pasture production, persistence and non-toxicity to grazing animals. These associations do not produce the toxic alkaloids lolitrem B and ergovaline but still produce the insect deterrent peramine, at similar levels to the wild-type strains.

They have proved resistant to most above-ground invertebrate pests deterred in wild-type associations (Figure 2) (Popay *et al.*, 1999) although they may be less resistant to black beetle (*Heteronychus arator*) than wild-type associations (Popay *et al.*, 2001).

Health and performance responses of lambs grazing ryegrass infected with AR1 were similar to those grazing endophyte-free pasture and significantly better than for lambs grazing wild-type endophyte treatments, for all parameters measured (Table 4) (Fletcher, 1999).

While the development of MaxQ and AR1 endophyte associations represent major advances in the control of endophyte toxicoses, the complexity, risks and constraints associated with these and future developments have also been exposed.

### **Issues with selected endophytes**

#### *Endophyte detection and chemotyping*

The increasing diversity of endophytes used in research and grazing systems, and protected by patent has highlighted the need for further development and refinement of rapid, reliable techniques for detection, identification and chemo-typing of endophytes. Currently immunoblot techniques are the most commonly used for non-quantitative detection of endophytes while most quantitative, or semi-quantitative, detection techniques are ELISA based. Immuno-blot techniques developed using monoclonal antibodies can distinguish between endophytes at the species level (Hiatt *et al.*, 1997a; Hiatt *et al.*, 1997b). More definitive, but slower and more costly, DNA and molecular based techniques are required to distinguish between sub species and strains (Doss *et al.*, 1998; Griffiths *et al.*, 1999). Simpler and more routine techniques need to be developed for identification at this level. Quantitative chemical profiles in grass/endophyte associations are currently determined using variations of HPLC and gas chromatography techniques (Shelby and Flieger, 1997; Lane, 1999; Lane *et al.*, 1999). Rapid, semi-quantitative techniques using ELISA have been proposed and are being developed for the detection of ergovaline but these are currently limited by cross reactivity with lysergyl compounds. Refinement of these techniques for greater specificity is a priority. This would speed up the screening and chemotyping of potentially useful endophytes and facilitate more comprehensive quality assurance programmes for 'non-toxic' endophytes released to the farming industry.

#### *Host-symbiont compatibility*

Incompatibility of targeted symbionts can be a major constraint in the selection and development of non-toxic grass/endophyte associations for pasture improvement, rendering many desired or potentially useful associations unachievable. These constraints will naturally be greatest in cross species inoculations but they can occur in the transfer of endophytes between populations of the same grass species.

Cross species inoculations could increase the potential for useful grass/endophyte associations and while they have been achieved experimentally, the issues of incompatibility, sustainability and unexpectedly high concentrations of some alkaloids, become more acute (Christensen, 1995; Chung and Schardl, 1997; Schardl *et al.*, 1997; Ferguson and Rice, 1998; Naffaa *et al.*, 1999).

Even when initial compatibility, after inoculation, appears acceptable, the transmission through to successive generations may be incomplete and unsatisfactory. This incomplete

transmission within a population can occur at the whole plant or individual tiller level (Ravel *et al.*, 1997), or within different branches of the same panicle (Wilson and Easton, 1997). Incomplete transmission has also been reported in some wild-type associations particularly in the case of tall fescue (Welty and Azevedo, 1993). While the mechanisms and factors associated with compatibility are as yet not well understood there has been considerable progress in identifying taxonomic groups of potential symbionts which are likely, or unlikely, to form compatible associations with a range of hosts (Naffaa *et al.*, 1999). There may also be opportunities to improve partial or incomplete compatibility and/or transmission through natural or improved selections among surviving associations, over time. A population of ryegrass infected with the selected strain AR1 showed considerable variation between families in vigour and pest tolerance (Easton *et al.*, 2001).

Genetically modified endophytes may also be exposed to some risk of incompatibility or incomplete transmission, although this is likely to be minimal where the modified endophyte is inoculated back into its original host genotype.

#### *Unexpected metabolites and extreme levels of known metabolites*

The risk of previously un-encountered toxic metabolites and unexpected extreme levels of known alkaloids also needs constant consideration and monitoring when manipulating grass/endophyte associations (Latch, 1994; Lane *et al.*, 1997b).

#### *Seed storage*

Endophyte in seed loses viability within months unless humidity and temperature are held low (Rolston *et al.*, 1986). Seed store and transport conditions are critical in managing grass seed infected with selected endophyte, especially under warm humid conditions. Progress has been made in defining minimum requirements. Critical upper limits in short term bulk storage for temperature, relative humidity (RH) and seed moisture are 10°C, 50% and 11% respectively.

Seed stored below 5°C, 50% RH and 11% seed moisture has maintained viable endophyte levels for up to 15 years. Conversely there are reports of viable endophyte levels reduced to zero in seed stored for less than 3 months at 25°C and 70% RH (M. P. Rolston *pers. comm.*).

Necessary conditions for endophyte survival in seed are relatively easy to achieve for longer term storage at purpose built facilities, or warehouses, but at considerable expense. It is likely to be more difficult to achieve and maintain these conditions during transit or on-farm storage. Development of suitable packaging to maintain seed at an acceptable low moisture is progressing (Rolston and Rowarth, 1995). In cooler drier climates such as most of New Zealand, storage and transport of ryegrass seed under normal conditions has been satisfactory for endophyte viability, provided seed is sown in the same year as harvest.

Harvest timing, conditions, and practices from cutting through to threshing, may affect endophyte transmission and long term viability and is a subject of current research.

#### *Pasture establishment and management*

Assuming the successful development of non-toxic grass/endophyte associations, strategies to successfully establish and manage pastures with these associations need to be developed to maximise their benefits to the livestock producer. The first priority is to establish the new pasture free of undesirable contaminants, especially plants of the same species infected

with toxic wild-type endophyte. Similar strategies will be required to maintain purity during seed increase (Hume, 1999). The proportion of wild-type contaminants may increase over time in some situations (Vught *et al.*, 1997; Gwinn *et al.*, 1998).

While some methods of establishment to minimise the risk of contamination are still being studied, others have already been prescribed (Hume *et al.*, 1997; Hume *et al.*, 1999).

Once the new pasture is established contaminant-free, maintenance over the life of the pasture must be considered. Major potential sources of contamination include hay, containing seed of contaminants, and faeces from animals which have recently grazed toxic pastures with mature seed heads present. The magnitude of these risks has been assessed in the case of contaminated faeces in grazing animals and is being assessed for contaminant hay being fed out on non-toxic pastures (Rolston *et al.*, 2001).

The risk of overgrazing is always a factor in pasture management but may be greater in the case of non-toxic grass/endophyte associations. In some circumstances, endophyte-free ryegrass is grazed more intensively than pasture infected with wild-type endophyte (Edwards *et al.*, 1993). Selected associations, free of the major toxins which tend to protect against overgrazing, may similarly be more palatable than their wild-type counterparts.

While good pasture management will be essential for maximisation of benefits from non-toxic grass/endophyte associations, most of the associated risks have been assessed and quantified and sound management techniques have been documented. Their success however will be in the hands of individual grassland farmers.

### **Priorities for development**

Research is revealing greater complexity of chemical defences and mammalian toxins produced by endophytes in conjunction with their host grass. However this complexity should not detract from opportunities to exploit or utilise endophytes for improvement of temperate grasslands. The bio-protective and stress tolerant properties imparted by endophytes have much to offer temperate grasslands especially in the more stressful environments.

To identify these opportunities, studies on the range of grass/endophyte/environment interactions in natural and developed grasslands must continue. More specifically the mechanisms and chemistry involved in bio-protection need further elucidation while the extent of bio-protective properties needs to be explored. This will include further exploration of the range of metabolites produced by grass/endophyte associations, establishing links between specific metabolites and bio-protective properties and, exploring and quantifying factors which influence the animal toxicity.

Drought, marginal fertility, and soil toxicity are major issues for many temperate grasslands throughout the world. Recent studies have identified changes in the rhizosphere chemistry, root morphology, root function, mineral uptake in marginal soils and tolerance of toxic and low pH soils associated with endophyte/grass associations. This presents exciting opportunities for future research, addressing endophyte/host communication, mineral uptake and sequestration mechanisms, and mineral use efficiency.

Dealing with latent or new stresses in target environments will also require continuing development and refinement of the ideal grass/endophyte association.

There are still opportunities to reduce toxicity to grazing animals of well adapted wild-type endophyte/grass associations in developed grazing systems, through animal remedies and management. These approaches will be important for areas where toxic associations can never be eliminated or replaced. Breeding or selection of grass germplasm which reduces the expression of

grass/endophyte toxins needs to continue especially in conjunction with other approaches i.e. management and animal remedies. However complete elimination of toxic principles through either selected or genetically modified endophytes is likely to be a more attractive and sustainable option.

Considerable progress has been made in developing selected non-toxic endophyte strains for grazing systems. Better understanding of the diversity of grass/endophyte associations, the specific interactions within them and the spectrum of the metabolites they produce is likely to open up even greater prospects for grass/endophyte improvement. The resources of grass and endophyte genotypes which could be used for bio-protection and increased stress tolerance in grasses have only begun to be explored.

As the understanding of grass/endophyte/animal/environment interactions increases, selection, quality assurance and evaluation criteria will be refined or changed as the perception of the ideal associations changes.

In addition to selecting suitable non-toxic naturally occurring endophytes with the required attributes, potential exists to more specifically 'tailor' the 'ideal' endophyte through genetic modification. While this approach has considerable attractions it is as yet unproven and may also be constrained by public acceptance.

"Grass/endophyte associations have been built over millennia, deliberate human intervention is only recent. The development of a more complete understanding of the complexity of grass/endophyte associations and their chemical ecology is a challenge to the scientific community. In return it has the potential for 'improved' grass/endophyte associations." (Lane, 1999).

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**Table 1** - Host-controlled variation in endophyte mycelium and ergovaline concentrations in ryegrass leaf sheath – glasshouse trial. (Easton et al, manuscript submitted for publication)

	Mycelial concentration (ELISA - arbitrary units)	Ergovaline concentration ( $\mu\text{g.g}^{-1}$ )
Maximum parent	60.0	14.8
Minimum parent	5.9	0.9
Maximum progeny family mean	45.5	15.5
Minimum progeny family mean	25.2	4.6
Parent-progeny regression	0.67	0.92
Heritability (Ex ANOVA mean squares)	0.68	0.56
Correlation ergovaline/mycelium in progeny family means		0.72

**Table 2** - Results of a lamb grazing trial during the spring grazing period for 1998 and 1999 at Eatonton, GA. (Bouton *et al* 2000).

<u>Cultivar</u>	Endophyte <u>Strain †</u>	<u>Forage</u>		<u>Animals</u>	
		Available <u>Yield</u> <i>lbs/acre</i>	Ergot <u>Alkaloids</u> <i>ppm</i>	Average <u>Daily Gain</u> <i>lbs/day</i>	Blood <u>Prolactin</u> <i>ng/ml</i>
Jesup	E+	1780	3.6a‡	0.19	4 b‡
Jesup	E –	1496	0 b	0.27	188a
Jesup	AR542	1837	0 b	0.29	217a
Georgia 5	AR542	1939	0 b	0.30	178a
LSD (<0.05)		NS	-----	0 04	-----

†E + = Contains *Neotyphodium* strain(s) naturally occurring in the cultivar; E – = Nil or *Neotyphodium* free cultivar; AR542 = Non-toxic AgResearch *Neotyphodium* strain not producing ergot alkaloids.

‡ Means within column followed by the same letter are not significantly different (p<0.05) by LSD comparisons calculated on data subjected to square root transformation.

**Table 3** - Beef steer performance trial at Eatonton, GA during the spring and fall grazing periods for 1999. (Bouton *et al* 2000).

<u>Cultivar</u>	<u>Endophyte Strains §</u>	<u>Spring †</u>		<u>Fall ‡</u>	
		<u>Average Daily Gain</u>	<u>Gain per Acre</u>	<u>Average Daily Gain</u>	<u>Gain per Acre</u>
-----lbs-----					
Jesup	E +	1.7	83	1.9	169
Jesup	E -	1.9	93	2.7	235
Jesup	AR542	2.5	123	2.6	223
Georgia 5	AR542	2.7	133	2.7	237
	LSD (<0.05)	0.7	36	0.5	42

† Stocking rate = 1.0 steer per acre; grazing period = 49 days

‡ Stocking rate = 1.5 steers per acre; grazing period = 58 days

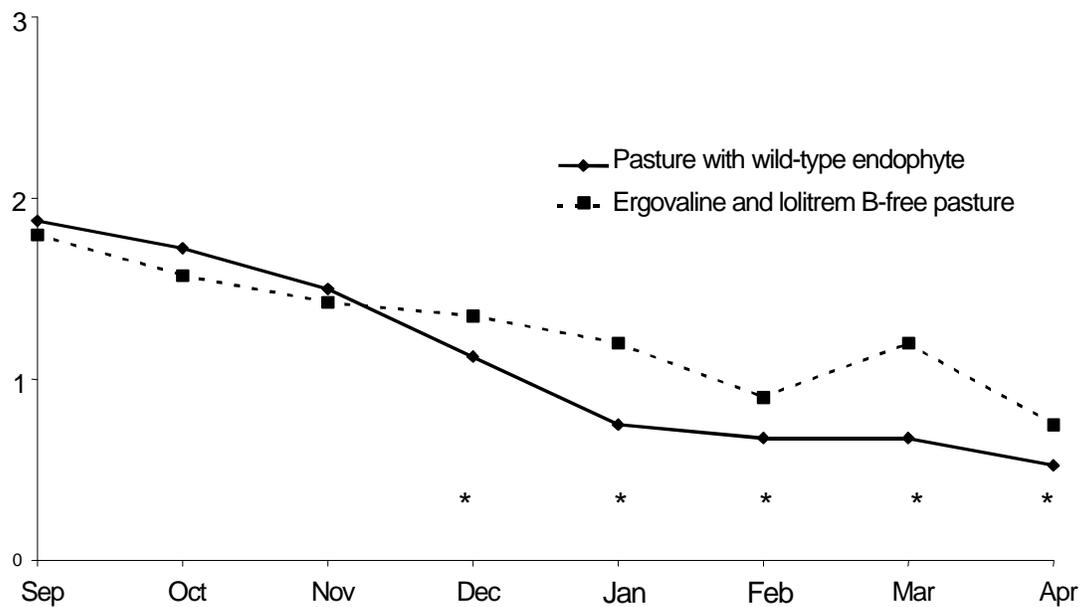
§ E+ = Contains *Neotyphodium* strain (s) naturally occurring in the cultivar; E - = Nil or *Neotyphodium* free cultivar; AR542 = Non-toxic AgResearch *Neotyphodium* strain not producing ergot alkaloids.

**Table 4** - Lamb responses to grazing Nui ryegrass with AR1 endophyte in summer and autumn. Means of 3 years. (Fletcher 1999).

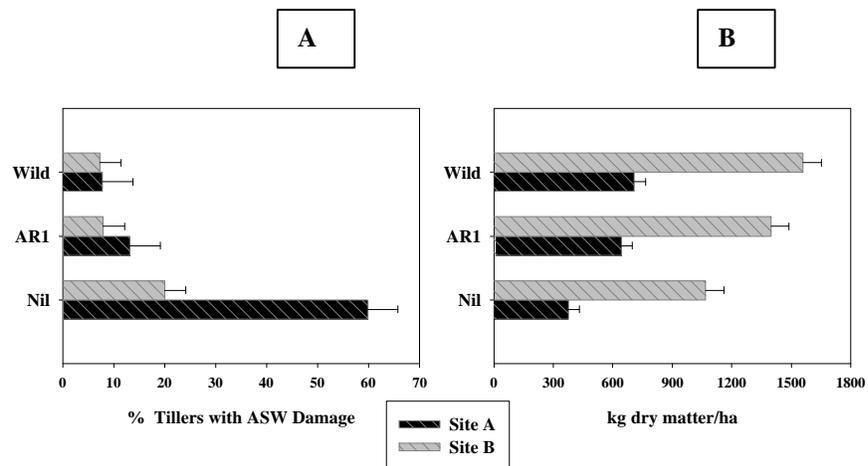
Mean	LWG g/hd/day	Rectal temp °C	Resp rate /min	PRL ng/ml	RGS 0-5 scale	Dags 0-5 scale
Nui/wild-type	23 <sup>a†</sup>	40.5 <sup>a</sup>	97 <sup>a</sup>	96 <sup>a</sup>	3.2 <sup>a</sup>	0.6 <sup>a</sup>
Nui/nil	120 <sup>b</sup>	40.0 <sup>b</sup>	73 <sup>b</sup>	185 <sup>b</sup>	0 <sup>b</sup>	0.2 <sup>b</sup>
Nui/AR1	131 <sup>b</sup>	40.1 <sup>b</sup>	79 <sup>b</sup>	203 <sup>b</sup>	0.3 <sup>b</sup>	0.3 <sup>b</sup>

† Values within a column with a letter subscript in common are not significantly different.

LWG= liveweight gain, Resp = respiration, PRL= prolactin, RGS = ryegrass staggers



**Figure 1** - Dairy milk solids production as affected by endophyte toxins. Reproduced with permission (Blackwell & Keogh 1999).



**Figure 2** - (A) Effect of the wild-type endophyte (wild), the novel endophyte, AR1, and endophyte-free (Nil) ryegrass on tiller damage by Argentine stem weevil (ASW) larvae at two sites in the North Island of New Zealand, and (B) the associated ryegrass yields after damage had occurred. Extracted with permission from Popay *et al* (1999).

