Endophytes improve ryegrass persistence by controlling insects

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Abstract
Fungal endophytes reduce populations and damage by several different insect pests which alone or in combination with each other can reduce ryegrass persistence. AR1, AR37, the Standard endophytes and two endophytes in tetraploid ryegrasses, NEA2 in cv. Bealey and Endo5 in cvs. Quartet II and Banquet II, are available to the New Zealand farmer. Their effects on insects depend on the spectrum and concentration of alkaloids they produce. Argentine stem weevil is controlled by all endophytes except perhaps Bealey NEA2; black beetle is controlled by all endophytes except AR1; pasture mealybug are controlled by AR1, AR37 and Standard and likely to also be controlled by Endo5 and NEA2; porina and root aphid are controlled by AR37. Increased persistence of ryegrass infected with endophyte is reflected in increases in endophyte infection rates in pasture, higher yields and tiller densities and reduced weed invasion, particularly where climatic stress is also present. AR37, with the protection it provides against a range of insect pests, is the most effective endophyte for improving persistence.
Keywords: alkaloids, Argentine stem weevil, black beetle, insect damage, *Neotyphodium*

Introduction
The fungal endophytes (*Neotyphodium* spp.) of grasses grow systemically within their hosts, have no external stage and are maternally transmitted. These symbiotic fungi produce a spectrum of alkaloids which have effects on both insect and mammalian herbivores. The type of alkaloid produced is a property of the strain of the fungus itself, while various factors, such as the environment and plant genotype, can modify the concentration of these alkaloids within a plant (Lane *et al*. 2000). All three major alkaloids produced by the Standard ryegrass endophytes (*Neotyphodium lolii* Glenn, Bacon & Hanlin) (also known as common or Wild-type strains), peramine, lolitrem B and ergovaline, have effects on insects (Popay & Bonos 2005) and the last two also cause toxicity in livestock. Different species of insects are affected by different alkaloids (Popay & Bonos 2005). Exploitation of the endophytes for the benefit of New Zealand agriculture has relied on acquiring new strains of fungi that can provide protection against insect pests while eliminating or minimising the adverse effects of the endophyte on grazing mammals.

Discoveries in the early 1980s that *N. lolii* caused ryegrass staggers (Fletcher & Harvey 1981) but also protected the plant from Argentine stem weevil (ASW) (*Listronotus bonariensis*) attack (Mortimer *et al*. 1982; Prestidge *et al*. 1992) launched a major research programme designed to harness the benefits of endophyte without harmful effects on livestock. AR1 met these criteria, providing strong protection against ASW (Popay *et al*. 1999) without causing either ryegrass staggers or heat stress in sheep (Fletcher 1999). During the development of AR1, another endophyte, AR37, came to the fore because of the resistance it conferred to a range of insect pests and the agronomic advantages associated with that. AR1, AR37 and two other endophytes, NEA2 and Endo5 in certain tetraploid cultivars, are now commercially available to New Zealand farmers.

Endophytes can increase ryegrass productivity. In a controlled environment, indoor experiment, Latch *et al*. (1985) found Standard-infected perennial ryegrass had 38% greater dry matter, more leaf area, tillers, pseudostems and roots than did the endophyte-free clones. These apparent direct effects of endophyte infection on growth, however, have not been replicated in outdoor experiments (Hume 1993; Barker 1997). Other studies have demonstrated endophyte-mediated increases in ryegrass productivity associated with insect resistance (Popay *et al*. 1999; Popay & Gerard 2007; Hume *et al*. 2007). Increased yields, however, do not necessarily translate to greater persistence. Persistence is about maintaining a sward with the desirable species with which a pasture was sown. Factors that reduce pasture persistence are those that cause mortality of sown species or reduce plant vigour to a point at which plants cannot recover. This allows ingress of unsown species, reducing both productivity and nutritive value of the pasture. In this paper we will review existing published information and present some new data on the effect of different endophyte strains on a range of insect pests and examine the evidence for increased persistence of ryegrass due to endophyte infection controlling these insects.

Effects of different endophytes on insects

Understanding the chemical basis of the effects of endophyte on insects has been an important part of the development of new endophytes. After the discovery that ASW was affected by the Standard endophyte, peramine was identified as the primary source of that resistance, strongly deterring adult weevil feeding (Rowan *et al*. 1990) and, to a lesser extent, the damaging larval stage (Dymock *et al*. 1989). Although ergovaline and lolitrem B also affected ASW in different ways (Prestidge *et al*. 1985; Popay *et al*. 1990), their toxicity to livestock negated any beneficial effects of their presence. The AR1 endophyte, which produces only peramine, provided control of ASW that was as effective as the Standard strains (Popay *et al*. 1999) with no adverse effects on sheep. Unexpectedly, however, another endophyte, AR37, proved to be just as effective in reducing ASW larval damage as AR1 (Popay & Wyatt 1995; Popay & Thom 2009) despite not producing peramine and not having any effect on adult weevil feeding or oviposition (Popay & Wyatt 1995). Observations (A.J. Popay, unpublished data) indicate that AR37 is toxic to young larvae but it is not known if epoxy-janthitrems produced by this endophyte are responsible for this effect.

Black beetle (*Heteronychus arator*), a sporadic but serious pest in parts of the North Island, particularly on well drained soils (Bell *et al*. 2011), is also affected by the Standard endophyte (Ball & Prestidge 1992). As for ASW, the Standard endophyte deters the adult, reducing egg laying in infected ryegrass pastures (Prestidge *et al*. 1994) and thus resulting in fewer of the root-feeding larvae that cause extensive damage. Ergopeptine alkaloids, including ergovaline, were identified as metabolites most probably responsible for resistance to black beetle while neither peramine nor lolitrem B had any effect (Ball *et al*. 1997). AR37 (as Lp14) similarly deters black beetle adult feeding (Ball *et al*. 1994), suppressing population increases in the field (Popay & Thom 2009). On the other hand AR1 has only a moderate effect on this pest (Popay & Baltus 2001) and populations increase faster on AR1-infected ryegrass than on either AR37 or Standard (Popay & Thom 2009).

Three other pests capable of inflicting damage to ryegrass that can be controlled by endophyte are a root aphid (*Aploneura lentisci*), pasture mealybug (*Balanococcus poae*) and porina (*Wiseana cervinata*). Pasture mealybug is controlled by AR1, AR37 and the Standard endophyte (Pennell *et al*. 2005) whereas strong protection against root aphid and porina is provided only by AR37 (Popay & Gerard 2007; Jensen & Popay 2004). The Standard endophyte also affects root aphid but effects appear to be transient (Popay *et al*. 2004). Ryegrass with AR1 often appears to be more susceptible to root aphid than endophyte-free ryegrass (Popay *et al*. 2004).

Although the spectrum of alkaloids is the property of the fungal strain, the amount produced is determined by the host plant-endophyte interaction and is a heritable trait in the host grass (Lane *et al*. 2000; Easton *et al*. 2002). This has been used in the development of the commercial endophyte, Endo5 in the tetraploid ryegrasses, Quartet II and Banquet II, which have low levels of the mammalian toxin, ergovaline, and moderate to high levels of peramine. NEA2, an endophyte that is used in association with a tetraploid cv. Bealey, is a mixture of two distinct endophyte strains, known separately in the NZ Plant Variety Rights system as 'NEA2' and 'NEA6', of which NEA2 is the dominant strain in Bealey. The NEA2 combination in Bealey produces ergovaline, peramine and lolitrem B. In Tolosa, a diploid cultivar from which Bealey was derived, peramine, ergovaline and lolitrem B occur at low concentrations, with peramine levels substantially lower than those in Samson infected with the Standard strains (van Zijll de Jong 2008). Peramine concentrations in tetraploids are significantly lower than their diploid relatives (Popay *et al*. 2003). Average leaf lamina concentrations of peramine for three samples taken at monthly intervals over summer from a field trial were low in Bealey NEA2, but moderate to high in the two cultivars infected with Endo5 (Table 1).

Ergovaline produced by both NEA2 and Endo5 ensures that these endophytes provide good protection against black beetle. In a pot trial, Banquet II and Quartet II with Endo5, Bealey NEA2 and Extreme AR6 had a significantly lower percentage of tillers damaged than the endophyte-free control (Table 1). The relative susceptibility of the NEA2 and Endo5/tetraploid associations to ASW is unknown but previous work with other endophyte-infected tetraploids shows that they can be significantly damaged by this insect (Popay *et al.* 2003). The peramine levels are a critical factor in this. It has been estimated that approximately 15 µg/g is needed to provide effective resistance against ASW (Popay & Wyatt 1995; Popay *et al*. 2003). On this basis, concentrations in Banquet II Endo5 may be marginal while clearly levels in Bealey appear too low deter the adult (Table 1). Ergovaline and lolitrem B produced in Bealey NEA2, albeit also at low concentrations, may give some measure of protection since both these alkaloids can also affect this insect (Prestidge *et al*. 1985; Popay *et al*. 1990), but experience in the field suggests that ASW can damage this association (Popay unpublished) .

Root aphid populations per plant in a 20 replicate pot trial with different endophytes were lowest on

Table 1 Peramine concentrations (mean of three field samples taken monthly over summer), level of damage by black beetle and mean number of root aphid/plant in two pot trials compared with a diploid cultivar (Extreme) infected with AR6; and mean root aphid infestation score in a field trial for two different tetraploid/endophyte associations.

Cultivar	Endophyte	Peramine (μg/g)	% tillers with black beetle damage	Mean no. root aphid/plant	Mean root aphid score
Extreme	AR6	20.1	0.6	271	-
Quartet II	Endo5	21.1	4.9	605	-
Banquet II	Endo5	11.0	4.4	222	0.94
Bealey	NEA2	3.1	6.5	378	2.78
Quartet Nil	-	-	20.3	455	-
SE			2.4	N/A[1]	0.53
Significance			<0.001	0.059	<0.05

[1] Analysis carried out on log transformed data

Banquet II Endo5 and highest in Quartet II Endo5 (P=0.059) (Table 1). In a field trial at Ballarat in Australia, in which samples were taken from plots and scored for root aphid infestation, the score for Banquet II was similar to that in Extreme AR37 and both were significantly less infested than Bealey NEA2 (A.J. Popay and R. Hill unpublished). Bealey NEA2 had lower infestations than Extreme AR1, however, reflecting the greater susceptibility of ryegrass infected with this endophyte to this insect (Popay *et al*. 2004). The effect of the endophytes in tetraploids on pasture mealybug is unknown but given this insect's sensitivity to endophyte it seems likely that they are effective.

Insect damage and pasture persistence

Most damage assessments regarding insects focus on losses in productivity and not pasture persistence, although there is some relationship between these two. Studies have helped define economic thresholds in terms of yield reductions (Zydenbos *et al*. 2011, this volume) but changes in pasture composition resulting in ingress of less productive species with low nutritional value, although known to occur (see Zydenbos *et al*. 2011, this volume), are not usually factored in. Some studies have used insecticides to manipulate pest populations and demonstrated both an increase in pasture production and persistence (Henderson & Clements 1979; Clements *et al*. 1991).

Insects that have the greatest effect on ryegrass mortality are those that damage the growing points and roots. Argentine stem weevil larvae destroy tillers by mining the central part of pseudostems and tiller growing points, while black beetle and grass grub (*Costelytra zealandica*) larvae feed on roots. Pasture mealybug and root aphid reduce plant vigour by sucking the nutrients out of plants. Insects that feed on herbage can be less damaging than these pests because grasses are adapted to grazing but porina, for instance, are wasteful feeders that often sever tillers close to ground level reducing plant survival. The damage insects do is exacerbated by environmental stress and grazing pressure from livestock. With the exception of porina, all these pests are capable of causing damage in the summer-autumn period, when drought and high temperatures also place ryegrass under considerable stress. Moisture stress is probably the most important. For those plants that have damaged roots, or reduced root growth as a result of reduced vigour, access to moisture will be severely compromised. Thus endophytes that protect against root-feeding insects are most likely to also influence persistence.

Little is known about the combined effects of several invertebrate pests, including plant-parasitic nematodes, where the population densities of each species may not exceed its economic threshold. Their damage is likely to be additive, if not compounded, in terms of reducing pasture productivity and persistence. This was illustrated in a trial at DairyNZ's Scott Farm where a trend in differences in ryegrass tiller densities between endophyte treatments (AR37 6040/m^2, Standard 5246/m^2, AR1 4574/m^2, Nil 4192/m^2) was starting to occur within just 2 years of establishing new pastures (Thom *et al*. 2008). None of the monitored insect species present, including black beetle, grass grub, root aphid and Argentine stem weevil, were individually present at economic threshold levels. The total population densities, however, of the two most damaging pests, grass grub and black beetle for AR37, Standard, AR1 and Nil respectively were 34, 38, 50 and 84/m^2. With significant levels of damage to Nil from ASW (average of 25% of tillers damaged in January) and pressure from root aphid infestations in 2, 10, 32 and 20% of samples for AR37, Standard, AR1 and Nil, the combined pest pressure reflected and may well account for the changes in tiller density (Popay & Thom 2009).

Persistence, endophyte and insect control

The benefits of endophyte for both ryegrass productivity and persistence were first recognised in 1982 when it was observed in a trial that swards with low endophyte infection were severely damaged by ASW. In this trial, tiller density of the hybrid ryegrass, 'Grasslands Manawa', with a 10% endophyte infection rate was reduced by 85% by ASW damage relative to an insecticide treated control (Prestidge *et al.* 1982). In comparison, there was a 30% reduction in untreated paddocks relative to treated for Ellett ryegrass with an endophyte infection rate >67%. Hunt *et al*. (1988) also used insecticide treated and untreated comparisons to show that ASW damage reduced ryegrass content of swards.

Since then, many studies have demonstrated poor survival of endophyte-free pastures due to ASW, as they quickly become replaced by endophyte-infected ryegrass (e.g Prestidge *et al*. 1984; Thom *et al*. 1999; Popay *et al.* 1999; Burggraaf & Thom 2000) or other species. Although the introduction in the early 1990s of a parasitoid, *Microctonus hyperodae*, has helped suppress ASW populations it has not obviated the need to have endophyte-infected grasses. This is due in part to the potential for ASW to still cause significant damage in seasons where the parasitoid is not highly effective, and also to the protection that endophyte can provide against insects other than ASW. Endophyte infection rates in plots sown with endophyte-free ryegrass, which have a low level of endophyte contamination, have been shown to increase after attack by black beetle (Popay & Thom 2009) and pasture mealybug (Pennell *et al*. 2005). In cooler climates and where there is little pest damage, infection rates in pastures sown as endophyte-free can stabilise at low levels (e.g. Eerens *et al*. 1998; McNeill *et al*. 2001, 2007).

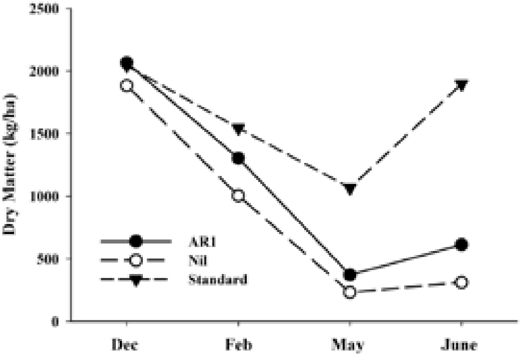
Over time, reductions in ryegrass content show that ryegrass is not persisting within a pasture. Two years after a severe infestation of pasture mealybug, there was 25% less ryegrass in Nil compared with endophyte-infected plots in a Canterbury trial (Pennell *et al*. 2005). Similarly, an AR37-infected sward had higher ryegrass content in spring, 2 years after sowing, than Nil and AR1 treatments in a paddock scale trial and by autumn the following year, ryegrass tiller densities in this trial were significantly higher in AR37 than in the Standard, AR1 and Nil pastures (Thom 2008). Such differences have been attributed to combined pressure of several different insect pests (Popay & Thom 2009). In a Hamilton trial, where both black beetle and root aphid were present and affecting AR1 more than the other two treatments, the Standard and AR37 endophytes had similar tiller densities that were 34% and 53% higher than for AR1, 3 and 5 years after sowing (Hume *et al.* 2007). Other changes in pasture composition such as ingress of weeds and the effect that endophyte has on this have been less well documented, although a decline in sown species is usually accompanied by an increase in volunteer species (Nie *et al*. 2004). Low endophyte ryegrass lines (low proportions of endophyte-infected seed) had more weeds, due to the loss of uninfected plants, than highly infected lines within 2 years of sowing (Francis & Baird 1989). Presence of endophyte in subtropical Queensland also suppressed weed invasion, consisting mainly of C_4 grasses (Lowe *et al*. 2008). AR37 substantially reduced reversion to kikuyu on farms in Northland compared with the Standard endophytes (Hume *et al*. 2009). A relationship also exists whereby endophyte-driven increases in ryegrass populations are associated with reduced clover content (e.g. Eerens *et al*. 2001; Thom *et al*. 2008).

Yield differences between treatments that increase with time are also indicative of differences in pasture persistence. In trials at Kerikeri and Hamilton, annual yield differences of perennial ryegrass cv. Samson infected with AR1, Standard or AR37 increased each year for the duration of each trial, with AR37 significantly out-yielding both AR1 and the Standard endophyte (Hume *et al*. 2007). At Kerikeri, the difference appeared to be due mainly to root aphid infestations, since no black beetle were found in the trial, whereas in Hamilton the differences were due to a combination of both root aphid and black beetle, exacerbated by drought. Similar examples of yield advantages to AR37 increasing with time have been demonstrated in several other trials in Northland and have been attributed to the insect protection provided by this endophyte (Hume *et al*. 2009).

Several trials have shown improved pasture persistence of endophyte-infected grasses where insect monitoring has not taken place (e.g. Nie *et al*. 2004; Lowe *et al*. 2008). In these cases no overt insect damage was present so it is not clear if endophyte-mediated insect protection was responsible for these effects.

Endophytes provide the greatest advantage in productivity during summer and autumn (Popay *et al*. 1999; Hume *et al*. 2004, 2007). This is the time when pressure from both pests and environmental conditions, particularly a lack of moisture, is greatest. Irrigation has been shown to slow the loss of endophyte-free plants in pasture (Francis & Baird 1989), while Hume *et al*. (2010) concluded that ryegrass persisted better at cool than at warm locations. In the DairyNZ trial near Hamilton mentioned above, a combination of pests and drought in 2008 reduced tiller densities to the extent that all six endophyte-free paddocks were replaced with endophyte-infected treatments, and three each of the AR1 and Standard treatment paddocks, but only one of the AR37 treatment paddocks had to be under-sown (Popay & Thom 2009). Plants that are able to survive drought conditions will be those for which pests have not impeded growth, particularly of roots which are

Figure 1 Average dry matter yields of four perennial ryegrass cultivars in a small plot trial sampled in between December 1999 and June 2000. Yields were determined by calibrated rising plate meter for sampling December, May and June samplings and by cutting in February.



critical for access to moisture. Endophyte infection may improve rooting depth (Crush *et al*. 2004) and AR37 has greater root growth than AR1, Nil or the Standard strains in pot trials (Popay 2004) and greater rooting depth in the field (Hume unpublished data). Other studies have shown no physiological responses to moisture deficit in endophyte-infected ryegrass (Barker *et al*. 1997). Grazing is another factor that can interact with insect damage and climatic factors to affect persistence. Ryegrass infected with the Standard strains can be grazed less heavily than ryegrass without endophyte (Edwards *et al*. 1993) providing it with a protective mechanism against overgrazing. New endophyte strains such as AR1 and AR37 appear to lack this protection.

A Case study

A trial was established at Ruakura in autumn 1999 to compare the performance of AR1 with that of endophyte-free and Standard-infected perennial ryegrass in several different cultivars. Plots were 3 x 2 m with four replicates of each treatment. Data are presented here for dry matter assessments made in December 1999, and February, May and June 2000. Plots were mown and a subsample dissected to determine composition in February while the other measurements were made using a calibrated rising plate meter. Plots rapidly became infested with black beetle as a result of an outbreak which had started in 1997/98. In February a visual estimate of black beetle larval damage was made on a scale of 0 – 10, based on the percentage of each plot that was damaged where 0 = no damage, and 5 = 50% damaged.

Results for the average of four diploid cultivars are presented here. Average endophyte infection status in November 1999 was 6% for endophyte-free and >90% for AR1 and Standard. Dry matter production was similar for the three treatments in early December but by late February the Standard had significantly greater yield than AR1, and both were significantly greater than Nil (Fig. 1). Total rainfall for December and January was 127.4 mm, 74% of the 30 year average of 183.5 mm for that period but sufficient to support good pasture growth. Differences between treatments mainly reflected differences in black beetle damage, which was lowest in Standard, intermediate in AR1 and highest in Nil plots (mean damage scores: 2.0, 2.6 and 3.0 respectively; SED = 0.384; $P<0.001$). Visible damage worsened considerably after the February cut with productivity declining dramatically in both Nil and AR1 treatments between February and May (Fig. 1). During this period, yield of AR1 was still significantly ($P<0.05$) better than for the endophyte-free, while in Standard plots production declined to a lesser extent and yield in May was markedly greater than either AR1 or endophyte-free ($P<0.001$). In February and March, only 6.1 mm and 31.8 mm of rain fell, representing 8% and 36% respectively of the long-term average, while total amounts in April and May were about normal. Despite the normal rainfall, differences between treatments became more evident by June with a marked recovery in productivity in the Standard plots but relatively little in either AR1 or Nil. By November 2000, endophyte infection rates had increased in endophyte-free plots to 33% from a level of 6% in November 1999. We conclude from that trial that high black beetle larval populations in Nil and AR1 destroyed the roots of the grasses between January and late March and that, combined with dry weather conditions, resulted in widespread plant mortality. Resistance to black beetle in ryegrass infected with the Standard strains greatly increased its persistence. Based on evidence reviewed above, AR37 would deliver similar benefits.

Conclusion

The evidence for endophytes improving ryegrass persistence mediated by their effects on insects is compelling. For such benefits to be realised, however, it is essential to choose the right endophyte for the pests in each area. For those areas at risk of black beetle damage, AR37, NEA2 and Endo5 as well as the Standard endophytes will clearly provide an advantage over AR1. AR37 has provided productivity advantages in other areas as well which are likely to also be reflected in greater persistence (Hume *et al*. 2004). Such advantages may be due to the effects of AR37 on root aphid and/or porina but we cannot be sure that there are not other minor insect pests that are affected by this endophyte. There will also be areas with benign climates and soil types that are less favourable for root aphid where AR1 will perform well. Persistence of tetraploid ryegrass will be improved by NEA2 and Endo5 particularly where black beetle is a problem. The Endo5 endophytes also offer protection against ASW and Banquet II Endo5 may reduce root aphid populations.

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