Stability and Purity of *Epichloë* Endophyte Infection in New Zealand Ryegrass Pastures

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Abstract

Perennial ryegrass (*Lolium perenne*) in New Zealand pastures is typically infected with the mutualist *Epichloë* fungal endophyte, which assists the plant in resisting biotic and abiotic stresses. Ryegrass that has naturalised in New Zealand is highly infected with the 'Standard' strain of endophyte. This strain provides good protection from a range of invertebrate pests but negatively impacts livestock productivity and health in the warmer seasons of the year. Grass-endophyte associations have been developed between elite perennial ryegrass cultivars and selected endophyte strains to provide protective properties to the host plant and lessen or eliminate the negative effects on animals. While *Epichloë*-ryegrass associations have been intensively monitored in experimental trials, little has been measured and reported from dairy pastures grazed in commercial settings, particularly with regards to ingress of Standard endophyte. Here we report on presence of endophyte infection and endophyte strain in ryegrass tillers from dairy pastures sampled for up to 7 years in regions of the North and South Islands. In general, infection levels were high (mean 86%). The sown, selected endophytes were the dominant endophyte strains present and these were stable over time. This demonstrates that the industry has been successfully delivering ryegrasses infected with selected endophytes on to dairy farms. The frequency of non-sown endophytes was greater in the North (mean 8%, and mostly Standard endophyte) than the South Island (2%), and Standard endophyte increased over time. There were some notable exceptions to these general effects. Of 23 pastures, two failed to achieve the 70% minimum infection for endophyte to effectively protect against invertebrate pests, and three out of 23 developed high levels of contamination from Standard endophyte. In two cases, the management practices could have contributed to the poor outcome but otherwise the drivers for the low infection or increases in Standard endophyte are unknown.

Introduction

In New Zealand, perennial ryegrass (*Lolium perenne*) is the most widely sown grass in pastoral farming and is typically infected with *Epichloë festucae* var. *lolii*, as this mutualist fungal endophyte assists the plant in resisting biotic and abiotic stresses (Hume et al. 2016). From the early 2000s, elite proprietary ryegrass cultivars infected with selected strains of *Epichloë* endophyte became widely available and are now an estimated 90% of the New Zealand perennial ryegrass market (Caradus et al. 2021). Selected endophytes displaced the use of the Standard, wild-type mammalian-toxic strain of endophyte in sown ryegrasses, as the Standard strain was known to cause significant financial losses to livestock farmers. These losses were due to endophyte alkaloids reducing in milk and meat production, as well causing animal health issues such as ryegrass staggers (Hume et al. 2016). The selected endophyte strains reduce or eliminate toxicity to livestock, while maintaining to varying degrees the positive agronomic benefits when the grass host is subject to stresses, particularly when pastures are under invertebrate pest pressure in New Zealand (Caradus et al. 2021).

While ryegrass-*Epichloë* endophyte associations have been intensively monitored in experimental trials, little has been measured and reported from pastures grazed in commercial settings. Concerns have been raised as to whether the seed supply chain and the pasture renewal process have been able to deliver ryegrass pastures that are highly infected with selected endophytes. In addition, as the ryegrasses that have naturalised in New Zealand are highly infected with the Standard endophyte, there was concern that Standard endophyte will re-invade pastures and undermine the benefits of the selected endophytes. Two farmlet endophyte grazing experiments and a long term small plot trial found only low levels of contamination from Standard endophyte (Hume and Barker 2005; Faville et al. 2020), but this may not be universal (McNeill et al. 2007). The ability of Standard endophyte to outcompete selected strains has been demonstrated in small plot agronomic experiments where Standard endophyte was sown in a 50:50 mix with the AR1 strain (Hume and Barker 2005). In these plots, Standard endophyte increased 10 percentage points per annum in the four experiments measured for up to 5 years.

Avenues by which a pasture can be contaminated with other endophytes are varied, ranging from sown seed, feeding hay, buried seed, natural reseeding, and incomplete kill of the old pasture, to transfer by animals e.g. in dung (Hume and Barker 2005).

Here we report on presence of endophyte and endophyte strain in ryegrass tillers from dairy pastures sampled for up to 7 years in regions of the North and South Islands. Most of the pastures used in this study were part of larger trials to investigate the drivers of pasture persistence and the value of renovation for dairy farmers in adjoining regions in the upper North Island (Waikato-Bay of Plenty) and in the eastern coastal region of the South Island (Canterbury-North Otago). This paper reports only data from the pastures that were newly (< 1 year) or recently (2-6 years old) renovated. Full details of the pasture renewal and persistence project, along with the initial endophyte results, are described by Rennie et al. (2011) and Taylor et al. (2012).

Methods

The endophyte infection frequency of ryegrass was determined using the tissue print immunoblot assay (Simpson et al. 2012) for tillers randomly selected from a pasture, each from a different plant. Where endophyte strain tests were completed, DNA was extracted from a subset of the tillers sampled for immunoblotting. The DNA was then analysed using simple sequence repeat (SSR) markers (Card et al. 2014) to determine absence/presence of *Epichloë* endophyte and the strain of *Epichloë*. The B10, B11 and ans025 markers enabled the detection of Standard endophyte and known commercial endophyte strains, with ~0.5% of tillers not aligning with known strains (data not presented).

For dairy pastures in the North Island, nine farms were monitored annually for a further 6 years than first reported by Rennie et al. (2011). Across these farms, a total of 13 pastures were tested in late spring/early summer for tiller endophyte infection by immunoblot (100 tillers per pasture). From the 100 immunoblotted tillers per pasture, subsets of 40 tillers per pasture were tested for endophyte strain by SSRs. By the final sampling, pastures ranged from 6 to 11 years of age.

Six pastures had been sown with the tetraploid cultivar ‘Bealey’ infected with a blend of NEA2 and NEA6 endophytes (marketed as ‘NEA2’) and, in two of those pastures, ‘Bealey’ was mixed with the diploid cultivar ‘Arrow’ infected with the AR1 endophyte. Three pastures were sown with the AR37 endophyte in diploid cultivars ‘Alto’, ‘Extreme’ or ‘Commando’. One pasture was sown with an unknown tetraploid cultivar with the AR5 endophyte.

A further paddock was sampled near Te Teko, eastern Bay of Plenty, North Island. This had inadvertently been sown in a seed lot of Bealey NEA2/NEA6 with a low proportion of viable endophyte (pasture was 37% infected as tested 6 months after sowing). The pasture was tested in autumn on seven occasions (4, 5, 6, 7, 8, 9 and 11 years after sowing) by immunoblotting (mean of 228 tillers per year) and on five occasions (5, 7, 8, 9 and 11 years after sowing) by SSRs (mean of 76 tillers per year).

The 10 dairy pastures in the South Island were sown in either autumn (n=8) or spring (n=2) of 2010, and all were irrigated as needed from late spring to mid-autumn (Taylor et al. 2012). They were measured annually in late summer/autumn using immunoblotting (50 tillers per pasture) and SSRs (40 tillers per pasture in each of 2012 and 2013 and 50 tillers in 2016, from the tillers immunoblot tested). Seven pastures were sown with the tetraploid cultivar ‘Halo’ infected with AR37 and three with the diploid cultivar ‘Samson’ infected with AR37.

For the North Island immunoblot data a semi-parametric regression was fitted to the response, with a random effect of Farm-Paddock and a smooth spline along Year. For the South Island immunoblot data a logistic mixed model was fitted to the response. The model had fixed effects of Year, Group (High, Moderate) and their interaction, and a random effect of paddock with a random coefficient of Year. For all SSR data, a logistic mixed model was fitted to the response, with a fixed effect of Year (an ordered factor) and a random effect of Farm-Paddock. The predicted means with multiple comparison were produced for the significant effects. All statistical analyses were done by R 4.21 and its package ‘predictmeans’ (Luo et al. 2021). 83.4% confidence intervals (CIs) were used to detect significant differences at the 0.05 level (Payton et al. 2003).

Results and Discussion

North Island

For the 13 pastures tested annually for presence of endophyte-infected tillers using immunoblot, infection rates were high, averaging 91% across all years (Table 1). There were differences between years (non-linear, $P<0.001$), with a small increase (4 percentage units) from the first year (2009) to the last year (2015) of measurement (based on non-overlapping CIs). On average, all 13 pastures had more than 70% endophyte presence in tillers, a level that is considered necessary for endophytes to provide sufficient protection to ryegrass from insect damage (data not shown) (Hume and Barker 2005).

The overall yearly mean of non-sown endophytes was 8% of the total tillers tested, with 2010 being lower than the other years (quadratic, *P*=0.001) (Table 1). Non-sown endophytes were detected in approximately three quarters of the samples (38 out of 51) taken over the 4 years, and were detected in all pastures in one or more years.

Table 1. Predicted means of tiller endophyte infection and endophyte strain from the upper North Island as tested by immunoblot (100 tillers) and a subset of tillers tested by SSRs (40 tillers); mean of 13 pastures

	Year						
	2009	2010	2011	2012	2013	2014	2015
	Tillers tested by immunoblot						
All endophytes	88%	95%	92%	87%	92%	91%	92%
	Tillers tested by SSRs						
Sown endophyte/s		80.3%*		74.5%	58.4%	76.0%	
Non-sown Standard endophyte		2.8%*		6.6%	8.1%	9.2%	
Non-sown other endophytes		0.4%*		1.6%	2.7%	1.1%	
All endophytes		83.5%*		82.7%	69.2%	86.3%	

*12 pastures measured

Standard endophyte was the major non-sown endophyte detected, with low numbers of AR1 and AR37 (a total of 268 non-sown endophyte-infected tillers, of which 252 were Standard, 7 AR1 and 9 AR37). The non-sown endophytes occurred primarily in three pastures (mean 39% of total tillers tested), and were almost all Standard endophyte (179 standard endophyte out of 182 non-sown endophyte-infected tillers) increasing from 19% to 55% of total tillers tested over time (*P*<0.001) (data not shown). For one pasture, under sowing with uncertified seed may have contributed to this increase. In comparison, unsown endophytes in the other 10 pastures were low (mean 5% of total tillers tested), mainly Standard (73 out of 86 non-sown endophyte-infected tillers) and showed no change over time (*P*=0.08) (data not shown). These differences between the high and low non-sown endophyte pasture groups appeared to be unrelated to pasture age, method of pasture renewal, sown endophyte strain and cultivar, or the level of Standard endophyte when measurements were first taken (usually 1-2 years after pasture renewal).

The additional eastern Bay of Plenty pasture, that had inadvertently been sown with a low viable endophyte seed line of Bealey NEA2/NEA6, increased 4 percentage units per annum in infected tillers from 24% to 52% of total tillers tested (years 4 to 11 post-sowing) (*P*<0.001). This increase could be attributed mostly to an increase in Standard endophyte from 9% to 23% of total tillers tested (years 5 to 11 post-sowing) (*P*=0.045).

Relative proportions of NEA2 to NEA6 (83:17) in cultivar Bealey did not change over time in the seven pastures sown to this cultivar, which was similar to the 85:15 ratio that was expected as mixed in the sown seed (Eady et al. 2017).

South Island

Endophyte tiller infection frequency averaged 80% for the 10 pastures tested annually using immunoblot (Table 2). The 10 pastures could be grouped as either highly infected pastures (n=5, predicted mean 87%) or moderately infected pastures (n=5, predicted mean 68%) (*P*<0.001). Both groups had a significant linear increase of 1 percentage unit per annum (*P*=0.007) but CIs overlapped between the first and last years of measurement. Two of the pastures in the moderately infected group (means 61% and 66%) were below the 70% level that is considered necessary for endophytes to protect pastures from insect damage. The cause of the moderate infection in one of the pastures (61%) is likely to be due to the pasture renewal process failing to kill all the high levels of endophyte-free plants from the old pasture as no chemical control was used on an organic farm (Taylor et al. 2012).

In contrast to the North Island pastures, frequency of non-sown endophytes was low (overall yearly mean of 2.4% of the total tillers tested) (Table 2). Non-sown endophytes were detected in all but one pasture, and in approximately half of the samples (14 out of 26) taken over the 3 years. Over half of the non-sown endophytes were Standard endophyte and the remainder AR1 (42 non-sown endophyte-infected tillers, of which 24 were Standard and 18 AR1). The maximum value recorded was 25% of total tillers tested, and this was all AR1, in a pasture that was known to have only partial herbicide kill during the pasture renewal process (Taylor et al. 2012). Total non-sown endophytes showed no change over time (*P*=0.09) but Standard endophyte did increase over time (linear, *P*=0.007).

Table 2. Predicted means of tiller endophyte infection and endophyte strain from the eastern South Island as tested by immunoblot (50 tillers) and a subset tested by SSRs (40-50 tillers); mean of 10 pastures sown in 2010

	Year						
	2010	2011	2012	2013	2014	2015	2016
	Tillers tested by immunoblot						
All endophytes	77%*	78%	79%	80%	81%	82%	83%
	Tillers tested by SSRs						
Sown endophyte			81.8%	74.1%			85.3%
Non-sown Standard endophyte			0.4%	1.4%			2.8%
Non-sown other endophytes			1.6%	0.2%			0.8%
All endophytes			83.8%	75.7%			88.9%

*8 pastures measured

Conclusions

This study has shown that in general the industry has been successfully delivering ryegrasses infected with selected endophytes on to dairy farms and these endophytes maintained a high frequency of infected ryegrass tillers in the pastures over time. Only a minority of the pastures (two out of 30) achieved less than the desired 70% infection minimum. For one of these pastures, a failure in the renovation process to effectively kill all the old pasture (organic farm) may have been a contributing factor.

As could be expected, due to the widespread nature of ryegrass infected with Standard endophyte, the major non-sown endophyte was Standard, and in some pastures this increased over time. This was of no real concern in the South Island where contamination was very low. However, in 3 of 13 North Island pastures, contamination increased over time to be on average over half of the tillers in a pasture. At this level, Standard endophyte is most likely to be reducing the animal production and health benefits of the sown selected endophytes. The study could not definitely attribute the occurrence and increases in Standard endophyte to any particular cultivar, endophyte and/or region, or pasture renovation practice.

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