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The effect of endophyte on the performance of irrigated perennial ryegrasses in subtropical Australia

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Abstract. The effect of fungal endophyte (*Neotyphodium lolii*) infection on the performance of perennial ryegrass (*Lolium perenne*) growing under irrigation in a subtropical environment was investigated. Seed of 4 cultivars, infected with standard (common toxic or wild-type) endophyte or the novel endophyte AR1, or free of endophyte (Nil), was sown in pure swards, which were fertilised with 50 kg N/ha.month. Seasonal and total yield, persistence, and rust susceptibility were assessed over 3 years, along with details of the presence of endophyte and alkaloids in plant shoots.

Endophyte occurrence in tillers in both the standard and AR1 treatments was above 95% for Bronsyn and Impact throughout and rose to that level in Samson by the end of the second year. Meridian AR1 only reached 93% while, in the standard treatment, the endophyte had mostly died before sowing. Nil endophyte treatments carried an average of ~0.6% infection throughout.

Infection of the standard endophyte was associated with increased dry matter (DM) yields in all 3 years compared with no endophyte. AR1 also significantly increased yields in the second and third years. Over the full 3 years, standard and AR1 increased yields by 18% and 11%, respectively. Infection with both endophytes was associated with increased yields in all 4 seasons, the effects increasing in intensity over time. There was 27% better persistence in standard infected plants compared with Nil at the end of the first year, increasing to 198% by the end of the experiment, while for AR1 the improvements were 20 and 134%, respectively. The effect of endophyte on crown rust (*Puccinia coronata*) infection was inconsistent, with endophyte increasing rust damage on one occasion and reducing it on another. Cultivar differences in rust infection were greater than endophyte effects.

Plants infected with the AR1 endophyte had no detectable ergovaline or lolitrem B in leaf, pseudostem, or dead tissue. In standard infected plants, ergovaline and lolitrem B were highest in pseudostem and considerably lower in leaf. Dead tissue had very low or no detectable ergovaline but high lolitrem B concentrations. Peramine concentration was high and at similar levels in leaf and pseudostem, but not detectable in dead material. Concentration was similar in both AR1 and standard infected plants.

Endophyte presence appeared to have a similar effect in the subtropics as has been demonstrated in temperate areas, in terms of improving yields and persistence and increasing tolerance of plants to stress factors.

Additional keywords: novel endophyte, alkaloids, yield, persistence, ergovaline, Lolitrem B, Peramine.

Introduction

Dairy farmers in the temperate regions of Australia make considerable use of highly productive pastures containing perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). These pastures produce high-quality feed for most of the year and persist for 5 or more years if management and environmental conditions are appropriate (Read *et al*. 1990). However, in the subtropical environment of south-eastern Queensland and north-eastern New South Wales (NSW), the growing conditions are less favourable and the hot, humid summers reduce the growth and persistence of perennial ryegrass (Lowe *et al*. 1999*a*).

Cultivar screening experiments in south-eastern Queensland (Lowe and Bowdler 1984, 1995) showed that alternative temperate perennial grasses including tall fescue (*Festuca arundinacea*) were more persistent during the warmer months and produced more dry matter (DM) yield than the more seasonally variable perennial ryegrass. However, a grazing trial conducted in the same environment demonstrated that milk production from tall fescue was less efficient (i.e. lower milk production per unit of forage on offer) than from perennial ryegrass (Lowe *et al*. 1999*b*) and perennial ryegrass is the grass favoured by most dairy farmers.

The presence of fungal endophyte in perennial ryegrass and tall fescue (*Neotyphodium lolii* and *N. coenophialum*, respectively) has been shown to confer varying degrees of protection against predation by insects and fungal leaf diseases in temperate environments (Popay and Bonos 2005) and there is also evidence that tolerance to drought stress and persistence are enhanced (Malinowski and Belesky 2000). The presence of

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endophyte can have a deleterious effect on animal production and health (Schmidt and Osborn 1993; Fletcher *et al*. 1999) through toxic alkaloids (such as ergovaline and lolitrem B) being produced in the grass foliage at certain times of the year. Novel endophytes, which still confer the positive attributes of greater plant production and persistence while eliminating/reducing the detrimental animal production effects, have been developed (Bouton and Easton 2005).

Improved drought tolerance and persistence are needed to improve the performance of perennial ryegrasses under subtropical conditions (Read *et al*. 1990) and the presence of endophytes might provide this improvement. This study aimed to determine if the positive effects of endophyte demonstrated in temperate regions can be shown to operate in a subtropical environment.

Materials and methods

Site

A field study was sown in 2002 into a fully prepared seedbed at Gatton Research Station in south-eastern Queensland (27° 34′S, 152° 20′E; alt. 95 m). There was no likelihood of contamination from endemic perennial ryegrass as the site had not grown ryegrass for over 30 years. The soil type was an alluvial, black clay (black earth, Stace *et al*. 1972; Ug 5.15, Northcote 1971) with a soil analysis of 119 mg/kg phosphorus (P) (Colwell extraction), 1.4 cmole/kg potassium (K), and a pH of 7.4 (H_2O).

Treatments and design

There were in total 12 treatments in a factorial design of 4 ryegrass cultivars, each with 3 endophyte status treatments. Three of the cultivars, Bronsyn, Meridian, and Grasslands Samson (hereafter referred to as Samson), were perennial ryegrasses (*Lolium perenne*), while the remaining cultivar, Grasslands Impact (Impact), was a long-rotation hybrid (*L.* × *boucheanum* syn. *L. hybridum*). Impact has production and persistence similar to the best perennial ryegrass cultivars at this site (Lowe *et al*. 2004). The endophyte treatments were each cultivar (*1*) free of fungal endophyte (Nil), (*2*) infected with the novel endophyte strain AR1, or (*3*) naturally infected with resident common toxic endophyte otherwise called standard (or wild-type). The standard endophyte may well have differed among these cultivars but they all produced the same endophyte alkaloids and showed no distinguishable differences in genetic analyses that have been completed to-date (B. A. Tapper, unpublished data). The treatments were laid out in a randomised block design with 3 replicates. The 36 plots of 10 m^2 each were sown by hand in April 2002 at 25 kg/ha.

Management

Plots received 50 mm of irrigation every 2 weeks, using hand-shift, overhead sprinklers to ensure that DM yields were not limited by soil moisture deficit. Irrigation schedules were maintained unless more than 25 mm of rainfall was received in the week before application. Urea was applied at a rate equivalent to 50 kg nitrogen (N)/ha after each defoliation. Sulfur was supplied by applying single superphosphate (18 kg P/ha, 22 kg S/ha) during the second and third spring periods.

Techniques

Harvesting of the experiment commenced in June, 9 weeks after sowing, and regrowth was assessed at 4-week intervals for 36 months. DM yield was measured by cutting to 5 cm, 5.9 m^2 from the central section of each plot, using a self-propelled harvester that defoliated, collected and weighed the samples in one operation. A subsample of harvested herbage was sorted into ryegrass and weed components and dried in a forced-draught oven at 80°C for 24 h to determine DM content and botanical composition. The remaining pasture residues on each plot were removed using a forage harvester. Seasonal DM yields were calculated by summing the sampling periods which fell within the following periods: Autumn, 1 March–31 May; Winter, 1 June–31 August; Spring, 1 September–30 November; and Summer, 1 December–28/29 February. A harvest was deemed to fall into a season if more than half the growth period occurred in that season.

Frequency of ryegrass plants was determined in late summer (late February) of each year and at the end of the experiment, using a fixed, 1 by 0.25 m quadrat, divided into a 100-square grid.

Rust (*Puccinia coronata*) incidence was visually assessed on all plots before each harvest. A 1–9 scale of decreasing damage to the leaf surface in increments of 10% was used, where 1 was greater than 80% of the leaf area within the plot affected by rust pustules and 9 was no damage.

Presence of endophyte in ryegrass tillers was checked using a tissue print immuno-blot assay. This assay uses polyclonal antibodies as first reported by Gwinn *et al*. (1991) but the procedure used was more closely aligned to that described by Hahn *et al*. (2003). To determine the viability of endophyte in the sown seed, 96 seeds of each seed line were planted in a commercial sand–peat potting mixture in a heated glasshouse in May 2002 in Palmerston North, New Zealand. Six weeks after sowing, a tiller from each seedling was tested for endophyte using the immuno-blot assay with 72–89 seedlings being assessed for each seed line. Ryegrass tillers in the field plots were tested for endophyte by immuno-blot on 3 occasions by randomly cutting 50 tillers at ground level from each plot. In Year 1, tillers of only 2 of the 3 replicates were sampled in late summer (7 February), 4 days after harvest. In Year 2, sampling occurred in mid-autumn (26 April), 4 days after harvest, with only AR1 and standard plots being tested. The final assessment in mid-autumn of Year 3 (26 April) tested all plots 15 days after harvest; however, Nil plots in 2 replicates had no, or very few, ryegrass plants left to sample, while those in some AR1 and standard plots had less than 50 plants remaining to sample. Differences between time of sampling and time of defoliation had little effect on the tillers sampled, because at these times of the year harvesting only removed small amounts of leaf lamina. Sampled tillers were therefore relatively uniform for the 3 harvest dates.

The ryegrass tillers, taken from the AR1 and standard field plots for the immuno-blot assay, were dissected into leaf blade, leaf pseudostem (leaf sheath and the enclosed emerging leaf blade), and dead matter. The dead matter was only that which was attached to the sampled tillers and is likely to be an underestimate of the total ryegrass dead matter in the plots. Dead material was not kept from AR1 plots in Years 1 and 2. Sorted herbage was kept frozen until freeze-drying, weighing and

milling through a 1-mm sieve. The milled plant material was analysed by HPLC for ergovaline and peramine, using procedures based on Spiering *et al*. (2002), and lolitrem B, using a procedure based on Gallagher *et al*. (1985). Ergovaline concentrations reported are the sum of measured ergovaline and ergovalinine. All plant parts were analysed for all alkaloids, except for lolitrem B and ergovaline in leaf and pseudostem of AR1-infected cultivars in Years 2 and 3. All alkaloid concentrations were corrected for the proportion of tillers infected with endophyte so that comparisons could be made between cultivars. The concentration in total tillers was calculated from the concentrations measured in each plant part and the dry weight (DW) of each plant part.

Statistical analyses

Data for DM yields and plant frequency were subjected to analyses of variance using the statistical package 'GENSTAT' (Genstat 8 Committee 2005), without requiring transformation. Angular transformations were performed on the Endophyte % data. Endophyte % and alkaloid concentrations were analysed by repeated-measurement analysis in GENSTAT, with data from endophyte treatments, using cultivar and year as factors. Endophyte % and peramine content were further analysed by adding endophyte treatment as an extra factor. Data from Meridian ryegrass were removed from all DM, rust, and persistence analyses and when analysing alkaloid concentrations in tissues infected with the standard endophyte because of the low levels of standard endophyte infection. It was retained in the analyses for AR1 endophyte as endophyte infection levels were similar to those in the other 3 cultivars. Generally, any interactions were small relative to the main effects, but are presented where significant.

Climate

Rainfall, evaporation, and relative humidity, recorded 100 m from the site, are presented in Table 1, and maximum and minimum temperatures in Fig. 1. Generally, monthly rainfall was below average in all 3 years, with considerably above-average values only occurring in February 2003, November 2004, and January and June 2005. Annual rainfall was about half the long-term average (790 mm) in 2002 and 2004. Above-average temperatures occurred in spring 2002, summer 2003–04, winter and spring 2004, and late summer and autumn 2005. Minimum temperatures were lowest in the winters of 2002 and 2004. A-pan evaporation values were highest in spring–summer 2002–03.

Results

Endophyte occurrence

Endophyte occurrence in the 4 ryegrass cultivars is presented in Table 2. Levels of viable endophyte in the sown seed closely matched the first test of tillers in the field plots 10 months after sowing, except for Meridian standard. In this treatment, infection was only 3% at the Year 1 sampling (11% mean for 3 years), with retrospective testing of the sown seed showing it was <1% infected with viable endophyte. All other standard and all AR1 treatments had high occurrence of endophyte-infected tillers. Bronsyn and Impact had >95% occurrence of standard and AR1 throughout the experiment, while Samson AR1 reached 93%. In Samson standard, endophyte occurrence increased ($P < 0.05$) from 91 to 99% and Samson AR1 from 93 to 99%. Meridian AR1 remained around 87%, although it was higher ($P < 0.05$) in 2004. Nil endophyte treatments had no endophyte or

Table 1. Monthly, annual, and long-term (38-year) average 9 a.m. relative humidity (%), rainfall (mm), and A-pan evaporation (mm) at Gatton Research Station in Years 2002 to 2005

Month	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Annual
						2002							
RH 9 a.m.	71	75	83	72	78	81	74	74	68	64	71	71	74
Rainfall	3	39	98	11	18	30	0	32	4	15	49	80	379
Evaporation	277	195	214	148	120	95	114	116	194	244	255	301	2274
						2003							
RH 9 a.m.	71	84	78	77	78	82	81	73	62	71	62	74	74
Rainfall	1	138	42	47	8	41	19	10	7	121	11	120	565
Evaporation	307	137	155	117	119	81	88	116	230	178	251	227	2007
						2004							
RH 9 a.m.	74	76	74	77	76	80	77	68	73	65	76	74	74
Rainfall	5	5	75	17	10	1	1	13	12	43	133	117	430
Evaporation	148	132	172	132	119	110	114	170	158	238	216	220	1930
						2005							
RH 9 a.m.	77	71	67	71	75	81	76	64	72	71	75	59	72
Rainfall	130	13	1	7	48	74	3	7	53	159	144	25	665
Evaporation	217	224	227	174	141	85	96	132	160	196	173	262	2086
						Average							
RH 9 a.m.	72	75	74	74	78	78	76	71	66	66	66	69	72
Rainfall	111	103	69	57	64	31	40	27	30	69	87	104	791
Evaporation	242	185	189	150	109	90	99	127	171	208	213	242	2008

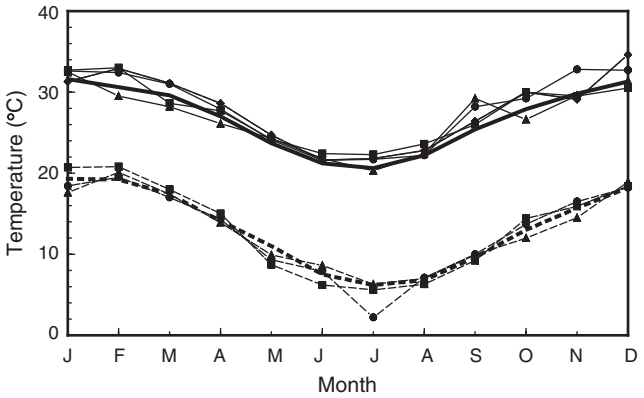

Fig. 1. Monthly and long-term (38-year average) maximum (unbroken line) and minimum (broken line) temperatures (°C) at Gatton Research Station in Years 2002 to 2005. ●, 2002; ▲, 2003; ■, 2004; ◆, 2005; —, long-term average.

remained low in endophyte throughout the experiment (mean 0.6%).

Dry matter yield

Endophyte absence/presence and endophyte strain affected the annual production of perennial ryegrass in the subtropical environment of southern Queensland in all 3 years. In Year 1, only the standard endophyte increased ($P < 0.05$) grass yields (Table 3). In Years 2 and 3, both endophytes increased ryegrass yields compared with no endophyte; the standard always produced higher yields than AR1 ($P < 0.05$) and this difference increased with time. Over the full 3 years, the standard significantly outyielded ($P < 0.05$) AR1 by 6%, while AR1 increased ($P < 0.05$) yields by 11% compared with no endophyte. Weed yields were reduced ($P < 0.05$) by the presence of endophyte (Table 3), with most of the weed production coming from C_4 grasses and most occurring in the

Table 2. Occurrence of endophyte-infected tillers, presented as the angular transformation (back transformed as % in parentheses) for three endophyte treatments and endophyte levels in seed of four ryegrass cultivars

Cultivar	Endophyte	Sampling period			
		Seed	Feb. 2003	Apr. 2004	Apr. 2005
Bronsyn	Nil	4.1 (0.5)	0.0 (0.0)	–[A]	12.4 (4.6)
	AR1	91.7 (100.0)	82.6 (98.3)	79.4 (96.6)	83.4 (98.7)
	Standard	78.5 (96.0)	90.0 (100.0)	77.3 (95.2)	83.4 (98.7)
Impact	Nil	9.9 (3.0)	0.0 (0.0)	–	0.0 (0.0)
	AR1	79.7 (96.8)	82.4 (98.3)	80.8 (97.4)	81.2 (97.6)
	Standard	84.6 (99.1)	79.0 (96.4)	81.7 (97.9)	78.8 (96.2)
Meridian	Nil	4.1 (0.5)	0.0 (0.0)	–	0.0 (0.0)
	AR1	65.8 (83.2)	69.0 (87.2)	74.4 (92.8)	68.7 (86.8)
	Standard	4.3 (0.6)	21.4 (13.3)	25.3 (18.3)	6.6 (1.3)
Samson	Nil	0.0 (0.0)	0.0 (0.0)	–	0.0 (0.0)
	AR1	73.5 (91.9)	81.9 (98.0)	84.5 (99.1)	75.0 (93.3)
	Standard	65.8 (83.2)	72.3 (90.8)	83.9 (98.9)	67.8 (85.4)
l.s.d. ($P = 0.05$)					
Time * cultivar * endophyte			10.4		
Same level of cultivar * endophyte			10.1		

[A]Endophyte not measured in Nil plots in 2004.

Table 3. Effect of endophyte on ryegrass and weed yield (t DM/ha) of pure ryegrass swards sown at Gatton in 2002
Within columns for endophyte or cultivar, means followed by the same letter are not significant at $P = 0.05$

Treatment	Year 1	Year 2	Year 3	3-year total	3-year total weeds
			Endophyte[A]		
Nil	17.72a	7.01a	3.93a	28.67a	11.36b
AR1	18.10ab	8.67b	5.03b	31.80b	7.11a
Standard	18.80b	9.21c	5.68c	33.69c	7.33a
l.s.d. ($P = 0.05$)	0.87	0.43	0.57	1.46	2.16
			Cultivar[A]		
Bronsyn	18.71b	9.48c	5.52b	33.71c	8.35
Impact	16.96a	7.48a	4.18a	28.61a	8.22
Samson	18.95b	7.94ab	4.95b	31.84b	9.23
l.s.d. ($P = 0.05$)	0.87	0.43	0.57	1.46	2.16

[A]Meridian not included because seed of the standard treatment contained little endophyte.

third year (data not presented). Cultivar × endophyte treatment interactions were not significant.

Bronsyn and Samson were the highest ($P < 0.05$) yielding cultivars in all 3 years, although Bronsyn was higher yielding than Samson in the 3-year total (Table 3). Yields of all cultivars were high in the first year but fell substantially in Years 2 and 3 while weed invasion increased. Three-year weed yields were unaffected by cultivar type.

Seasonal yields

The effect of endophyte on seasonal yield of perennial ryegrass increased as the pastures aged (Table 4). There was no significant ($P > 0.05$) effect in the first autumn, but in the 2 subsequent autumn periods, endophyte increased ($P < 0.05$) grass yield. In the first and second winters there were no effects on yield, but by the third winter, endophyte presence increased ($P < 0.05$) grass yield. Significant ($P < 0.05$) effects of endophyte commenced in the first spring when the presence of both endophytes increased yield. There was a similar result in the following 2 spring periods, although in the third spring the standard endophyte was more ($P < 0.05$) efficacious. Only in the second year was summer yield increased ($P < 0.05$) in the presence of both endophytes.

Apart from the first autumn when establishment vigour was the dominating effect, Bronsyn was generally the highest ($P < 0.05$) or equal highest, and Impact the lowest or equal lowest yielding cultivar in all seasons (Table 4). No one cultivar consistently showed superior performance in any particular season.

Persistence

Persistence of perennial ryegrass at the end of each summer period (as measured by the percentage frequency in fixed quadrats) was higher in the presence of the standard endophyte compared with no endophyte, the effect reaching significance ($P < 0.05$) in all but the second summer (Table 5). There was 27% better persistence by standard infected plants at the end of summer of Year 1, increasing to 198% by the end of summer of Year 3. While persistence in the presence of AR1 was not significantly ($P > 0.05$) lower than the standard (increasing it by 20 and 134%, respectively), it was also not significantly higher ($P > 0.05$) than persistence when no endophyte was present, except at the end of the experiment.

All cultivars showed similar ($P > 0.05$) persistence throughout the experiment (Table 5). Percent frequency data showed no cultivar × endophyte interactions.

Rust

Perennial ryegrass infected with either AR1 or the standard endophyte always carried similar ($P > 0.05$) levels of infection (Table 6). The level of rust on ryegrass without endophyte was inconsistent. It was more severe ($P < 0.05$) than standard in October 2004, less severe ($P < 0.05$) than AR1 in December 2002, but similar ($P > 0.05$) at other times. Cultivars had a greater effect on rust than endophyte treatment, and significant differences occurred on all occasions when rust was present, with Samson consistently having the lowest rust infection ($P < 0.05$).

There were significant ($P < 0.05$) cultivar × endophyte interactions in 3 of the 5 instances where rust affected ryegrass foliage (March 2003 and April and October 2004). On these occasions, Bronsyn always reacted differently from the general trend (data not presented); on 2 occasions, AR1-infected plants carried higher ($P < 0.05$) levels of rust while on the other, Nil carried more ($P < 0.05$) rust than the standard. Impact also behaved differently on 2 occasions; once with AR1 plants carrying more ($P < 0.05$) rust and, on the other, Nil plants carrying more ($P < 0.05$) rust.

Presence of endophyte alkaloids

Peramine concentrations were similar for all AR1-infected cultivars ($P > 0.05$), except for Meridian, which contained higher ($P < 0.05$) peramine in leaf, pseudostem, and green tiller (Table 7). Peramine was consistently higher ($P < 0.05$) in Year 1 (late summer) than in Years 2 and 3 (mid-autumn) in AR1 plants, while peramine in standard varied between years, depending on plant part. Peramine in dead matter was at or below the limit of detection. There were no significant ($P > 0.05$) differences in the peramine content of the leaf (corrected for endophyte content) in standard or AR1 plants (23 µg/g) but, in the pseudostem, peramine content was higher ($P < 0.05$) in AR1 plants (22 and

Table 4. Effect of endophyte on the seasonal ryegrass yields (t DM/ha) of four perennial ryegrass cultivars in south-eastern Queensland
Within columns for endophyte or cultivar, means followed by the same letter are not significant at $P = 0.05$

Treatment	Year 1				Year 2				Year 3				Year 4
	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
						Endophyte[A]							
Nil	0.20	7.14	5.29a	3.87	1.42a	1.50	2.99a	1.03a	1.50a	1.59a	1.26a	0.57	0.52
AR1	0.23	6.94	5.73b	3.73	1.69b	1.60	3.25b	1.48b	2.35b	1.95b	1.49b	0.82	0.77
Standard	0.25	7.04	6.05c	3.85	1.87c	1.55	3.44b	1.66b	2.56b	2.21b	1.81c	0.77	0.90
l.s.d. ($P = 0.05$)	0.05	0.31	0.31	0.60	0.13	0.15	0.26	0.24	0.29	0.17	0.13	0.39	0.32
						Cultivar[A]							
Bronsyn	0.21	7.10b	6.10b	3.85	1.66ab	1.82c	3.81b	1.62b	2.23	2.02b	1.74c	0.92	0.84
Impact	0.23	6.75a	4.96a	3.68	1.57a	1.36a	2.96a	1.10a	2.06	1.69a	1.27a	0.60	0.62
Samson	0.23	7.27b	6.01b	3.93	1.74b	1.47ab	2.90a	1.44b	2.13	2.03b	1.54b	0.64	0.73
l.s.d. ($P = 0.05$)	0.05	0.31	0.31	0.60	0.13	0.15	0.26	0.24	0.29	0.17	0.13	0.39	0.32

[A]Meridian was not included because little endophyte was found in the seed of the standard treatment.

Table 5. Effect of endophyte on the persistence (% frequency) of perennial ryegrass plants at the end of each summer and the end of the experiment

Within columns for endophyte or cultivar, means followed by the same letter are not significant at $P = 0.05$

Treatment	End of summer			End of experiment
	Year 1	Year 2	Year 3	May 2005
		Endophyte[A]		
Nil	48.1a	40.1	5.6a	5.0a
AR1	57.6ab	45.1	13.1ab	14.3b
Standard	61.2b	49.0	16.7b	15.6b
l.s.d. ($P = 0.05$)	10.8	9.7	6.8	6.5
		Cultivar[A]		
Bronsyn	50.3	40.7	13.1	14.0
Impact	61.3	48.0	10.1	9.4
Samson	55.2	45.6	12.1	14.0
l.s.d. ($P = 0.05$)	10.8	9.7	6.8	6.5

[A]Meridian was not included because little endophyte was found in the seed of the standard treatment.

Table 6. The effect of endophyte on the level of crown rust infection in ryegrass foliage (on a 9 to 1 basis, where 9 is no rust evident and 1 is greater than 80% of the area of leaf laminae affected by pustules)

Within columns for endophyte or cultivar, means followed by the same letter are not significant at $P = 0.05$

Treatment	Dec. 2002	Mar. 2003	Apr. 2003	Apr. 2004	Oct. 2004
		Endophyte[A]			
Nil	5.3b	6.6	5.9	7.6	6.9a
AR1	4.32a	6.2	6.4	7.2	7.2ab
Standard	4.7ab	6.6	5.9	7.4	7.4b
l.s.d. ($P = 0.05$)	0.6	0.4	0.8	0.3	0.3
		Cultivar[A]			
Bronsyn	4.3a	6.4b	5.4a	7.2a	6.7a
Impact	4.0a	6.0a	5.1a	7.0a	6.7a
Samson	6.0b	7.1b	7.3b	7.9b	8.1b
l.s.d. ($P = 0.05$)	0.6	0.4	0.8	0.3	0.3

[A]Meridian was not included because little endophyte was found in the seed of the standard treatment.

25 μg/g, respectively). These responses were not affected by year or cultivar (data not presented).

Plants infected with the standard endophyte had very little or no detectable ergovaline in dead tissue (Table 7). In contrast, lolitrem B was relatively high in dead material (Table 7). Leaf had ~20% of the concentrations of ergovaline and lolitrem B that occurred in pseudostem but similar levels of peramine. There were no significant ($P > 0.05$) cultivar differences for alkaloid concentrations in leaf or pseudostem. Ergovaline levels in Years 2 and 3 were higher than in Year 1, while for lolitrem B there were no consistent differences between the 3 years of sampling. In Year 1, analysis of leaf and pseudostem of AR1-infected cultivars showed no detectable ergovaline or lolitrem B.

The dead component comprised close to half the DW of the total tiller harvested, and of the green component, leaf and pseudostem were approximately similar in proportion (data not presented). Total tiller (leaf, pseudostem and dead) alkaloid concentrations were ~45% lower than green tiller (leaf and pseudostem) concentrations for ergovaline (standard) and peramine (standard and AR1) but 22% higher for lolitrem B (standard) because of the differences in concentrations in the dead component (Table 7). Peramine concentrations in the green tiller of the standard and AR1-infected plants were similar ($P > 0.05$) to those of the leaf and pseudostem components.

Discussion

Plant production and persistence

The presence of endophyte in the tissue of perennial ryegrass significantly improved the performance of perennial ryegrasses under subtropical conditions. While both the standard and novel endophyte improved DM yield and persistence, the standard, overall, was more effective and also on some occasions improved the resistance of the plants to infection by rust. The effect was shown as higher seasonal yields and this effect increased with time.

Our results are similar to those reported in the temperate climate of New Zealand, where perennial ryegrass infected with endophyte is often higher yielding than when endophyte-free, particularly during summer and autumn (Popay *et al*. 1999; Pennell *et al*. 2005), and endophyte may also enhance ryegrass persistence and decrease weeds (Prestidge *et al*. 1985; Francis and Baird 1989). There are relatively few studies in Australia that have directly trialled the effects of endophyte. Results from temperate areas of southern Australia, (South Australia, Valentine *et al*. 1993; Victoria, Quigley 2000; NSW, Launders *et al*. 1996; Wheatley 1998), support our findings from the subtropics, with others in Victoria showing variable results (Cunningham *et al*. 1993). Indirect evidence also points to enhanced persistence due to endophyte infection in south-eastern Australia (Hume and Barker 2005).

Mechanisms by which endophyte enhances plant performance in ryegrass and tall fescue are primarily centred on protection from insect attack (Popay and Bonos 2005) and drought stress (Malinowski and Belesky 2000). In New Zealand, the timing and magnitude of enhanced ryegrass performance are most closely linked to presence and severity of insect attack, as well as the proportion of ryegrass tillers infected with endophyte (Popay *et al*. 1999). Such a close relationship of insects and plant performance may not occur in Australia, with McDonald *et al*. (1993) believing that endophyte did not confer sufficient protection to ryegrass plants from Australian insect pests. In contrast to tall fescue, there is little or no evidence for endophyte enhancing drought tolerance *per se* in ryegrass (Barker *et al*. 1997), although this could play a role in some areas of Victoria along with heat stress (Cunningham *et al*. 1993; Latch 1994).

The effect of endophyte in our study appears to have occurred in the absence of any major insect pests. Drought stress also appears to be an unlikely mechanism. Soil moisture levels may have been limiting growth during the peak evaporative periods in summer–autumn despite regular irrigation, but soil moistures would not have been low enough to be considered drought. Endophyte effects also occurred at cooler times of the year

Table 7. Concentrations (μg/g) of endophyte alkaloids in the tissues of four perennial ryegrasses grown under subtropical conditions in response to the infection by either the standard or AR1 endophyte, sampled at the end of summer (Year 1), or mid-autumn (Years 2 and 3)

Alkaloid contents have been adjusted for % endophyte occurrence. Within columns for endophyte or cultivar, means followed by the same letter are not significant at $P = 0.05$. Endophyte differences: ** $P < 0.01$; n.s., not significant. Detection limit is 0.1 μg/g for ergovaline and lolitrem B, and 2 μg/g for peramine

Cultivar/Year	Endophyte%	Ergovaline content (μg/g)					Lolitrem B content (μg/g)					Peramine content (μg/g)				
		Leaf	Pseudostem	Dead	Green tiller[C]	Total tiller[D]	Leaf	Pseudostem	Dead	Green tiller	Total tiller	Leaf	Pseudostem	Dead	Green tiller	Total tiller
AR1 endophyte																
Cultivar																
Bronsyn	97.5b	0.0[A]	0.0[A]		0.0[A]		0.0[A]	0.0[A]		0.0[A]		23a	25a	1[E]	23a	12[E]
Impact	97.3b	0.0	0.0		0.0		0.0	0.0		0.0		20a	25a	1	23a	13
Meridian	87.2a	0.0	0.0		0.0		0.0	0.0		0.0		29b	29b	1	29b	15
Samson	95.7b	0.0	0.0		0.0		0.0	0.0		0.0		26b	24a	1	24a	13
l.s.d. ($P = 0.05$)	3.6											4	3	n.s.	3	
Year																
1	93.2	0.0[A]	0.0[A]		0.0[A]		0.0[A]	0.0[A]		0.0[A]		27 b	29b		28b	
2	95.0											23a	25a		23a	
3	95.1											23a	23a	1[E]	23a	13[E]
l.s.d. ($P = 0.05$)	n.s.											3	3		3	
Standard endophyte[B]																
Cultivar																
Bronsyn	96.3	0.3	1.6	0.1	0.9	0.5	0.56	3.0	2.3	1.7	2.1	24	24	1	24	13
Impact	96.8	0.3	1.4	0.1	0.8	0.5	0.47	3.0	2.4	1.7	2.1	22	23	1	22	12
Samson	91.1	0.3	1.3	0.0	0.7	0.4	0.47	2.2	1.9	1.3	1.6	24	19	0	21	11
l.s.d. ($P = 0.05$)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Year																
1	92.9	0.2a	0.8a	0.0a	0.4a	0.2a	0.6b	2.7	2.6b	1.6	2.1b	27b	25b	1c	26b	14b
2	95.5	0.3b	1.6b	0.1b	1.0b	0.6b	0.3a	2.6	2.6b	1.5	2.1b	21a	24b	2b	23b	12a
3	95.7	0.4b	1.9b	0.0a	1.0b	0.6b	0.5b	2.9	1.4a	1.6	1.5a	21a	18a	0a	20a	11a
l.s.d. ($P = 0.05$)	n.s.	0.1	0.3	0.0	0.2	0.1	0.2	n.s.	0.5	n.s.	0.5	4	4	0	4	2
Endophyte differences	**											n.s.	**		n.s.	

[A]Ergovaline and lolitrem B tested in tissues of AR1-infected cultivars only in Year 1.

[B]Meridian not tested due to very low (8%) endophyte occurrence in Meridian standard plots.

[C]Green tiller contains both leaf and pseudostem.

[D]Total tiller adds dead to green tiller data.

[E]Dead analysed for peramine for AR1-infected cultivars only in year 3, enabling total tiller values to be calculated for Year 3 only.

when evapotranspiration rates would have been less and soil moisture levels higher. Endophyte may assist plant growth through other less well studied abiotic and biotic factors (Malinowski and Belesky 2000; Popay and Bonos 2005). Some of these factors, such as protection from plant nematodes and root diseases, improved nutrient uptake, and overall rooting structure (Crush *et al*. 2004), may be operating in this subtropical environment.

Ryegrass plants infected with the standard endophyte produced superior yields to those infected with AR1, as has also been reported in New Zealand (Hume *et al*. 2004; Cooper *et al*. 2006). These effects may be attributed to differences in pest protection offered by these 2 endophytes (Popay and Bonos 2005), but again the lack of an obvious insect presence at the Gatton site leaves the mechanism for this endophyte strain difference unanswered. We also report no interaction between endophyte and cultivar. Endophyte–cultivar interactions are possible (Latch *et al*. 1985) and may be related to differences in the effectiveness of the various endophytes in conferring tolerance to abiotic stresses (Malinowski and Belesky 2000) or in the type, or level, of alkaloids produced in the cultivars (Latch 1994). Persistence of perennial ryegrass in this experiment is lower than the average in this environment but conditions were more severe (Lowe *et al*. 2008*a*). However, tripling the persistence under extreme conditions has raised persistence to around that expected under average conditions and suggests that all perennial ryegrass seed sown under subtropical conditions must contain endophyte.

The increase in the standard endophyte-infected plants of Meridian over time (to around 20% by the end of the 3 years) also emphasises the value of endophyte in improving persistence. The increase can be attributed to a greater loss of plants not containing endophyte as there is no possibility of plant recruitment or endophyte infection.

Endophyte alkaloids

The ability of perennial ryegrasses to produce alkaloids in response to endophyte infection appears to be generally similar under subtropical and temperate conditions (see below). Fletcher *et al*. (2001) report that temperatures were positively correlated with alkaloid production in temperate regions and it is possible that this may be enhanced further under subtropical temperatures. While endophyte has a high optimum temperature for growth in culture of 20–25°C (Latch *et al*. 1984), in-planta growth of endophyte may be limited by the ryegrass host being beyond its optimum temperature, as occurs in south-eastern Queensland/north-eastern NSW in summer and early autumn. There is also evidence that endophyte alkaloids may be reduced at high temperatures in ryegrass (G. A. Lane *et al*., unpublished data) and tall fescue (Adcock *et al*. 1997). Alkaloid production at other times of the year in south-eastern Queensland, when temperatures are relatively cooler, is currently subject to investigation (K. F. Lowe and D. E. Hume, unpublished data).

In general, our results of alkaloid concentrations in different plant tissues confirm those of Di Menna *et al*. (1992), Davies *et al*. (1993), Keogh *et al*. (1996), Ball *et al*. (1997*a*, 1997*b*) and Watson *et al*. (1999). Knowing these effects and the dry weights of each tissue component, valid comparisons can be made with other data (as discussed below) when height of sampling, time of sampling, and tissue type are clearly described.

Lolitrem B concentrations in standard infected cultivars in February and April at Gatton were similar to those reported in the Waikato region of New Zealand (temperate climate) (Hawkes *et al*. 1995) but lower than several other measurements in New Zealand (Di Menna *et al*. 1992; Davies *et al*. 1993; Keogh *et al*. 1996; Watson *et al*. 1999; Fletcher *et al*. 2001). In the south-west region of Victoria, Reed *et al*. (2004) reported similar mean values to ours, once adjusted for the occurrence of endophyte infection. However, their sampling height (below the crown) may have resulted in greater concentrations (Ball *et al*.1997*b*). Lolitrem B is the primary causal agent of ryegrass staggers, a neurological disorder occurring in sheep and cattle when pasture lolitrem B concentrations are above 1.8–2.5 μg/g as measured to ground level (Di Menna *et al*. 1992; Tor-Agbidye *et al*. 2001). Mean cultivar values over the 3 years at Gatton were 1.6–2.3 μg/g and are likely to be higher than this as the sampling technique did not gather all the dead matter in the pasture. At these concentrations, ryegrass staggers is likely to occur but has rarely been reported in this region of Australia. This may be due to the high contamination of ryegrass swards by C_4 grasses at this time of the year, reducing total pasture concentrations of lolitrem B below the threshold values for staggers to occur.

Ergovaline concentrations in February at Gatton are ~4-fold lower than those in New Zealand reports, but similar to those in south-western Victoria depending on cultivar (Woodburn *et al*. 1993). The concentrations in April were more aligned to those recorded in New Zealand (Bluett *et al*. 2005*a*) and Victoria by Reed *et al*. (2004) but 3-fold greater than those recorded by Woodburn *et al*. (1993) in the same region of Victoria. Unlike lolitrem B, ergovaline concentrations at Gatton were 2–3 times higher in April than in February. While this appears to be against the general trend of decreasing alkaloids with decreasing autumn temperatures (Fletcher *et al*. 2001), more measurements would be needed to define monthly seasonal changes in alkaloid profiles in the subtropics. Cattle, sheep, and horse responses to lysergyls (as measured via ergovaline) in tall fescue and perennial ryegrass are essentially linear and appear to have no safe threshold below which animal liveweight gain (or abortion in horses) is unaffected (Schmidt and Osborn 1993; Layton *et al*. 2004). Low levels in February at Gatton are likely to have few discernible effects on animal production and heat stress, while those recorded in April would, depending on the heat and humidity stress experienced by grazing animals at the time (L. R. Fletcher, pers. comm.). However, as with lolitrem B, dilution of total pasture alkaloids by C_4 grasses in the animals' diet will be an important factor as will collection of all dead matter in the pasture when assessing pasture alkaloid concentrations.

In contrast to the other alkaloids, peramine was usually at similar concentrations to those reported for comparative herbage and months in New Zealand (e.g. Fletcher *et al*. 2001; Bluett *et al*. 2005*a*), followed the same seasonal decline (Fletcher *et al*. 2001), but did not show large variation between years as occurred in the study of Bluett *et al*. (2005*a*). Reed *et al*. (2004) in south-western Victoria also reported similar but a much wider range of values.

AR1 produced no measurable ergovaline or lolitrem B in plant tissues but high levels of peramine in green tissues, in keeping with reports from the temperate climate of New Zealand (Fletcher 1999; Bluett *et al*. 2005*a*, 2005*b*). This alkaloid profile in AR1-infected plants should ensure none of the detrimental animal health (ryegrass staggers) or production effects that are associated with standard endophyte in ryegrass (Fletcher 1999; Bluett *et al*. 2005*a*, 2005*b*).

In the New Zealand studies, considerable variability (up to 2-fold) occurs between years at the same site, and between sites within the same year. Fletcher *et al*. (2001) could not successfully predict from climate data much of the short-term variation or unusually high concentrations. Cultural factors may play an important role here, with factors such as N and water affecting alkaloid production, although not necessarily in a consistent and predictable way (Lane *et al*. 1997). In comparison, the variations in concentrations for the 2 April samplings at Gatton were small, which may be attributed to the regular and controlled applications of N and water. Many of the temperate sites where alkaloids have been measured would have less or no control of soil moisture and less frequent N applications.

Rust

The effects of endophyte on rust infection were inconsistent, with both positive and negative responses. The only report, previous to our study, of endophyte affecting crown rust in perennial ryegrass is from Clay (1990) in the USA, with Latch (1994) noting that this effect has not been found in New Zealand. Compared with endophyte-mediated host resistance to insects, there are few reports of *Neotyphodium* endophytes affecting foliar diseases in ryegrass and tall fescue (Popay and Bonos 2005). Wheatley *et al*. (2000) reported that standard endophyte protected 4 perennial ryegrass cultivars from infection by *Pyrenophora semeniperda* at Orange in the central Tablelands of NSW, Australia. Christensen (1996) has shown antifungal effects with ryegrass in a plant tissue assay, and Vincelli and Powell (1991), effects in turf ryegrass in the USA. The mechanism for any antifungal activity has not been elucidated (Popay and Bonos 2005). The inconsistent effect of endophyte on rust in the current experiment has been confirmed in subsequent research at this site (K. F. Lowe and D. E. Hume, unpublished data). In practise, choice of cultivar will still be more important in reducing the incidence of rust than will choice of endophyte (absence/presence and strain).

Implications

In practical terms, these increases in yield (11 and 18% for AR1 and standard, respectively) and persistence (190% and 210%, respectively) are quite substantial but do not appear sufficient to make perennial ryegrass perform as well as tall fescue or prairie grass (*Bromus willdenowii*) in subtropical conditions. For example, the best perennial ryegrass swards can achieve a 3-year total of around 41 t/ha while the best tall fescue cultivars and Matua prairie grass produce around 54 and 45 t/ha, respectively (Lowe *et al*. 2008*a*). Equivalent figures for persistence of perennial ryegrass and tall fescue are around 20 and 80%, respectively, from around 10 experiments (Lowe *et al*. 2008*a*). However, it may be sufficient to improve the performance under grazing (Lowe *et al*. 1999*a*) or in mixtures with tall fescue and white clover (Lowe *et al*. 2008*b*) where perennial ryegrass cultivars need to be oversown each year to replace the ~40% of the sward lost each summer (Lowe *et al*. 1999*a*).

The above increases in ryegrass yields also have important implications for experimentation that measures the relative performance of ryegrass cultivars in this environment. Yield differences between perennial ryegrasses evaluated at Gatton can be as much as 50% (Lowe and Bowdler 1995), but are typically 10–25%. Whether the seed supplied for evaluation is endophyte-infected or endophyte-free could therefore alter the relative rankings between cultivars. The experience with Meridian standard in the current experiment also indicates that seed should be tested shortly before sowing into evaluation experiments to confirm its level of viable endophyte, or better still, the sown plots should be tested for endophyte occurrence.

While current usage of perennial ryegrass in the Australian subtropics means that animals are rarely likely to ingest sufficient toxins to cause clinical effects, subclinical effects can be as important. For this reason, we do not advocate standard endophyte-infected cultivars being sown. On the other hand, the use of AR1-infected cultivars provides most of the agronomic efficacy provided by endophyte presence without the detrimental effects on animal health and production.

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