Grasslands Legacy – a new, large-leaved white clover cultivar with broad adaption

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Abstract
White clover (*Trifolium repens* L.) continues to play a pivotal role in the Australasian pastoral industry, despite increased use of nitrogen fertiliser on farms. Improved white clovers for dairy farming must be well adapted to the farm systems they are intended for, including increased rates of fertiliser nitrogen, higher stocking rates and access to irrigation. The breeding objective was to develop a white clover cultivar in evaluation systems that simulate modern farming practices, and test that cultivar in both New Zealand and Australia for adaptation and agronomic merit. This included breeding and early generation evaluation at research farms in the Manawatu and Waikato, with subsequent evaluations in these locations and farms in Southland and Victoria, Australia. This resulted in 'Grasslands Legacy', a new large leaved white clover cultivar bred for New Zealand and eastern temperate Australian pastures, which has shown significant (P<0.05) improvement on New Zealand cultivars including 'Kopu II' and 'Kotare'. 'Grasslands Legacy' offers good utility for modern intensive pastoral systems, particularly through yield, persistence, and improved adaptation to abiotic stress.

Keywords
Breeding, abiotic stress, Australasia, genotype × environment, *Trifolium repens*

Introduction
The dairy and intensive red meat industries make a significant economic contribution to New Zealand, with dairy exports making up almost a third of the annual agricultural exports. Pastures are a cost-efficient source of feed for these industries, reducing risk associated with the volatility in input and commodity prices. White clover (*Trifolium repens* L) is the main forage legume species used in combination with perennial ryegrass (*Lolium perenne* L.) across New Zealand and in high rainfall regions of temperate Australia (Jahufer *et al.* 2012). It adds to the low cost pasture base through its capacity to fix nitrogen, its high nutritive value, ability to improve animal feed intake and utilisation in mixed swards, and compatibility with other forage grasses and herbs where it complements seasonal growth patterns (Annicchiarico *et al.* 2015). The proportion of white clover in swards is generally lower than the longstanding optimum of 30–40% recommended by Davies & Levy (1931) and the 50–60% of diet to optimise per animal production supported by more recent research (Harris *et al.* 1998). Suggested reasons for this low content are the lack of vegetative persistence under summer moisture stress (Jahufer *et al.* 2002) excessive fertiliser nitrogen application, and management systems that favour grass growth over legume persistence (Annicchiarico *et al.* 2015). Despite these challenges, the rate of genetic gain for white clover content in mixed swards has been estimated at approximately 1% per year which is ahead of many pasture species (Brummer & Casler 2014). This is augmented by substantial parallel advances in seed yield potential (Widdup *et al.* 2004).

The objective of the research described here was to develop a white clover cultivar better adapted to current dairy and intensive farming practices in New Zealand and Australia, and to compare under grazing the seasonal growth, leaf size, cyanogenesis and stolon density trait expression of 'Grasslands Legacy' with a relevant range of marketed cultivars including medium ('Huia' and 'Apex') and medium large to large leaved ('Aran', 'Kopu II', 'Kotare', 'Sustain', 'Tribute', 'Trophy' and 'Weka') types.

Materials and Methods
The development of 'Grasslands Legacy' included early generation breeding and evaluation in the Manawatu region of New Zealand, and breeding with subsequent advanced synthetic and cultivar evaluations in the Waikato and Southland regions of New Zealand, and south-west Victoria, Australia, which we report on here.

Breeding History
A first generation synthetic population was developed in 1999/2000 by polycrossing ten plants from cultivars 'Kopu II,' 'Barblanca', and three experimental breeding lines. A progeny bulk was tested for agronomic potential under sheep grazing in Manawatu (Trial 1) with further selection for agronomic merit and seed yield potential to develop the advanced breeding line GC239. Ten trials under sheep or cattle grazing from 2002 to 2015

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provided data that led to the advancement of GC239 as the cultivar ‘Grasslands Legacy’. We report here on the initial trait data for GC239 under sheep grazing in the Manawatu (Trial 1), and five further trials under dairy or beef cattle grazing.

Initial evaluation and development of GC239

Trial 1 was at AgResearch Grasslands Research Centre, Palmerston North (40.38°S, 175.61°E) was under rotational sheep grazing management. The trial included 84 elite breeding lines and 17 control cultivars, including GC121 later released as ‘Grasslands Trophy’ (Ayres *et al*. 2007). Seedlings were tested after seven weeks to ascertain the level of hydrogen cyanide (HCN) using the picrate method (Corkill 1940). The trial was transplanted in August 2002 as 11-week-old seedlings into single 1 m rows of ten genotypes, with four replicates in an established grass sward of ‘Grasslands Impact’ long rotation ryegrass (*Lolium perenne* L.) containing a wild type endophyte. Agronomic data collection commenced October 2002 including visual assessment for white clover growth (1–10, 1=within lowest perceived growth decile in trial at that timepoint, 10=highest) prior to each grazing. Data on leaf size (score 1=within smallest perceived quintile in trial at that timepoint, 5=largest) after year one, and leaf width (mm) for ten representative leaves per plot were collected in year two. Lateral spread was assessed in year one, and stolon density (stolons/m^2) was measured each autumn by counting stolons in a 12 × 16 cm quadrat in each plot.

Trial 2 was managed under rotational grazing by dairy cows in the Waikato at AgResearch Ruakura (37.77°S, 175.31°E). The trial included GC239 and 39 other breeding lines and cultivars. Seedlings were transplanted into a sward of ‘Extreme’ perennial ryegrass infected with AR1 endophyte. The sward was established in 2004 and sprayed twice with dicamba selective herbicide to remove white clover seedlings and other broadleaf weeds in the season prior to transplanting. The trial was established as four reps in July 2005 and ended July 2007 due to the severe summer temperatures and drought, and the effect of an intense clover root weevil (*Sitona lepidus*) infestation during 2006/07. Plots were visually assessed for white clover growth prior to each grazing as described for Trial 1.

In 2009, the GC239 synthetic population underwent final parental seed yield selection at AgResearch Lincoln Farm, using published methods (Woodfield *et al*. 2004). Three hundred plants were assessed in the first year for seed yield, presence of foliar diseases, and plant morphological characteristics. Twenty-seven selected plants were cloned and crossed using bumble bees (*Bombus terrestris*) to produce the second synthetic generation seed, which was then increased in an isolated field at Lincoln to produce the third synthetic generation seed for further agronomic evaluation to ensure the parental selection and further cycles of recombination had not reduced agronomic potential.

Multi-site Evaluation of ‘Grasslands Legacy’

The name ‘Grasslands Legacy’ was assigned to the third generation synthetic population of GC239, prior to multi-site field testing in the Waikato (Trial 3), Southland (Trial 4), and Glenormiston, Victoria, Australia (Trial 5) between 2009 and 2013, and in the Manawatu (Trial 6) between 2011 and 2015. Trials in Waikato, Southland and Victoria, Australia were rotational grazed using dairy cattle while the Manawatu trial was rotational grazed by beef cattle.

Trial 3 was established in the Waikato (37.77°S, 175.31°E) with 100 breeding lines and cultivars transplanted as four reps into a sward of ‘Extreme’ perennial ryegrass with AR37 endophyte sown in spring 2008 at 20 kg/ha. The sward was kept free of white clover and broad leaf plants using dicamba 500SL and Nufarm MCPB 400 selective herbicides at recommended rates, with three applications in the season before planting. Nitrogen (urea) was applied twice annually at the rate of 50 units N/ha. Visual score data for white clover growth and leaf size were collected prior to each grazing over 3 years, as per methods in Trial 1.

Trial 4 was established in Southland at Tussock Creek (46.25°S, 168.43°E) with 75 breeding lines and cultivars each transplanted as 0.25 m^2 plots in four replicates during early September 2009 into previously established ‘Ceres One50’ perennial ryegrass with AR1 endophyte, with management and data collection as described for Trial 1.

Trial 5 was established September 2010 at Glenormiston (38.15°S, 142.96°E) in southwest Victoria, Australia included 20 entries as 1.8 m × 3 m plots in a row-column design with three replicates. White clover was sown at 5 kg ha^{-1} together with an unspecified perennial ryegrass at 15 kg ha^{-1}. The trial was run over 3 years finishing April 2013, with white clover yield data collected as described for Trial 1.

Trial 6 was established in April 2011 at the AgResearch Aorangi research farm (40.34°S, 175.46°E) in the Manawatu. It contained 26 entries sown at the rate of 5 kg/ha of white clover seed in plots 1 × 3 m, with 3 m between plots in four replicates. Plots were immediately oversown with ‘Ceres One50’ perennial ryegrass containing AR1 endophyte at 20 kg/ha. The pH at sowing was 5.8 with an Olsen P of 29, soil samples varied from pH 5.4 to 6.2 and Olsen P 27–34 from trial establishment through to May 2015. Plate meter readings were completed weekly to monitor

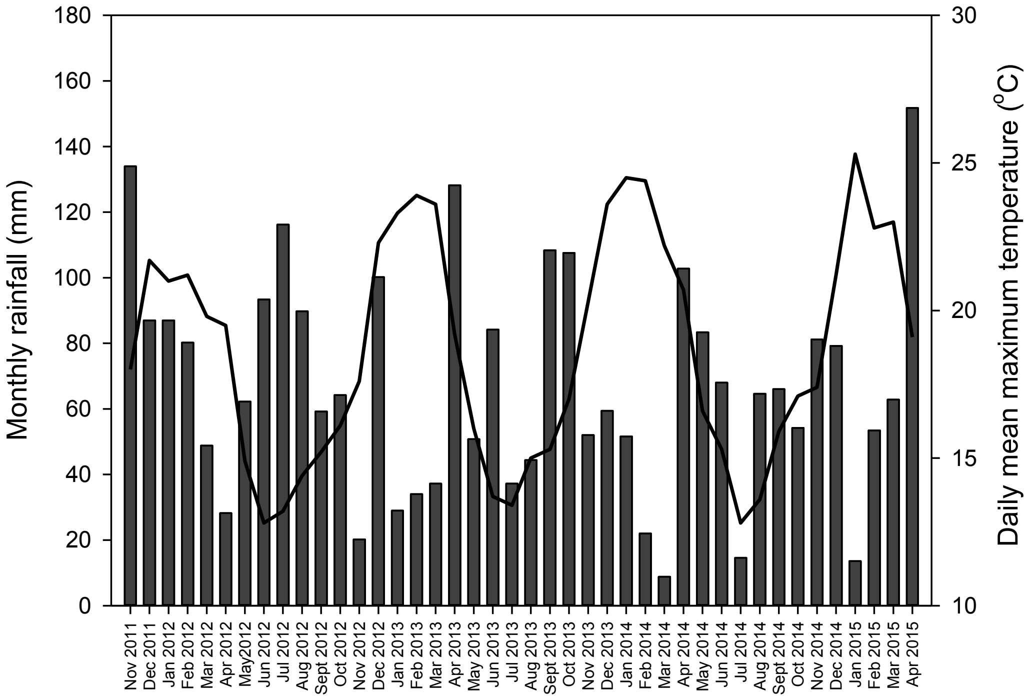

Figure 1 Manawatu mean maximum air temperature and monthly precipitation records for Trial 6 site in the Manawatu plains 2011 –2015.

growth rate and aid in timing for visual assessments. Trial 6 results include data as collected for Trial 1, augmented by estimated dry matter yields (kg DM/ha) over time calculated from dry matter yield measured on quadrat cuts of a representative subset of 15 or more plots after each visual growth score. Using a 25 x 25 cm quadrat for sampling, selected plots were harvested using electric shears to a height of 1–2 cm, each sample was dissected into white clover and grass components which were dried and weighed separately. This allowed calculation of estimated white clover dry matter yield for all plots. Nitrogen was applied four times annually, at 50 units urea N per application. Maxi Super 15 was applied annually in the autumn of each year from 2011 until 2015. Lime was applied in years 2014 (2500 kg/ha) and 2015 (100kg/ha).

Statistical Analysis

Analyses within and among trials were carried using mixed linear models using REML analysis in Genstat version 16.1.

Weather data

Trial 3 rainfall ranged from 1087 mm (2010) to 1395 mm (2011) and annual mean daily maximum air temperature 17.7 °C (2012) to 19.5 °C (2010). During the period February to April 2010, 70 mm of rain was recorded. Trial 4 was more consistent with annual mean daily maximum between 15.1 and 15.8 °C, with annual rainfall consistent ranging from 857 mm to 990 mm. Trial 6 weather records indicate this trial went through relatively dry periods during autumn 2012, February–April 2013, and December 2014–February 2015 (Figure 1).

Results and Discussion

Trial 1

Over 3 years to 2005, GC239 exhibited a significant ($P<0.05$) yield advantage over 'Kopu II', GC121, and all other cultivars with adaptation to sheep grazing management in this Manawatu-based mixed sward trial. Stolon density after 3 years was significantly ($P<0.05$) higher for GC239 than 'Kopu II' and all other cultivars apart from 'Tribute' (Table 1). 'Tribute' is known to exhibit high stolon growing point density relative to its leaf size under rotational sheep grazing (Woodfield *et al.* 2003). The discovery of high stolon density with relatively large leaf size was pivotal in the early identification of GC239 agronomic potential. Stolon density has a marked influence on production and persistence in intensively grazed pastures (Caradus & Chapman 1996), but is generally negatively correlated with leaf size and growth (Annicchiarico *et al*. 2015), although some data indicate recent New Zealand cultivars have improved that relationship (Widdup & Barrett 2011). GC239 had a similar cyanogenic percentage as 'Kopu II' (Table 1), a trait strongly implicated in persistence and productivity in New Zealand (Crush & Caradus 1995).

Trial 2

In the 2005 trial at Ruakura, GC239 was significantly ($P<0.05$) different to all cultivars assessed in this trial, with a 57% growth advantage over 'Kopu II' (Table 1). This trial was severely infested with clover root weevil in 2006 onwards and by drought stress in the summer and autumn of 2007. While GC239 was the

Table 1 Leaf size, stolon density and growth score means of seven white clovers relative to 'Kopu II' (100%) in two trials rotationally grazed with sheep in Manawatu (Trial 1) and Ruakura (Trial 2) which included GC239 as an experimental line. Cyanogenesis data for each population for percentage of plants that produced hydrogen cyanide (HCN) are presented. Missing data are indicated as '-'.

	Manawatu				Ruakura
Cultivar	HCN %	Leaf Size (mm)	Stolons / m^2 Year 3	Mean Growth	Mean Growth
Apex	97	82	132	92	-
GC121	100	85	109	92	143
GC239	73	92	156	110	157
Huia	72	81	106	76	81
Kopu II	77	100	100	100	100
Sustain	67	88	109	86	101
Tribute	88	89	159	95	116
LSD (0.05)		8	45	7	16
Kopu II Mean		21.5	889	6.2	4.9

Table 2 Leaf size and mean seasonal growth score data for eight white clover cultivars compared to 'Kopu II' under rotational dairy grazing in small plot Trials 3–5 in the Waikato, Southland and Victoria Australia, respectively. Missing data are indicated as '-'.

	Leaf Size	Mean Growth		
Cultivar	(mm)	Waikato	Southland	Victoria (Aus)
Apex	68	87	110	-
Huia	73	39	97	-
Kopu II	100	100	100	100
Kotare	96	80	86	106
Legacy	96	122	106	152
Sustain	72	80	82	-
Tribute	78	90	85	136
Trophy	72	97	84	156
Weka	83	82	81	103
LSD (0.05)	19	8	3	28
Kopu II mean	4.2	5.3	6.7	2.2

Table 3 Leaf size and seasonal, annual and trial average growth of eight white clover cultivars relative to 'Kopu II' (100%) under beef cattle grazing in the Manawatu (Trial 6) from 2011-2015.

	Leaf Size	Mean Growth								
Cultivar	(mm)	Spring	Summer	Autumn	Winter	Year 1	Year 2	Year 3	Year 4	Overall
Apex	75	123	96	91	111	113	94	111	87	104
Huia	80	66	68	65	56	59	53	72	79	66
Kopu II	100	100	100	100	100	100	100	100	100	100
Kotare	105	139	124	121	116	136	130	127	111	124
Legacy	106	157	150	138	138	150	148	158	148	146
Sustain	83	69	70	70	61	68	58	74	77	70
Tribute	79	104	107	96	100	100	93	109	101	101
Trophy	78	112	98	75	106	105	90	95	73	95
Weka	96	138	131	125	110	137	137	118	121	126
LSD (0.05)	19	25	20	28	29	15	18	16	23	17
Kopu II mean	4.2	5.6	5.9	6.1	5.4	5.2	5.5	6.2	6.8	5.8

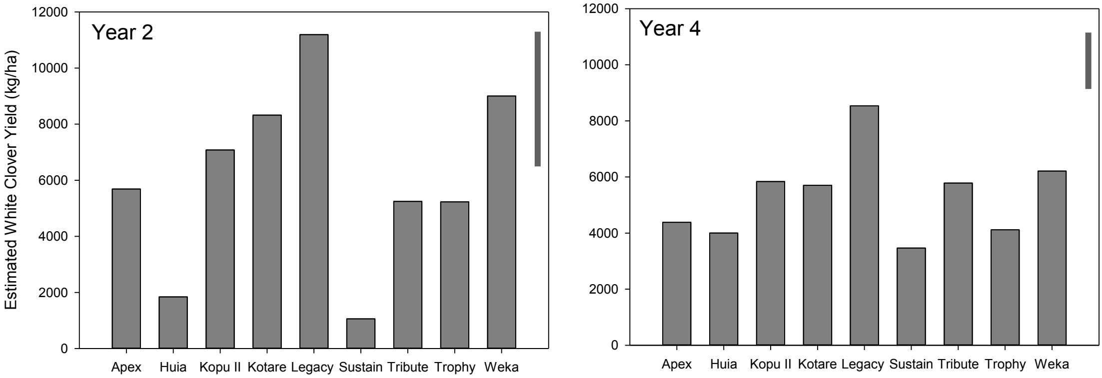

Figure 2 Estimated dry matter yield of white clover in small plot Trial 6 at years 2 and 4. Bar at right hand side of each chart is LSD at a 5% significance threshold.

entry with the highest growth, the drought tolerant line GC121 (Jahufer *et al.* 2009), the forerunner of 'Trophy' was the next best performing entry. Both GC239 and GC121 were significantly ($P<0.05$) better than 'Tribute' and 'Sustain' which have similar leaf size to the two experimental lines, indicating a genetic advance was made (Table 1).

Trials 3–5

While the early evaluations under sheep and dairy cattle grazing were promising, multi-site testing of potential cultivars is important to gain insight into genotype × environment interactions. This enables assessment of broad adaptation to Australasian grazing systems, and provides seed industry and farmers with information to support adoption. Trials 3–5 are from three disparate pastoral environments in Australia. In each trial, the cultivar had significantly higher estimated clover yield ($P<0.05$) than 'Kopu II', with a yield advantage of 22% in the Waikato, 6% in Southland, and 52% at Glenormiston, Victoria. There were also significant ($P<0.05$) differences to all other cultivars tested in the Waikato including two recently released cultivars 'Kotare' and 'Weka' (Table 2). The moisture stress and insect pressure in the summer and autumn of 2010 in Trial 3 in the Waikato tested adaptation to biotic and abiotic stress, which may have contributed to the relative advantage of 'Grasslands Legacy' over other cultivars in that trial.

In Southland, the dry matter yield of 'Grasslands Legacy' was less than 'Apex' but significantly ($P<0.05$) superior to all other cultivars in Trial 4 including 'Tribute', 'Kotare' and 'Weka' (Table 2). The conditions in Southland are different to other regions of Australasia where in most years weather conditions are favourable for clover, pest numbers are low, and the absence of winter grazing favours pasture recovery.

In Trial 5, the cultivar 'Trophy', bred in hot and dry conditions of New South Wales, was the top ranked entry for clover growth at Glenormiston, Victoria, Australia after 3 years under grazing (Table 2). However, it was not significantly ($P>0.05$) different from either 'Grasslands Legacy' or 'Tribute'. The mean growth score of 'Grasslands Legacy' over the 3 years in Trial 5 was significantly better than 'Kopu II', 'Kotare' and 'Weka' (Table 2).

Trial 6

The estimated seasonal dry matter yield of 'Grasslands Legacy' in the Manawatu were significantly better than 'Kopu II' by 57% in the spring and 38% in autumn and winter across the 4 years of evaluation. This emphasises the cool season activity advantage this cultivar offers farmers where it is significantly ($P<0.05$) better than 'Kopu II' in maintaining production throughout the year (Table 3). 'Grasslands Legacy', 'Kotare' and 'Weka' all exhibited significantly ($P<0.05$) more growth in spring and summer than 'Kopu II'.

In addition to the improved cool season activity, 'Grasslands Legacy' showed superior performance to all cultivars assessed in this trial for mean annual yield in the third and fourth years ($P<0.05$). 'Grasslands Legacy' was 58% and 48% better than 'Kopu II' in years three and four, respectively. 'Weka' and 'Kotare', the next best performing cultivars, were not significantly ($P>0.05$) different to 'Tribute' and 'Apex' in the third year, or to 'Tribute' and 'Kopu II' in the fourth year. The oldest cultivars in this trial were 'Grasslands Huia' (released in 1957) and 'Grasslands Sustain' (released in 1996). They were the poorest performing for yield and persistence over the duration of the trial.

The mean dry matter yield of the white clover cultivars in Trial 6 varied significantly ($P<0.05$, Figure 2). There is a dry matter yield advantage for 'Grasslands Legacy' in both years, estimated at 1.5 tonnes/ha in the fourth year. 'Grasslands Legacy' produced 600 kg/ha more dry matter than the four next best cultivars 'Kotare', 'Kopu II', 'Tribute' and 'Weka' in year three as well (Table 3). These very high yields of white clover are attributed to the dry summers advantaging white clover over the grass component of the trial, in which some plots effectively became clover monocultures as the trial progressed. These may not be representative of results in larger plots and swards, where further agronomic evaluation is warranted to assess the impact at that scale.

'Grasslands Legacy' was the highest yielding white clover in Trial 6, and was able to compete well with perennial ryegrass in the presence of nitrogen fertiliser application. The trial experienced periods of summer moisture stress during 2013, 2014 and 2015 (Figure 1). During these periods, which coincided with the natural summer dominance of white clover, lower perennial ryegrass densities were observed in Trial 6 (data not shown). This may have further reduced competition and enabled increased stolon branching and stolon numbers which influence white clover yield over time (Brock & Hay 1996), further contributing to the high estimates for white clover yield.

Sward yield (kg/ha) from calibrated rising plate meter (RPM) readings before and after each grazing give an indication grazing management practices in Trial 6 (Table 4). The plate measure recordings are an estimate of the total yield of plots in the trial, rather than white clover content. White clover yield scores and regression cuts were assessed when total yield on the RPM was over 3000 kg/ha with post-grazing residuals averaging 1650 kg/ha to favour grass growth and apply added stress to the white clover. The r^2 values between yield scores and regression cuts were between 0.78 and 0.93 per harvest (data not shown), indicating a reliable correlation between visual assessment and dry matter yields.

While the RPM data are of use in grazing management for the trial, the small plot size and constraints of the trialling systems to accommodate large numbers of entries mean these data may not be representative of pasture mass in larger plots and at pasture and farm scale. Further agronomic work to assess the relative yield and adaptation at scale and under varying management is warranted.

Conclusions

Over a decade of experimental activity across generation and locations was used to develop and evaluate adaptation of 'Grasslands Legacy' in comparative trials under representative pasture management, using replicated, small plot evaluation formats.

In breeding trials 'Grasslands Legacy' has repeatedly exhibited high growth and persistence in Australasian environments. In early breeding it had higher seasonal yield across 3 years at the Manawatu and Waikato sites, compared to the widely used cultivar 'Kopu II'. After parental selection similar agronomic potential was seen in mean seasonal yield, under simulated dairy farm grazing management at Waikato, Southland and Glenormiston in Australia in the later years of testing.

These data indicate broad adaptation of 'Grasslands Legacy', with advantages over cultivars such as 'Kopu II', 'Kotare', and 'Weka' in these trials. Relatively higher tolerance to moisture stress was observed under field conditions in the Waikato, Manawatu, and Glenormiston, where it was better able to survive and recover than other cultivars in the hot and dry conditions the trial experienced.

The performance of 'Grasslands Legacy' under relatively high nitrogen application in the Manawatu trial under cattle indicated its adaptability to respond positively to more intensive farm practices. 'Grasslands Legacy' also exhibited agronomic advantages with the lower levels of 100 units of N applied per annum, suggesting it has adaptation to lower input systems as well.

Availability of 'Grasslands Legacy' in Australasia will offer dairy farmers a broadly adaptive large-leaved cultivar with excellent cool season activity, high stolon density and above average persistence. This provides a new cultivar option for the farmers in Australasia seeking to improve white clover yield and persistence in pastures under dairy grazing management.

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Table 4 Estimated mass (kg/ha) of seasonal pre-grazing herbage, post-grazing residual, and estimated consumption based on calibrated rising plate meter (kg/ha) measurements in Trial 6 in the Manawatu from 2011 to 2015.

	Sum1	Aut 1	Wint1	Spr1	Sum2	Aut2	Win2	Spr2	Sum3	Aut3	Win3	Spr3	Sum4	Aut4
Pre grazing	3478	6086	6753	6698	5906	6103	6018	6995	5566	5247	2861	6922	7945	3087
Post grazing	1752	3424	3572	3584	3376	3591	3006	3536	3425	3348	1574	3644	3562	1781
Consumption	1726	2662	3181	3114	2530	2512	3012	3459	2141	1899	1287	3278	4383	1306
Grazings/season	1	2	2	2	2	2	2	2	2	2	1	2	3	1

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