Agronomic evaluation of white clover selected for increased floral condensed tannin

V.T. BURGGRAAF[1], P.D. KEMP[2], E.R.THOM[1], G.C. WAGHORN[3], D.R. WOODFIELD[3] and S.L. WOODWARD[1]
[1]*Dexcel, PB 3221, Hamilton*
[2]*Institute for Natural Resources, Massey University, PB 11222, Palmerston North*
[3]*AgResearch, Grasslands Research Centre, PB 11008, Palmerston North*
vicki.burggraaf@dexcel.co.nz

Abstract
The agronomic performance of an experimental white clover (HT) selection bred for increased floral condensed tannin (CT) production and extended flowering season was compared with Grasslands Huia white clover, under dairy cow grazing in the Waikato. Pure clover swards were sown at 5 kg/ha in autumn 2001. Seedling establishment was better in HT than Huia, but stolon growing point densities from January 2002 to May 2003 were 13 to 62% greater in Huia than HT. Huia swards had higher clover contents than HT, and produced 0.8 t/ha more DM (dry matter) in the first year. CT concentrations in the flower heads varied over the season, but were similar in the two clovers (1 to 8% of DM). Higher flower densities in HT resulted in higher clover CT concentrations (peak of 0.6 vs 1.2 % of DM for Huia and HT, respectively). Further testing is required to determine any effects of HT on animal performance.

Keywords: condensed tannins, flowering, growing point density, *Trifolium repens*, white clover

Introduction
White clover (*Trifolium repens*) is an important component of temperate pastures because of its high nutritive value, and its ability to fix nitrogen and withstand grazing. However, limitations exist because of its propensity to cause bloat, and the large wastage of protein due to rapid degradation to ammonia in the rumen (Howarth *et al*. 1978). These problems may be overcome by increasing the condensed tannin (CT) concentration in white clover. CT are phenolic compounds produced in a range of plant species (Jones *et al*. 1976). CT bind to plant proteins, reducing the formation of the protein based foams in the rumen that cause bloat (Kendall 1966). The presence of CT in various forages has been shown to increase milk and milk protein yield, wool growth, fecundity and animal livewieght gain, while also reducing bloat in cattle. Benefits of CT to nutrition arise from reduced plant protein degradation to ammonia by rumen microbes, thereby increasing the by-pass of plant protein to the intestines, where they are absorbed (Waghorn *et al*. 1987).

More than 25 years of international research has failed to provide white clover containing foliar CT. White clover does, however, naturally produce CT in its flower heads (Jones *et al*. 1976), although the current concentrations may be too low to affect animal performance. Attempts by AgResearch to capitalise on the natural occurrence of floral CT in white clover resulted in the development of a high floral tannin (HT) selection.

A white clover with increased CT has the potential to improve animal performance, however, increased flowering and CT production may place agronomic limitations on the plant. In temperate regions, white clover persists primarily by the production of stolons (Harper 1978). As flowers are produced at the same site as new stolons (Thomas 1980), increased flowering may reduce plant persistence. CT production also limits the energy and substrates available for growth and primary metabolism (Chew & Rodman 1979). This research compares herbage accumulation, stolon density, flowering intensity and seasonal CT concentrations of HT and Grasslands Huia white clover over 2 years from sowing.

Materials and methods
Breeding history
Development of the HT selection used in this experiment involved three phases: (i) divergent selection for floral CT content; (ii) selection for extended flowering and high floral tannin content; and (iii) seed multiplication without selection. In the first phase four cycles of divergent selection for floral CT content were undertaken in Grasslands Huia white clover resulting in development of high and low floral CT lines which differed by 66% in CT content (Woodfield *et al*. 1998). To make this CT available to grazing dairy cattle over a greater period of the year, phase two involved the high floral CT Huia selection being crossed with plants from the cultivar Siral, which has an extended flowering duration (early spring to late autumn). Two cycles of selection for increased floral CT content and extended flowering were undertaken. The CT concentration in the resulting F3 generation was 32% higher than the mean CT

concentration of the two parental cultivars (Grasslands Huia and Siral). In phase three, two generations of random intermating without selection for CT content or flowering duration were undertaken under stringent isolation to produce the required seed quantities for this experiment. The CT content of the resulting nucleus generation was only 19% higher than the mean of the parental cultivars (Woodfield unpublished data) indicating that some of the selection gains had been lost during seed multiplication.

Trial design and pasture management

The experiment was conducted at Dexcel's Lye Farm, Hamilton, on a Te Rapa humic silt loam soil. Three replicates of each treatment (swards of HT white clover or Grasslands Huia white clover) were arranged in a randomised block design. Each replicate was divided into two 0.5 ha HT paddocks and two 0.5 ha Huia paddocks.

The existing pasture was sprayed in November 2000 with glyphosate and dicamba herbicides, then cultivated and fertilised with superphosphate (90 and 110 kg/ha of phosphorus (P) and sulphur (S), respectively for replicate 1, and 59 and 72 kg/ha of P and S, respectively for replicates 2 and 3), to bring the soil Olsen P concentration of all replicates up to 35 µg/ml. An initial attempt at establishing the clover swards in spring 2000 failed, and the paddocks were left fallow until April 2001. Paddocks were then resprayed and cultivated before repeating the roller drilling at 5 kg/ha on 26 April 2001.

Two months after drilling, emerging weeds were sprayed with Preside herbicide (52 g/ha flumetsulam plus 1 L/ha uptake oil). Further herbicides (Gallant NF, Asulox, Jaguar and 2,4-DB) were applied at the recommended rates during the experiment to control grasses, broad-leaved dock (*Rumex obtusifolius*) and other pasture weeds. Maintenance dressings of fertiliser (MagPhos, muriate of potash, urea, potassic sulphur super and super 10) were applied throughout the experiment. Clover treatments were grazed simultaneously by dairy cows when herbage mass reached approximately 2 to 2.5 t DM/ha, or as required for a concurrent cow grazing experiment. Swards were cut for silage on 18 October 2001, 3 January 2002 and 8 January 2003.

Clover density

Five and 11 weeks after sowing, clover seedlings were counted in 60, 0.12m^2 quadrats per paddock. Clover flowers were counted in October 2001 in 15, 0.12 m^2 quadrats per paddock. From November 2001 to July 2002, clover stolons (growing points), flowers and seedlings were counted bi-monthly in 15 quadrats (5 x 20 cm) per paddock. The frequency of flower, seedling and stolon growing point counts was increased to monthly between July 2002 and May 2003.

Herbage dry matter accumulation

Twelve 0.5 m^2 exclosure cages were placed in each treatment (two per paddock) in December 2001, and the herbage within each cage trimmed to 2 cm. Every 3 to 8 weeks, herbage mass was estimated by cutting a 0.2 m^2 quadrat to 2 cm within each cage, after which the cage was relocated, avoiding areas containing weeds or grasses. The cut herbage was dried for 36 hours at 95°C before weighing.

Condensed tannins and botanical composition

CT concentration was measured bi-monthly in white clover flower heads from November 2001 to July 2002. The following season (September 2002 to April 2003), CT concentrations were measured monthly when sufficient flowers were present. A sample was collected from each paddock by cutting to just above ground level (so as to avoid stolons). Each sample was dissected into clover flower heads, the remainder of the clover plant, and other species. The fresh weight of each component was recorded, then a subsample was dried to determine DM%. The proportion of each component was then calculated on a DM basis for each paddock. The CT concentration in the total clover herbage was determined by multiplying the CT content of the flower heads by the proportion of flower heads in the herbage, and is referred to as clover CT concentrations, as opposed to the concentration measured in the flowers, which is referred to as floral or flower head CT.

A 50 g sample of fresh clover flower heads from each paddock was freeze-dried and ground, and the CT content determined using the butanol HCl colorimetric technique (Terrill *et al.* 1992) to measure free, protein bound, and fibre bound CT. Once 60 samples had been analysed using this method, samples were scanned to establish a calibration curve for CT analysis by near infrared spectroscopy (NIRS) (Corson *et al.* 1999).

Statistical analysis

Significant ($P<0.05$) treatment differences were determined by analysis of variance, using Genstat 5 (version 4.1). Data were analysed separately for each date, as a randomised complete block design. For botanical composition data, the percentage of DM as flower head could not be analysed for July and September 2002 due to most values being zero.

Figure 1 Density of clover growing points in pure swards of Grasslands Huia and HT white clover up to 2 years after sowing. Error bars represent LSD (P<0.05).

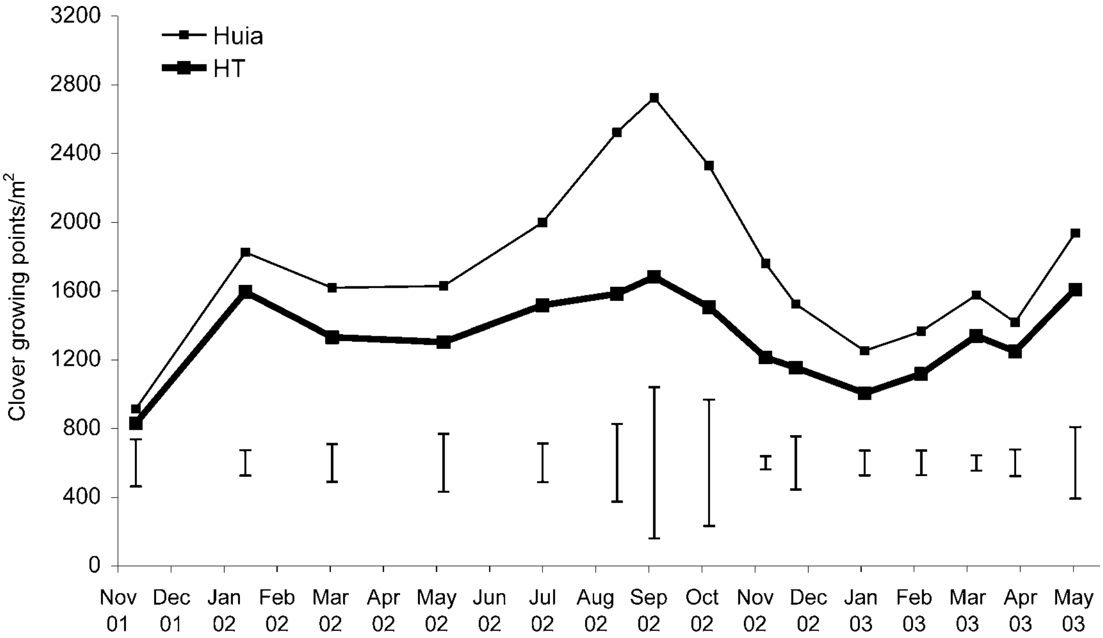
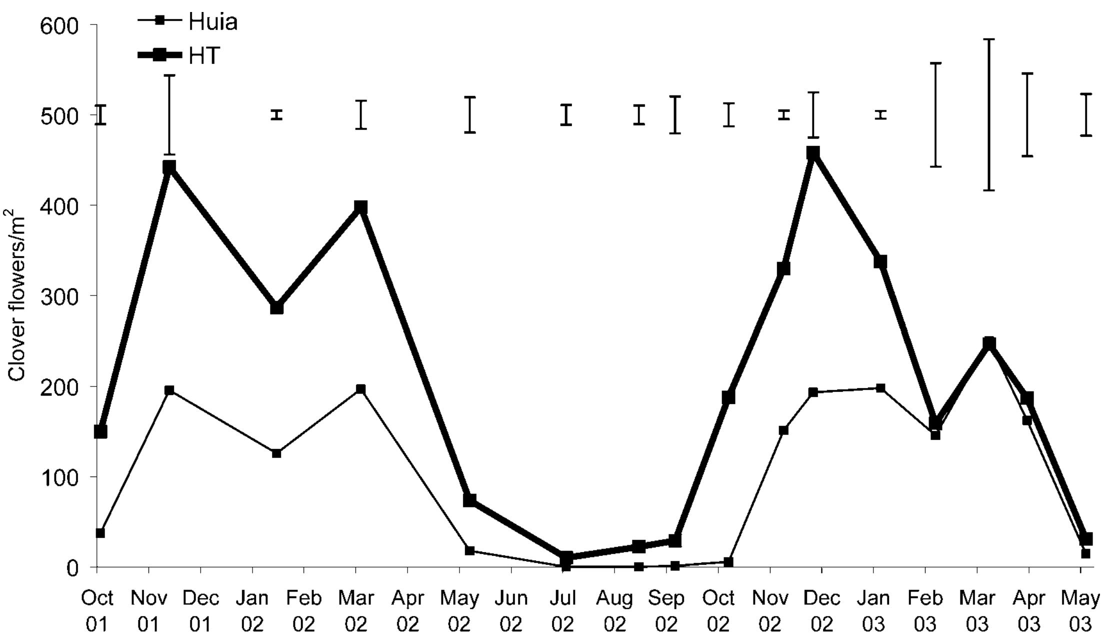


Figure 2 Density of clover flowers in pure swards of Grasslands Huia and HT white clover up to 2 years after sowing. Error bars represent LSD (P<0.05).



Results

Clover flower, seedling and stolon growing point density

Clover seedling densities were greater in HT than Huia both 5 weeks (352 versus 204 seedlings/m^2; P<0.05) and 11 weeks (313 versus 187 seedlings/m^2; P<0.01) after sowing. The following autumn (2002), clover seedling densities were 203 vs 69 seedlings/m^2 in HT and Huia, respectively. Comparable data in autumn 2003 were 537 vs 440 seedlings/m^2, but differences in the latter 2 years were not significant.

Stolon growing point density was similar between treatments at the first measurement in November 2001, after which the treatments diverged, with Huia having between 13 and 62% higher growing point densities than HT for the remainder of the experiment (Figure 1).

Flowers were present throughout the year in HT, but were only evident from October to May in Huia (Figure 2). In the first year, flower densities in HT were more than double those of Huia, peaking in November at 442 and 196 flowers/m^2, respectively (P<0.01). The start of the second flowering season was similar to the first, but from February to May 2003, no differences were detected between treatments.

Table 1 Seasonal clover herbage accumulation (kg DM/ha) of Grasslands Huia and HT white clover swards from 1 December 2001 to 28 February 2003.

Season	Huia (kg DM/ha)	HT (kg DM/ha)	SED
Summer 1 (Dec 01 to Feb 02)	4886	4342	126*
Autumn (Mar 02 to May 02)	1949	1925	10^NS^
Winter (Jun 02 to Aug 02)	194	410	32*
Spring (Sep 02 to Nov 02)	3302	2810	57*
Year 1	10331	9487	160*
Summer 2 (Dec 02 to Feb 03)	3611	3069	77*

NS = not significant, * = P<0.05.

Table 2 Flower head content, and condensed tannin (CT) concentration in clover flower heads, and whole clover (leaf plus flower) as a % of DM for Grasslands Huia and HT white clover from November 2001 to April 2003.

Month	Flower head %			Flower head CT%			Clover CT%		
	Huia	HT	SED	Huia	HT	SED	Huia	HT	SED
Nov 01	3.6	9.9	0.69*	6.5	7.0	0.23NS	0.23	0.70	0.10*
Jan 02	4.9	15.3	0.59**	7.0	7.9	0.26NS	0.34	1.21	0.07**
Mar 02	13.2	21.2	1.35*	3.9	4.0	0.68NS	0.50	0.87	0.14NS
May 02	1.2	2.9	0.42NS	4.5	5.3	0.57NS	0.06	0.15	0.01**
July 02	0.0	0.1	-	-	-		-	-	
Sep 02	0.0	0.5	-	-	-		-	-	
Oct 02	0.0	0.7	0.06**	-	4.6[a]		0.00	0.03	
Nov 02	3.2	10.0	0.27**	7.5	7.9	0.22NS	0.24	0.79	0.04**
Dec 02	4.7	14.1	2.01*	7.1	7.5	0.28NS	0.33	1.07	0.15*
Jan 03	12.8	22.0	0.96*	3.3	3.0	0.22NS	0.43	0.65	0.04*
Feb 03	12.6	14.2	0.45NS	4.7	4.7	0.15NS	0.57	0.65	0.03NS
Mar 03	11.5	14.4	0.94NS	2.8	3.0	0.21NS	0.32	0.42	0.03NS
Apr 03	5.2	7.7	0.45*	1.7	1.3	0.12NS	0.09	0.10	0.01NS

[a] One bulk sample collected across all HT paddocks.
NS = not significant, * = P<0.05, ** = P<0.01.

Herbage accumulation

Annual clover dry matter accumulation for Huia exceeded that for HT (P<0.05; Table 1). HT grew approximately 0.5 t DM/ha less than Huia in both summers, and in spring 2002. Autumn yields were similar between treatments, and winter yields were higher for HT.

Botanical composition and condensed tannin concentration

Non-sown species contributed less than 5% of DM for both treatments in the first year. In the second year clover contents were always greater in Huia than HT swards. Weeds contributed 0-11% of DM for Huia and 1-19% for HT, whilst grass accounted for 0-4% for Huia and 0-12% for HT. Clover content was lowest in July 2002, with 84 and 68% of DM as clover in Huia and HT, respectively (P<0.05). The remainder of pasture was *Poa annua* and chickweed (*Stellaria media*).

The flower head proportion of clover plants was consistently higher in HT, peaking in March 2002 and January 2003 (Table 2). There was large seasonal variation in the concentration of CT in flower heads, being highest in late spring/early summer, and low in autumn, with little difference between treatments (Table 2). Clover CT concentrations were higher in HT than Huia, with a maximum of 1.2% for HT, and 0.6% of DM for Huia (Table 2).

Discussion

Condensed tannin concentration

This experiment has shown that white clover CT concentrations in the sward can be increased by plant selection. This was achieved primarily through increased flower production with no differences in floral CT concentration between the Huia and HT treatments. The absence of any significant difference in floral CT between treatments was not expected. Tests from various generations of the HT selection showed that there was a loss of CT during the seed multiplication, but the floral CT level of HT was still 26% higher than Huia (Woodfield unpublished data). Further work is needed to determine whether differences between initial plant testing and the field experiment is due to genetic x environmental effects or other factors.

The clover CT concentrations in HT (maximum of 1.2 % of DM) were low compared to bloat safe legumes such as lotuses (2-8%), sulla (*Hedysarum coronarium*; 3-12%) or sainfoin (*Onobrychus viciifolia*; 5-8%) (Waghorn *et al.* 1998). However, the optimum concentration of dietary CT varies with type and diet, with an approximate upper limit of 4-6% of DM for improving the nutritional value of forages (Waghorn *et al.* 1998). Although Li *et al.* (1996) suggested 0.1 – 0.5 % of CT in the DM could prevent bloat, this estimate has not been tested and some of their data were based on CT measured using the acidified vanillin technique, which accounts for only about half of the total CT in legumes (Terrill *et al.* 1992). Even though the CT in HT clover rarely exceeded 1% of clover DM, similar concentrations (0.7% from an acidified vanillin analysis) have lowered rumen ammonia concentrations in cows fed white clover with maize silage, and appear to have 'over protected' dietary protein for milk production relative to autumn clover containing 0.2% CT in the DM (Stockdale & Dellow 1995).

The peak period for bloat deaths in New Zealand is October-November, and to a lesser degree, March (Reid 1974). Stockdale (1994) reported a marked reduction in the incidence of bloat when white clover contained more than 5% DM as flowers, and attributed this to the presence of condensed tannins. High flower densities may also affect bloat and nutrition by reducing the metabolisable energy, digestibility, and protein concentration of the diet (Stockdale 1999). In this experiment, flower densities were very low in October, and are unlikely to be adequate for bloat control, but the higher densities in November and March may prove beneficial.

Seasonal variation in CT concentration is not well understood, and although it is increased in *Lotus* species by temperature and soil moisture stress (Duarsa *et al.* 1993), this is not the case in other species. Stockdale & Dellow (1995) reported a similar reduction to ours in white clover floral CT between summer and autumn. Variation in flowering intensity and CT production, and the dilution of CT when clover is grown in mixed pastures will limit its value for bloat prevention and nutrition.

Clover production and persistence

Seedling densities in both clover treatments were above the recommended establishment target of 150 plants/m^2 (Haggar *et al.* 1985), but the subsequent agronomic performance of HT was inferior to that of Huia. The HT selection has not undergone any selection for agronomic performance and hence may lack adaptation under intensive grazing. Siral white clover, which was used as a parent due to its unusually prolonged flowering, was developed in Australia from an Algerian ecotype. It has good winter growth and good persistence under moisture stress in Australia but has poor agronomic performance in New Zealand. Woodfield & Caradus (1994) compared Siral to Huia under sheep grazing and found lower growing point densities, a lower proportion of clover in the pasture and lower clover DM production for Siral than for Huia. In New Zealand, Mediterranean clovers are often winter active and become summer dormant during periods of high temperatures and drought (Caradus 1994). The lower summer yields of HT would be a disadvantage on dairy farms, as improved summer growth of white clover is important for maintaining milk production when ryegrass quality is declining (Woodfield *et al.* 2001).

The other major factor in the poor agronomic performance of HT is the trade off between stolon growing point density and increased flowering. In selecting for increased flowering, a reduction in stolon growing point density was expected since a stolon apical bud can give rise to either a stolon branch or a flower head but not both (Thomas 1980). High stolon growing point densities are important for clover persistence, allowing quicker recovery after droughts or pasture damage, and quicker colonisation of pasture gaps, minimising weed invasion (Cooper *et al.* 1997). The poor agronomic performance of HT further emphasises the importance of high stolon growing point densities under dairy grazing, and suggests that any future breeding efforts must be undertaken in germplasm with high stolon growing point densities. The reduced stolon density in HT enabled the invasion of weeds, resulting in lower clover contents than for Huia swards. This also suggests that HT may be less competitive than Huia when sown in mixed swards.

Conclusions

Flower densities in HT clover swards were usually higher than in Huia swards, resulting in higher clover condensed tannin concentrations. However, HT swards had more weeds, lower stolon growing point densities, and produced less herbage than Huia swards. Further testing with grazing dairy cows will determine whether these CT concentrations reduce bloat and rumen protein breakdown, and increase milk production. Beneficial responses would require a better understanding of the control of CT production in white clover, and further plant selection in germplasm with improved adaption to dairy grazing.

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