Development of white clover populations with higher concentrations of water soluble carbohydrate

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Abstract

Water soluble carbohydrate (WSC) provides readily available energy in the rumen that improves the efficiency of crude protein (CP) utilisation, partitioning of dietary N towards animal growth, and reduces the loss of N as urea. Divergent selections within three populations of white clover were made using near infrared reflectance spectroscopy (NIRS) estimates of WSC and CP concentrations in leaves from potted plants in the Manawatu. After four selection cycles (2001-2004), the high sugar (HS) populations had 35% greater WSC concentration and 28% lower CP concentration than the cycle average. The magnitude and rate of response to selection was equivalent in all three source populations. The HS and low sugar (LS) populations together with two modern cultivars were field tested in Canterbury. Some HS populations had significantly ($P<0.001$) higher WSC and concomitantly lower CP concentrations than the LS populations and cultivars during all seasons except winter. HS plants from different populations were inter-crossed to restore plant vigour which had declined during the selection process. The resultant progenies were evaluated in mixed clover/ryegrass swards in Canterbury and Manawatu for 2 years. Individuals from families exhibiting HS and vigour comparable to cultivars were identified and inter-crossed within medium and large-leaf size classes.

Keywords: soluble carbohydrates, protein, plant vigour, plant breeding

Introduction

Increasing the feeding value of forage offers potential benefits in animal production as well as the opportunity to reduce a farm's environmental footprint. White clover (*Trifolium repens*) has superior nutritive value compared to the main forage grasses (Ulyatt 1997) but there may still be scope to improve its already high feed quality through changes in concentration of water soluble carbohydrate (WSC) and crude protein (CP). Perennial ryegrass (*Lolium perenne*) cultivars selected for higher levels of WSC are available in New Zealand (Easton *et al.* 2009) and some studies have shown an advantage for animals feeding on these in terms of reduced loss of nitrogen (N) and in some cases, an increase in animal performance (Edwards *et al*. 2007). In high WSC feeds, the enhanced levels of readily available energy improve the efficiency of CP utilisation, and partition dietary N toward animal growth thereby reducing losses to the environment as urea (Cosgrove *et al*. 2007).

Compared to other forages, white clover foliage has a high concentration of CP and a relatively low concentration of WSC (Cosgrove *et al.* 2006). There are both animal production and environmental consequences of this high protein diet (Pacheco & Waghorn 2008). Woodfield *et al.* (2001) tested a range of white clover cultivars and found that foliar WSC concentrations varied among individuals from 135-205 g/kg DM, with a trend for large-leaved plants to have higher concentrations of WSC than small-leaved types. These data suggest that gains in herbage quality may be possible using a selection program aimed at increasing the concentration of WSC in white clover foliage, potentially leading to animal production and environmental benefits in mixed white clover/ryegrass pastures.

The objectives of this research were to: a) alter WSC concentrations in white clover leaves, b) identify lines with high WSC and high agronomic performance, and c) characterise seasonal and geographic effects on WSC accumulation in white clover.

Materials and Methods

Plant material and WSC selections

The conventional plant breeding practice of divergent selection was used to develop white clover lines with modified WSC concentrations. High sugar (HS) and low sugar (LS) lines were developed through parallel recurrent selection within three white clover populations, New Zealand large-leaved (NZLL), USA large-leaved (USLL) and New Zealand small-leaved (NZSL). In each selection cycle, 100-140 plants within each population were tested. Each plant was grown in a pot containing peat and soil mix in a glasshouse during winter at Palmerston North. Pots were positioned outside in August and leaves from each pot were sampled on one day during late October or early November. At sampling, 20-30 leaf lamina were removed from each plant between 8:00 and 10:00 am, sealed in a plastic bag and snap-frozen in liquid N. Samples were freeze-dried, finely ground and analysed for WSC and CP concentration using an MPA Brucker Near Infra Red Spectrophotometer (NIRS; FeedTech, AgResearch Grasslands, Palmerston North). In each cycle, the six groups of selected plants were each isolated with bumblebees (*Bombus* sp.) to carry out cross pollination, resulting in six populations. Typically, 20-25 plants with either HS or LS were identified per cycle in each population sample. Four selection cycles within each population were completed from 2000 to 2004.

Seasonal patterns of WSC and CP concentration

Seed from the fourth selection cycle of populations NZLL-HS, NZLL-LS, USLL-HS and USLL-LS, together with cultivars 'Kopu II' and 'Tribute' were sown in trays containing a peat and soil mix in a glasshouse at AgResearch Lincoln in July 2005. Plants were transferred to the field on 6 October and established in a randomised complete block design with four replicates. Plants were spaced 0.7 m apart, using 10 plants per population per replicate. Soil test results based on samples taken from the trial site indicated adequate soil fertility (pH 5.6, Olsen P 37, K quick test 20 and S level 9).

Seasonal samplings of foliage for chemical composition analysis were taken during the morning on the following dates: 20 December 2005 and 21 February, 30 March, 2 June, 12 October and 28 November 2006. At each sampling, 10 leaves were removed from each plant and bulked among plants within replicate within population, and snap-frozen. The leaves were prepared and analysed using NIRS as described above.

Additional selection for vigour restoration under grazing

The cultivars and fourth selection cycle plants in the seasonal pattern trial were assessed visually for growth (scored on a 1 = low to 9 = high) during each sampling event, then cut to 3 cm above ground level with a mower. Throughout the course of the experiment both the HS and LS populations exhibited reduced plant vigour compared with the commercial cultivars (data not presented). This yield reduction may have been a result of the sampling strategy used or inbreeding depression during the recurrent selection process. To decrease inbreeding, multiple HS plants from the fourth cycle populations were selected and pair-crossed in three combinations (NZLL x USLL, USLL x NZSL and NZLL x NZSL) during summer 2006-07, giving rise to 60 new families.

Plants from each of the 60 new families together with parent lines and cultivars were transplanted into plots in a randomised complete block design with four replicates at sites in Canterbury and Manawatu in September 2007. Each plot consisted of 12 genotypes planted in a 1 x 0.25 m wide plot. Clover vigour in each plot was assessed visually (1-9 scale) every 4-6 weeks for 2 years. Following each assessment the trials were grazed with sheep in Canterbury and cattle in Manawatu.

In October 2008, stolon segments from vigorous plants were sampled from plots of the better progeny at both sites. In total, 220 stolon segments from within 24 progenies, and four stolon segments from each of unselected control plants sampled from 'Kopu II' and 'Tribute' were harvested and individually transplanted into three litre pots containing a soil and peat mixture and placed outside at AgResearch Lincoln. In early December, leaves were removed from each plant during mid-morning and prepared then analysed by NIRS as above. Forty six plants with high levels of WSC and agronomic merit were divided into two leaf size classes (medium and large) and allowed to inter-pollinate within leaf class.

Statistical Analysis

Data from the four selection cycles were subjected to analysis of variance, and tested for significant treatment (high versus low) selection effects within cycle. Data from the seasonal levels trial and replicated field trials were subject to analysis of variance using unadjusted means of cumulative yield and quality performance data for each experimental unit. Main (genotype) and interaction (genotype x environment) effects were tested.

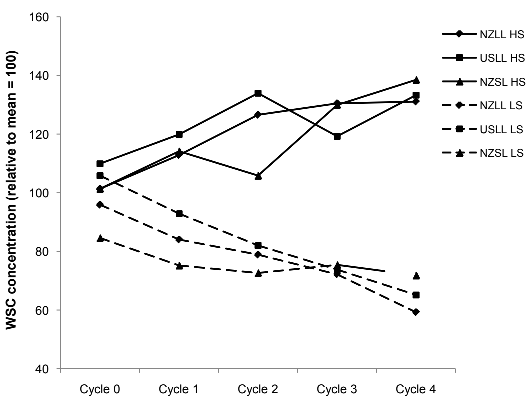
Results

WSC selections

Over four cycles of selection the HS and LS population's levels of WSC diverged significantly ($P<0.05$) from the initial unselected populations (cycle 0) and continued to diverge in each successive generation (Fig. 1A). By the fourth cycle of selection the HS lines were 35% higher than the cycle average, and the LS lines were 35% lower than the cycle average. The magnitude and pattern of response to selection was generally consistent in each of the three source populations. The CP concentrations for HS and LS populations were inversely proportional to the WSC concentrations (Fig. 1B). The HS populations showed declining CP concentrations in each successive generation and had 28% lower CP concentrations by cycle 4. Initial mean WSC concentrations of the HS populations were 230 g/kg DM rising to 310 g/kg DM in later cycles, while the mean CP concentrations decreased from 320 g/kg DM to 250 g/kg DM by cycle 4.

Figure 1 Relative water soluble carbohydrate (WSC) and crude protein (CP) concentrations in spring of leaves from white clover plants grown in pots. The six populations are from a divergent selection programme over four cycles conducted in Manawatu. The trait data are expressed relative to the within cycle mean. The $LSD_{(0.05)}$ values between HS and LS populations in cycles 1 – 4 are 1.87, 2.99, 1.94 and 2.66 respectively. For CP the $LSD_{(0.05)}$ values for cycles 1 – 4 are 1.99, 1.44, 1.58, 2.02 respectively; indicating significant differences were observed between the HS and LS selections in each cycle.

A) Water soluble carbohydrate



B) Crude protein

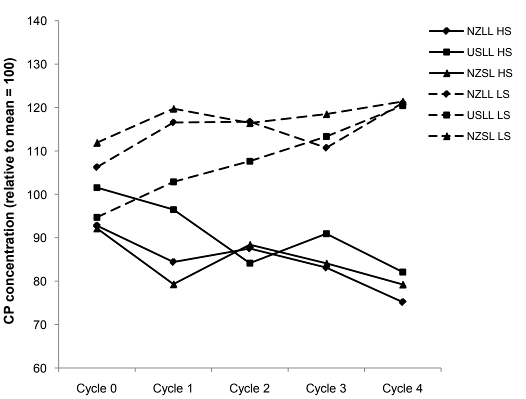
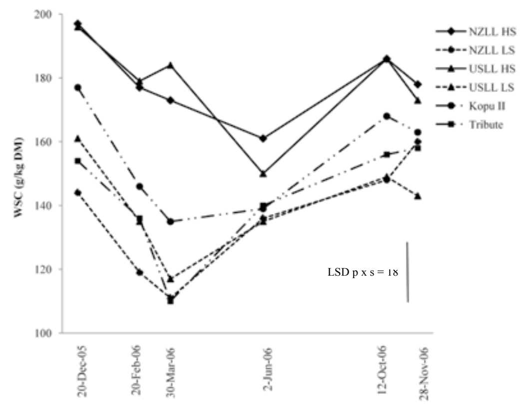
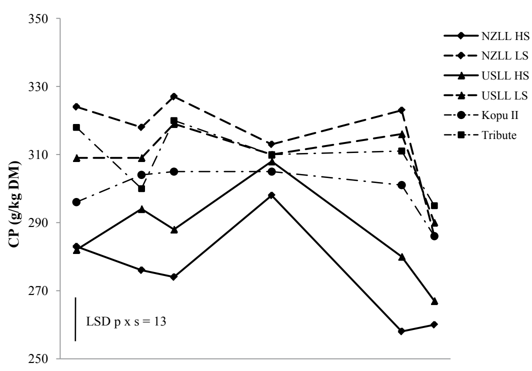


Figure 2 Seasonal water soluble carbohydrate (WSC) and crude protein (CP) concentrations in leaves sampled from four white clover populations and two cultivars ('Kopu II' and 'Tribute') grown as spaced plants in the field in Canterbury. The $LSD_{(0.05)}$ values for the population x season interaction are presented, and indicate season and population specific significant differences.

A) Water soluble carbohydrate



B) Crude Protein



Seasonal patterns of WSC and CP concentrations

In Canterbury, there were significant ($P<0.001$) among season and among population differences in WSC and CP concentration of clover leaves (Fig. 2A & 2B, respectively). The population x season interaction was significant ($P<0.001$) for both traits. For WSC, as measured mid-morning, levels were greatest in summer, declined in autumn and winter, increasing again in spring (Fig. 2A). Some HS populations from both the New Zealand and USA sources exhibited significantly higher concentrations of WSC compared to other populations in every season, except winter. The LS populations consistently exhibited the lowest concentrations of WSC, and the cultivars 'Kopu II' and 'Tribute' were intermediate. The season x population interaction was most obvious in winter when compared with other seasons. The population NZLL-HS had significantly lower concentrations of CP in all seasons except winter when all populations were similar (Fig. 2B); and the population USLL-HS was significantly lower in all but the late summer and winter measurement. The LS populations tended to have the highest concentrations of CP while the cultivars 'Kopu II' and 'Tribute' were intermediate, but these differences were not significant.

Combining herbage yield and WSC concentration

The plant growth vigour of the fourth cycle USLL population was significantly inferior ($P<0.05$) to the first cycle population in the first year at both sites, but in the second year the differences were non-significant

Table 1 The annual vigour score under sheep grazing (Canterbury) and cattle grazing (Manawatu) of high water soluble carbohydrate (HS) selected white clover after the first and fourth selection cycle, and of progeny of crosses between fourth cycle HS populations. The vigour scores are relative to cultivar 'Tribute'.

	Canterbury		Manawatu	
	Year 1	Year 2	Year 1	Year 2
HS Selections				
NZLL 1st cycle	79	76	88	96
NZLL 4th cycle	67	63	79	71
USLL 1st cycle	80	79	88	89
USLL 4th cycle	48	51	64	62
Mean of HS Cross Progeny				
NZLL x USLL (22)[1]	82	73	83	84
USLL x NZSL (21)	86	77	85	90
NZLL x NZSL (17)	81	78	73	75
Best HS Cross Progeny				
NZLL x USLL (#10)[2]	128	108	115	115
NZLL x USLL (#51)[2]	83	59	103	121
USLL x NZSL (#19)	117	118	95	91
USLL x NZSL (#35)	86	78	97	126
NZLL x NZSL (#20)	116	108	93	80
NZLL x NZSL (#56)	85	94	94	121
Cultivar				
'Kopu II'	103	75	104	121
'Tribute'	100	100	100	100
'Crusader'	105	100	92	82
'Bounty'	105	116	92	86
'Demand'	95	90	78	89
LSD_{P} [3]	20	33	21	27
$LSD_{p \times s}$	21	30	21	30

[1] The number of progeny making up the mean value
[2] The progeny identity that had the highest value in either Canterbury or Manawatu
[3] The $LSD_{0.05}$ for progeny (p) and the progeny x site (p x s) interaction

(P>0.05, Table 1). The fourth cycle NZLL population had less growth (but not significant) compared to the first cycle population at both sites and years. The mean vigour score of the 60 progenies from the three crossing groups at both sites was similar to the NZLL and USLL first cycle populations (Table 1) indicating good recovery in vigour was achieved.

Overall, the mean vigour score of the progenies in the three crossing groups showed agronomic performance similar to 'Tribute' at both sites. However, there was a significant (P<0.05) progeny x site interaction, with the highest agronomic performance being shown by different progenies under the contrasting managements imposed at each site (Table 1). For example, progenies 10, 19 and 20 which had a small-medium leaf size were best in Canterbury under sheep grazing, while large-leaved progenies 35, 51 and 56 were best in the Manawatu under cattle grazing. At each site the best progenies equalled or were better than the commercial cultivars in agronomic performance.

Table 2 Levels of water soluble carbohydrate (WSC) and crude protein (CP), and the WSC: CP ratio (mean ± standard error) in leaves of white clover plants sampled from two cultivars and 24 high sugar (HS) cross progenies growing in Canterbury and Manawatu mixed-sward field trials; and of the final HS plants selected for crossing and seed increase within two leaf size categories. N = number of plants.

	N	WSC (g/kg DM)	CP (g/kg DM)	WSC: CP ratio
'Kopu II'	4	89 ± 4	293 ± 15	0.30 ± 0.03
'Tribute'	4	107 ± 16	292 ± 17	0.37 ± 0.07
HS cross progenies	220	114 ± 3	286 ± 2	0.41 ± 0.01
HS small-leaf selection	22	179 ± 5	273 ± 4	0.66 ± 0.02
HS medium-leaf selection	26	169 ± 4	267 ± 5	0.64 ± 0.02

The mean WSC value for the 220 HS cross plants tested mid-morning was relatively low in absolute terms (Table 2). The nutrient analysis of plants sampled from the best agronomic families revealed on average only a small increase in WSC and decrease in CP compared to cultivars. However, the mean level of WSC among the 46 selected plants from these 220 was 79% higher than the cultivars tested, while CP concentrations decreased by 8% (Table 2). These trends are also reflected in the WSC:CP ratios (Table 2).

Discussion

Divergent selection for herbage quality traits produced white clover populations with changes in WSC, CP and their ratio. The consistent magnitude of response to selection in the three base populations suggests a strong additive genetic effect for both traits. The continued and substantial response over the four cycles suggests that in the populations used there is still residual genetic variation that may be used to further improve WSC concentrations in these white clover populations.

The sampling was done either early or mid-morning, when the WSC levels are rising rapidly as photosynthesis converts atmospheric carbon dioxide into WSC. The measures were made at this time to accommodate workforce availability, but are likely to be under-estimates of the actual maximum WSC levels achieved in the afternoon of sunny days. In the field trial, the highest concentration of WSC measured from both HS populations in the morning was 200 g/kg DM during the summer, with an associated CP concentration of 280 g/kg DM and WSC:CP ratio of 0.7. Cosgrove *et al.* (2006) measured WSC and CP concentrations of 140 and 280 g/kg DM, respectively, (WSC:CP = 0.5) in the morning from a commercial clover cultivar. Pacheco & Waghorn (2008) suggested that a CP content of about 200 g/kg DM would be the desirable level for animal nutrition while Edwards *et al*. (2007) proposed that the WSC:CP ratio for forage needs to be greater than 0.7 to lie within the range where better use of dietary N for milk production and reduced N losses in urine may be achieved. The HS clover selections in these experiments are showing improved WSC concentrations compared to commercial cultivars but the CP levels, although reduced, remain higher than optimal levels for animal diets.

Some HS selections and cultivars of perennial ryegrass have shown strong genotype x environment interaction (Parsons *et al*. 2004) contributing to mixed animal response to the trait (Edwards *et al*. 2007). This is not always the case, as Easton *et al*. (2009) found consistent differences among ryegrass cultivars across seasons and sites. In the divergent populations of white clover, the HS plants selected in pots in the Manawatu maintained their relative advantage over LS populations and cultivars when grown under field conditions in Canterbury. The relative advantage was also consistent across years and throughout the growing season, albeit with suppressed expression in winter. It is not known to what extent the maximum (i.e. sunny afternoon) WSC accumulation is sensitive to genotype x environment interaction. We did, however, observe genotype x environment interaction for agronomic performance, which is typical of observations in multi-site trials of white clover (Jahufer *et al*. 2002).

The divergent breeding strategy used here resulted in a uniform loss of vigour in both the HS and LS progeny in all three backgrounds, particularly in first year growth. This may have been the result of a genetic bottleneck, or inadvertent selection for agronomically inferior types during the pot plant recurrent selection cycles. The inter-crossing between HS populations appeared to restore the vigour of the selected material to levels comparable with modern cultivars. This suggests that it is possible to combine increased WSC, decreased CP, and high agronomic performance in a single population; and that it is essential to design and follow a breeding strategy that will achieve all three goals.

Before providing this material to farmers, it is essential that the HS trait is carefully characterised so it is known to what extent it will be expressed and influenced by site, season, and pasture management; and to what extent it will affect animal performance, environmental footprint, and product characteristics at various proportions of clover in the diet. An initial investigation (Higgs *et al*. 2010) of the impact of the trait on N utilisation and partitioning in the rumen used the fourth cycle divergent selections described in this manuscript. That research indicates the HS line substantially reduced protein degradation indicator metabolites in the rumen. The authors also found that the HS trait in white clover had a downwards influence on the levels of urinary N and milk urea-nitrogen when clover formed a major part of the diet.

Our data suggest that selection for HS in white clover has resulted in populations that have increased concentrations of WSC, and that agronomic vigour and HS can be combined in white clover. The initial indications are that the HS expression in white clover is stable across populations, locations, years and seasons. The germplasm warrants further work to develop a commercially suitable cultivar of HS white clover that is well characterised for its trait expression and on farm value.

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REFERENCES

Cosgrove, G.P.; Burke, J.L.; Death, A.F.; Lane, G.A.; Fraser, K.; Pacheco, D. 2006. The effect of clover-rich diets on cows in mid lactation: production, behaviour and nutrient use. *Proceedings of the New Zealand Grassland Association 68*: 267-273.

Cosgrove, G.P.; Burke, J.L.; Death, A.F.; Hickey, M.J.; Pacheco, D.; Lane, G.A. 2007. Ryegrasses with increased water soluble carbohydrate: evaluating the potential for grazing dairy cows in New Zealand. *Proceedings of the New Zealand Grassland Association 69*: 179-185.

Easton, H.S.; Stewart, A.V.; Lyons, T.B.; Parris, M.; Charrier, S. 2009. Soluble carbohydrate content of ryegrass cultivars. *Proceedings of the New Zealand Grassland Association 71*: 161-166.

Edwards, G.R.; Parsons, A.J.; Rasmussen, S.; Bryant, R.H. 2007. High sugar ryegrasses for livestock systems in New Zealand. *Proceedings of the New Zealand Grassland Association 69*: 161-171.

Higgs, R.J.; Cosgrove, G.P.; Burke, J.L.; Lane, G.A.; Pacheco, D.; Fraser, K.; Death, A.F.; Ford, J.L. 2010. Effect of white clover containing either high or low concentrations of water-soluble carbohydrate on metabolic indicators of protein degradation in the rumen of dairy cows. *Proceedings of the New Zealand Society of Animal Production 70*: 23-28.

Jahufer, M.Z.; Cooper, M.; Ayres, J.F.; Bray, R.A. 2002. Identification of future research to improve the efficiency of breeding strategies for white clover in Australia – a review. *Australian Journal of Agricultural Research 53*: 239-257.

Pacheco, D.; Waghorn, G.C. 2008. Dietary nitrogen – definitions, digestion, excretion and consequences of excess for grazing animals. *Proceedings of the New Zealand Grassland Association 70*: 107-116.

Parsons, A.J.; Rasmussen, S.; Xue, H.; Newman, J.A.; Anderson, C.B.; Cosgrove, G.P. 2004. Some high sugar grasses don't like it hot. *Proceedings of the New Zealand Grassland Association 66*: 265-272.

Ulyatt, M.J. 1997. Livestock Improvement Corporation lecture. Can protein utilization from pasture be improved? *Proceedings of the New Zealand Society of Animal Production 57*: 4-8.

Woodfield, D.R.; Clifford, P.T.P.; Cousins, G.R.; Ford, J.L.; Baird, I.J.; Miller, J.E.; Woodward, S.L.; Caradus, J.R. 2001. Grasslands Kopu II and Crusader: New generation white clovers. *Proceedings of the New Zealand Grassland Association 63*: 103-108.