Improving quality characteristics of white clover

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Abstract

White clover (*Trifolium repens* L.) is included in temperate pastures because it is able to improve soil nitrogen status through N-fixation, and it also improves the quality of forage eaten by ruminants. It is a highly nutritive forage legume with good persistence under grazing. However, there are some anti-quality characteristics associated with white clover. The most widely publicised is the frequent occurrence of bloat in ruminants fed high clover diets. One option being investigated to alleviate the occurrence of bloat involves screening clovers for the presence of condensed tannin synthesis in leaves. Plant characteristics which increase the amount of plant protein able to by-pass the rumen will improve nutritive value and may reduce the incidence of bloat. These may include an increase in flower production in spring and the condensed tannin content of flowers, increasing epicuticular wax content, increased cell wall and leaf tissue strength to reduce the rate of protein degradation. However, care must be taken not to increase the proportion of petiole with consequent reductions in nutritive value. Recently, circumstantial evidence has suggested that oestrogen-like compounds can be present in white clover and that these can have detrimental affects on animal fertility. These include zearalenone, a metabolite of saprophytic fungi that is stable in soil and can be absorbed by forage plants such as white clover. Coumestans are plant metabolites produced in response to infection by plant pathogenic fungi and have detrimental effects on fertility. White clover has low concentrations of formononetin which is better known as a phyto-oestrogen associated with infertility in ewes grazing red clover. In New Zealand strong agronomic performance of white clover cultivars has been associated with increased cyanogenesis. Cyano-glucosides occur in some cultivars of white clover and are hydrolysed to form free hydrogen cyanide (HCN) when ingested by ruminants. The amount of HCN produced from leaf tissue of white clover cultivars can vary 10 fold and high concentrations can affect livestock performance. For example high levels of HCN can exacerbate the effects of selenium deficiency, iodine deficiency and in extreme cases sulphur deficiency. Determining the extent of genetic variation for these characteristics in white clover is the first step towards incorporating the traits into commercially available cultivars to improve nutritive value of this already high nutritive forage legume.

Additional key words: *Bloat, cyanogenesis, oestrogens, wax, leaf tissue strength, non-degradable protein, genetic variation, fungal pathogens.*

Introduction

White clover is generally considered a high quality forage. Its feeding value for young sheep is 50-100% greater than that of forage grass species and 15-35% greater than that of other forage legume species (Ulyatt, 1981). This is the result of greater intake, rapid particle breakdown, digestion, absorption and metabolisable energy content of white clover. The nutrients arising from digestion and absorption of white clover result in a high efficiency of metabolisable energy utilisation for live weight gain. Despite these attributes there are several quality components of white clover that require improvement. Long-standing issues include the associated incidence of bloat and the possible impact on animal health of oestrogenic compounds and of hydrogen cyanide production from some cultivars. In addition, white clover quality may be improved by increasing the concentrations of non-degradable protein and therefore reducing protein N wastage. Our aim is to determine the genetic variation in these characteristics as a first step towards incorporating these nutritional attributes into commercially available white clover cultivars.

Improved forage quality

Plant characters associated with increasing non-degradable protein concentrations

Increasing the proportion of protein by-passing the rumen for absorption in the intestine has important implications for protein supply to the animal and for animal productivity. An increased protein supply will benefit milk production, wool production and muscle growth and may also reduce the incidence of bloat in cattle.

Selection for increased concentrations of non-degradable protein: Most New Zealand pastures contain 12-25% crude protein with the range largely due to variations in clover content. However, rumen fermentation results in a substantial wastage of high quality plant proteins (Waghorn and Barry, 1987). The rumen microflora degrade plant proteins to amino acids which are either incorporated into microbial protein or further degraded to ammonia which is used for microbial synthesis or is absorbed into the blood and excreted as urea in the urine. When fresh forages, such as white clover, containing high quantities of crude protein are fed to ruminants about 70% is degraded in the rumen and only 30% reaches the small intestine for absorption. Recent evidence suggests that removal of ammonia to urea in the liver also involves a substantial catabolism of absorbed amino acids which will further reduce the supply of amino acids for production (Beever, 1993). The extensive ruminal degradation of plant protein therefore has a double penalty for the ruminant, and increasing the proportion of plant protein escaping ruminal degradation, which will increase absorption from the small intestine, is an important objective for plant breeders.

Levels of non-degradable protein were estimated both *in situ* and *in vitro* using methods described by Waghorn and Caradus (1994). In a comparison of 30 white clover cultivars with two replicates there was no significant difference between cultivars for the *in situ* measurement of degradability. However, based on percentage nitrogen converted to ammonia *in vitro* cultivars Buyucua, Will, California, Ladino Gigante Lodigiano, Osceola, and Polyploid ladino had the lowest values and cultivars Smalbladet, Tahora, Prestige and G49 the highest values (Table 1). There was no significant difference among cultivars for percentage petiole to total leaf but there was a significant difference for leaf percent nitrogen concentration which was positively correlated to ammonia release ($r = 0.71$, $p<0.001$, $df = 27$). Therefore, potentially low protein degradability is more likely for cultivars with low crude protein concentration.

Amino acid loss during *in situ* digestion was determined from a duplicated sub-set of 11 of these 29 cultivars. There were no significant differences among cultivars in the concentrations of 17 amino acids and in total essential or non-essential amino acid concentrations. *In situ* digestion resulted in a similar proportion of essential and non-essential amino acids lost for all 11 cultivars of white clover, so differences between cultivars in the rate of conversion to ammonia must have been due to cell structure rather than protein composition *per se.*

Condensed tannin synthesis in leaves: Condensed tannins are present in the leaf tissue of non-bloating forage legumes and appear to prevent bloat by either

Table 1. Percentage petiole to total leaf (laminar plus petiole), percentage nitrogen in total leaf, and percentage nitrogen converted to ammonia *in vitro* of white clover cultivars.

Cultivar	Origin	% Petiole	% N	% N converted to NH_3 (*in vitro*)
Alban	Denmark	33	4.0	25.6
Bagé	Brazil	29	4.2	24.5
Bayucua	Uruguay	32	3.7	21.1
Beta	Sweden	33	4.4	30.5
California	USA	32	4.1	22.4
Clarence	Australia	36	4.3	29.5
Daeno K	Denmark	33	4.4	26.7
De Brasov	Romania	33	4.0	27.5
Demand	NZ	33	4.1	27.6
El Lucero	Argentina	37	4.0	25.2
G. 49	NZ	34	4.3	34.1
Gwenda	UK	36	4.2	24.0
Karina	Germany	40	4.4	26.5
Kopu	NZ	34	3.9	26.1
Ladino G.L.	Italy	31	3.8	22.9
Lune de Mai	France	34	4.2	28.0
Nesta	UK	40	4.0	23.7
Osceola	USA	34	3.9	22.6
Ovcak	Czech	37	4.3	27.4
Pastevec	Czech	33	4.3	28.5
Polyploid ladino	USA	36	4.0	22.5
Prestige	NZ	36	4.3	38.2
Retor	Netherlands	35	4.4	27.2
Smalbladet	Denmark	34	4.6	39.5
Sonja	Sweden	36	4.3	27.1
Tahora	NZ	39	4.3	34.6
Tillman	USA	37	4.0	26.3
Viglasska	Slovakia	33	4.1	25.4
Will	USA	31	3.8	22.6
p		ns	***	**
$LSD_{p<0.05}$		-	0.4	7.7

acting as protein precipitants and/or by inhibiting microbial invasion and digestion. In the mid-1970s and again more recently, after a mutagenesis programme, several tens of thousands of white clover genotypes were screened for leaf tannins. No genotypes were found that contained condensed tannins; however, some genotypes contained phenolic or tannin-like compounds. These genotypes were tested for protein degradability by measuring the percentage of nitrogen converted to ammonia *in vitro* when incubated with or without polyethylene glycol (PEG). PEG suppresses the effect of tannins and increases the extent to which N is converted to ammonia in plants with condensed tannins. In this experiment PEG significantly increased the percentage nitrogen converted to ammonia for *Lotus pedunculatus* Cav. indicating the presence of tannins (Table 2) but there were no effects on the white clovers which contained phenolic compounds. However, one white clover genotype (MINT 200) had a very low percentage of nitrogen converted to ammonia, not significantly different from lotus and significantly lower than all other white clovers tested. Whilst the low degradation of nitrogen was not caused by the presence of tannins this genotype will complement the project aimed at identifying germplasm with a reduced rate of protein degradation in the rumen.

Table 2. Net conversion of nitrogen (N) to ammonia (NH_3) *in vitro* incubation of fresh minced white clover genotypes and lotus, with and without polyethylene glycol (PEG) to remove the effects of condensed tannin. Incubations were facilitated by rumen liquor from a cow grazing pasture and each value is the mean of 5 replicates.

	% N converted to NH_3		
Genotype	Control	PEG	P
MINT 117	17.7	17.5	ns
MINT 200	6.9	7.1	ns
MINT 137	18.6	19.4	ns
MINT 52	26.0	24.3	ns
MINT 212	20.0	17.1	ns
cv Kopu	29.7	27.4	ns
cv Bagé	19.5	19.2	ns
Lotus pedunculatus	4.3	10.4	*
P	***	***	
$LSD_{p>0.05}$	6.6	7.0	7.0

Floral tannin content and time/duration of flowering: Tannins are present in white clover petals and the tannin concentration is under genetic control. One cycle of selection within Grasslands Huia for both high and low concentrations of tannin in petals has resulted in a 30% difference in the two progeny groups (Table 3). To have an effect on nutritive value and bloat, this high floral tannin content needs to be combined with an earlier and longer flowering period so that flowers are present earlier in spring when bloat incidence is highest.

Cell wall and leaf tissue strength: Leaf cells of many bloat-safe legumes are more resistant to mechanical rupture than cells of bloat causing legumes (Lees *et al.*, 1981) possibly due to thicker epidermal cell walls (Lees, 1984). Selection for increased non-degradable protein levels in lucerne has also resulted in breeding lines with increased leaf tissue strength (Goplen *et al.*, 1993). While this appears to be a promising area of research there is no documentation of genetic variation in epidermal or mesophyll cell wall thickness in white clover.

Epicuticular wax content: Epicuticular waxes are lipids that possess surface active properties (Holloway, 1969). They are of interest for two reasons: (a) they are the first line of defence against pathogens (Eglinton and Hamilton, 1967) and (b) they may act as anti-foaming agents in animals grazing bloat-inducing crops. In pearl millet, increased epicuticular wax content reduced the *in vitro* dry matter digestibility rates of intact leaf sections and slowed the rate of passage of ingesta through the gastrointestinal tract of heifers (Hanna *et al.*, 1974). Increased epicuticular wax content also slowed microbial penetration during digestion.

Table 3. The concentration of tannins in white clover flowers of populations from genotypes selected for high and low levels of condensed tannin compared with an unselected population of Huia.

Population	floral tannin content (mg/g fresh weight of petals)
Low selection	14.3
Huia	16.9
High selection	19.4
P	***
$LSD_{p>0.05}$	2.1

An examination of white clover populations in the UK has identified a three-fold range in epicuticular wax content among 10 white clover populations (Moseley, 1983). It was concluded from that study that selective breeding could increase epicuticular wax levels to a sufficiently high level to provide a natural source of anti-foaming material for ruminants grazing normally bloat-inducing pastures.

Oestrogenic compounds

Recent evidence (Bland, 1994) has suggested that oestrogen-like compounds can be present in white clover and that these can have detrimental affects on animal fertility. These compounds fall into three categories:

1. Phyto-oestrogens such as formononetin which can be produced in high concentrations in some forage legumes such as red clover. A recent survey of white clover cultivars has shown that low levels of formononetin are present (Table 4). Biochanin A and genistein, other phyto-oestrogens are also present, but again in small amounts.

2. Plant metabolites produced in response to infection by pathogenic fungi. Of major concern in this group are coumestans produced in response to infection by pepper spot (*Leptosphaerulina trifolii*) and leaf-spot (*Pseudopeziza trifolii*) fungi (Wong and Latch, 1971; Wong *et al.*, 1971). The most appropriate method of dealing with this potential problem will be to identify germplasm resistant to these fungal diseases. However, a recent screening for resistance showed no significant differences in susceptibility / resistance to pepper spot among the white clover cultivars Prop, Tahora, Pitau, Kopu, Huia, Prestige, Demand, Challenge and Sustain. When breeding lines were generated from genotypes selected for both high and low incidence of pepper spot in a spaced plant field trial, there were significant differences in the incidence of pepper spot (Table 5). The breeding line developed from genotypes having a low incidence of pepper spot had significantly more progeny genotypes without any incidence of pepper spot and significantly less in the category for high incidence of pepper spot, than the breeding line developed from genotypes having a high incidence of pepper spot.

3. Fungal metabolites absorbed by plants. Zearalenone is a metabolite of saprophytic fungi which has oestrogenic activity and can be absorbed by forage plants including white clover (Table 6). It would be appropriate to identify white clover plants less capable of absorbing zearalenone than current cultivars.

While each of these oestrogen sources on their own may not be of great concern the simultaneous occurrence of two or more, perhaps in conjunction with a high endophyte (wild-type) perennial ryegrass, could conceivably cause infertility problems in cattle and sheep.

Table 4. Concentration of phyto-oestrogens, on a dry weight (DW) basis, in white clover cultivars grown in trays of potting mix outside.

Cultivar or Selection(s)	Origin	Formononetin (% DW)	Biochanin A genistein (% DW)
Pilgrim	USA	0.153	0.20
Brown loam	USA	0.140	0.20
SRVR	USA	0.095	0.19
Ranger(s)	NZ	0.115	0.25
Steerbarn(s)	USA	0.148	0.26
Kopu	NZ	0.142	0.26
Prestige	NZ	0.148	0.23
Prop	NZ	0.177	0.25
P		ns	ns

Table 5. Percentage of progeny from breeding lines developed from genotypes selected for high or low incidence of pepper spot, in either (a) no incidence of pepper spot, (b) less than 5 visible 'spots' per leaf, (c) less than 10, (d) less than 20, and (e) 20 or more 'spots' per leaf.

	Percent genotypes in pepper spot severity class				
Breeding line	(a) none	(b)	(c)	(d)	(e) high
High incidence	11	24	20	20	24
Low incidence	17	26	20	20	17
P	***	ns	ns	ns	***
$LSD_{p>0.05}$	3	-	-	-	4

Cyanogenesis

In New Zealand improved agronomic merit has been associated with increased levels of cyanogenesis (Caradus and Williams, 1989). Resistance to some insect pests has also been associated with high levels of cyanogenesis (Ellsbury *et al.*, 1992). Cyanoglucosides occur in some cultivars of white clover and are hydrolysed to free hydrogen cyanide (HCN) when ingested. High levels of HCN production have been implicated as a factor exacerbating the effects of selenium deficiency (Gutzwiller, 1993), iodine deficiency (Greer *et al.*, 1966) and in extreme cases sulphur deficiency since sulphur is involved in the detoxification of HCN to thiocyanate (Mlingi *et al.*, 1993). A recent survey of commonly used white clover cultivars (Crush and Caradus, 1995) showed that HCN contents ranged from 120 to 1110 µg HCN/g dry matter. Despite the suggestion by Coop and Blakely (1950) that clovers exceeding 700 µg HCN/g dry matter should not be bred, there are no recorded instances of mortality due to cyanide toxicity in stock in New Zealand. However, the potential of sub-clinical effects of high HCN diets must be recognised. The goitrogenic effects of highly cyanogenic white clover on animals fed low iodine diets has been documented (Butler *et al.*, 1957; Greer *et al.*, 1966) and this may have important implications in many New Zealand situations because of the low iodine status of our swards (Waghorn and Northover, 1992). Cyanogenic clover cultivars may also exacerbate selenium deficiency in sheep (Gutzwiller, 1993) which has been associated with periods of active clover growth (Caple *et al.*, 1980). New Zealand soils are markedly deficient in selenium, with consequent widespread incidence of white muscle disease in lambs unless selenium supplements are given (Grace, 1994). Additionally, ruminants, such as sheep, detoxify HCN with sulphur containing compounds such as cysteine, a high sulphur amino acid that promotes wool growth. Therefore, while direct toxicity effects of HCN from high cyanogenic clovers is unlikely, indirect effects on iodine, selenium and sulphur metabolism of animals is probable and may have serious consequences for nutrient availability so that cyanogenesis levels of newly released cultivars must be monitored.

Table 6. Zearalenone concentration in forages in autumn.

Species	Cultivar	Concentration (µg/g)
Perennial ryegrass	Nui E-	0.30
	Nui E+	0.34
	Ellett	0.37
Browntop	Muster	0.46
Tall fescue	Roa	0.40
	Advance	0.44
White clover	Huia	0.86
	Kopu	1.19
Chicory	Puna	0.27

Concluding comment

While white clover is correctly considered a high quality component of grazed temperate pastures there is the potential for improvement. Various strategies may result in increasing the concentration of non-degradable protein, reduction in presence of oestrogen-like compounds and an understanding of the indirect effects of high cyanogenic white clover cultivars on trace element availability for ruminants. Determining the genetic variation of these characteristics is the first step towards breeding white clover cultivars with improved nutritive quality.

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