The development and plant characteristics of interspecific hybrids between white and caucasian clover

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Abstract

A series of *Trifolium ambiguum* x *T. repens* hybrid populations has been developed at the hexaploid (four *T. repens* and two *T. ambiguum* genomes) and pentaploid (four *T. repens* and one *T. ambiguum* genome) level. When tested for effectiveness of symbiotic nitrogen fixation, the 6x and 5x hybrids nodulated with either *T. repens* or *T. ambiguum* strains of rhizobia but only formed an effective N-fixing symbiosis with a mix of *T. repens* rhizobia strains. When grown in the field, the 6x and 5x hybrid plants had a similar morphology to white clover in that the hybrids grew surface stolons (fewer numbers than white clover) and no underground rhizomes. The advantage of the 6x hybrid was deeper roots and a greater proportion of root, a characteristic considered important for greater drought tolerance and persistence than for white clover. The 6x hybrid indicated 55% of the seed production potential of white clover, but there was large variation between plants in all reproductive traits. The 5x hybrid showed poor levels of seed set. The growth pattern of the 6x hybrids in the field indicated lower herbage yield in the first year but improved performance compared with white clover into the second year. At this early stage, the hybrid breeding populations consist of unselected and novel hybrid combinations. Variations in growth and reproductive characteristics exist between hybrid plants, thus providing scope for improvement through selection and breeding.

Key words: caucasian clover, interspecific hybrid, morphology, nitrogen fixation, seed production, white clover

Introduction

White clover (*Trifolium repens* L. $2n=4x=32$) is one of the most important and widely grown forage legumes in temperate regions of the world. However, perennial stands of white clover often decline

markedly in the second or third year of growth due to the death of the taproot (Westbrook & Tesar 1955), as well as susceptibility to a number of stress factors including drought (Brock 1988), various viral diseases (Barnett & Gibson 1975), nematodes (Mercer 1988) and other root chewing insects (Gaynor & Skipp 1987). Caucasian clover (*T. ambiguum* M. Bieb), on the other hand, is tolerant to several viral diseases (Barnett & Gibson 1975), spreads by means of underground rhizomes rather than aboveground stolons and has the ability to persist under dry conditions due to its deep, well-developed root and rhizome system (Taylor & Smith 1998). However, caucasian clover exhibits poor establishment, particularly when sown with perennial grasses (Hill & Mulcahy 1995, Black *et al.* 2002) and with slow growth during the first 2 years of a pasture (Moss *et al.* 1996). Both of these factors impact on the acceptance of caucasian clover by farmers. Interspecific hybridisation between caucasian and white clover offers the potential for the improvement of drought tolerance and persistence in white clover by combining forage yield with a well-developed root system.

This paper describes the development and nitrogen fixation potential of interspecific hybrids, and then presents agronomic characteristics of hybrid plants evaluated in the field.

Hybrid development

The first crosses between white and caucasian clovers were difficult (Williams & Verry 1978), and required embryo rescue with low rates of success. When the current project was initiated, only one partially fertile F_1 *T. ambiguum* x *T. repens* hybrid was available (Williams *et al.* 1990). This hybrid (designated 4x H-435) had low fertility (9% pollen viability determined using acetocarmine stain) and was only cross fertile with the *T. repens* parent in

backcrosses. The chromosome number of 4x H-435 was doubled using colchicine, and the derived 8x H-435 showed improved fertility in comparison with the tetraploid clones (30% pollen stainability) (Hussain & Williams 1997). The steps involved in the hybridisation programme are schematically presented in Figure 1.

Figure 1 The steps involved in the development of interspecific hybrids between caucasian and white clovers.

The 8x H-435 was used in reciprocal backcrosses with both parental species (Hussain & Williams 1997). Reciprocal backcrosses of 8x H-435 with 4x and 6x *T. ambiguum* were not successful, whereas reciprocal backcrosses to *T. repens* were successful and resulted in the 6x BC₁F₁ hybrid progeny. These backcrossed plants were cytologically evaluated for ploidy level and were confirmed to be hexaploids (6x) with four genomes of white clover and two genomes of caucasian clover (Hussain & Williams 1997). The initial fertility and pollen stainability of these BC₁F₁ hybrids varied from 30 to 58%. Further intercrossing of these plants resulted in increased fertility that approached the parental fertility level. The 6x BC₁F₁ hybrids were also backcrossed to the parent *T. repens*, resulting in 5x BC₂F₁ progeny with four genomes of white clover and a single genome of caucasian clover; however, their fertility level was low (Hussain & Williams 1997). 'Huia', 'Demand', 'Sustain', 'Prestige' and 'Kopu' white clover cultivars were used in the development of the current set of interspecific hybrids.

Over the last 4 years, a wide range of 6x intercrossed progeny have been developed that have shown

desirable characteristics from both parental species. However, at this early stage in the programme, the hybrid breeding populations consist of unselected and novel hybrid combinations that will require further screening and selection to improve the agronomic merit of the genetic material.

Nitrogen fixation in the hybrids

Improvement of N-fixation is a potential problem in legume hybrids (Williams 1987). Therefore, a key requirement of *T. ambiguum* x *T. repens* is a high fixation capability, from a successful symbiotic relationship. Howieson *et al.* (2000) reported that interspecific hybridisation produced plants that were unpredictably specific in their symbiotic requirements. Caucasian clover has highly specific requirements for rhizobia (*Rhizobium leguminosarium* biovar *trifolii*). Rhizobia nodulating white clover, found widely in the agricultural soils of New Zealand, do not form effective nodules on caucasian clover (Hely 1957).

Nodulation and nitrogen fixation of *T. repens* x *T. ambiguum* hybrids and their parents, inoculated with strains of rhizobia effective on either white clover or caucasian clover, have been assessed to determine host/*Rhizobium* specificity (Lowther *et al.* 2002). As expected, rhizobia from white clover formed nodules on white clover plant roots but did not nodulate caucasian clover. However, rhizobia from caucasian clover nodulated both caucasian clover and white clover plants. All the hybrid plants were nodulated irrespective of strain of rhizobia used for inoculation.

The percentage of hybrid plants with effective symbiotic nitrogen fixation following inoculation with the strains of rhizobia was more specific than nodule formation (Table 1). The majority of the 8x hybrids formed an effective symbiosis with rhizobia from white clover or caucasian clover. The majority of 6x and 5x hybrids formed an effective symbiosis with white clover rhizobia, but not with caucasian clover rhizobia. However, the hybrids, particularly 8x and 6x, required a mix of white clover strains for an effective N-fixing symbiosis. Lowther *et al.* (2002) and Pryor (unpublished) have identified hybrid germplasm with similar symbiotic N-fixation to white clover, and this characteristic was included as an essential selection parameter.

Table 1 Percentage of plants with effective N-fixing symbiosis following inoculation with rhizobia single strain or a mix of strains (adapted from Lowther *et al.* 2002).

Germplasm	Rhizobia effective on <i>T. repens</i>		Rhizobia effective on <i>T. ambiguum</i>	
	Single strain	Mix of strains	Single strain	Mix of strains
<i>T. repens</i>	100	100	0	0
<i>T. ambiguum</i>	0	0	100	100
8x H-435	6	100	80	nd
6x hybrids (BC ₁ F ₂)	8	83	0	0
5x hybrids (BC ₂ F ₁)	78	89	9	0

nd=no data

Morphological, reproductive and growth characteristics of the hybrids

Materials and methods

A series of glasshouse-raised hybrid plants, together with the white and caucasian clover parent species, were planted as spaced plants, 0.75m apart, in the field at Lincoln, Canterbury in October 2001. An inoculum slurry containing a mixture of white clover *Rhizobium* strains had previously been applied to plants while in the glasshouse. The hybrid material consisted of 6x BC₁F₃ (40 lines), 6x BC₁F₆ (23 lines) and 5x BC₂F₁ (3 lines) that contained 'Huia', 'Demand', 'Prestige' and 'Kopu' white clover parentage. The spaced plants from these lines were evaluated for reproductive features during January–February 2002, morphological features in October 2002 and herbage yield (visual score of growth) from January 2002 until May 2003. Plants were grazed by sheep every 6–8 weeks following measurements. Leaf, stolon and root dry matter (DM) measurements were taken from a separate set of 18-month old 6x BC₁F₃ hybrid, white and caucasian clover spaced plants.

Results and discussion

Morphology

A major feature of the earlier BC₁F₁ hybrids was the occurrence and severity of chlorotic leaf abnormality in plants, which affected their growth and vigour (Hussain pers. comm.). The percentage of chlorotic plants has declined with each successive intercross, so that by the time the BC₁F₆ hybrid was grown in the Lincoln trial, there were no chlorotic plants. However, the BC₁F₃ hybrids containing 'Kopu' white clover parentage had the worst symptoms, where 70% of plants were chlorotic and remained small, dome-like

plants throughout the duration of the trial.

The hexaploid confirmation of the 6x hybrids with a complete set of four genomes from *T. repens* together with two genomes from *T. ambiguum* resulted in plants with a dominant expression of white clover features (Table 2). The 6x hybrid plants produced fewer but thicker stolons than white clover, with pointed leaves intermediate in size between white and caucasian clover. The plant diameter of the 6x hybrids indicated less stolon spread than white clover. The nodal rooting along the 6x hybrid stolons was less frequent than in white clover (Table 2), but occasional nodes formed deep roots and a secondary crown, which produced many new stolons. Over time, some of these stolons broke from the original parent plant and formed a ring of plantlets. The 5x hybrids were closer to white clover in appearance, even showing enhanced stolon numbers and spread. None of the hybrids produced functioning rhizomes, which contrasts with the hybrids developed by Abberton *et al.* (1998).

Seed production

The reproductive features of the hybrid and parent species are shown in Table 2. The 5x and 6x hybrids produced about 70% (i.e. 116 v 170) of the mean number of flowers compared with white clover. Fertility, as depicted by seeds/flower head, was much greater in the 6x than the 5x hybrid material (80% and 20% that of white clover, respectively). The unbalanced chromosome makeup of the 5x hybrid was probably the major determinant of the low fertility demonstrated by these hybrids. Overall, the 6x hybrid seed production potential was approximately 55% that of white clover and must be improved further for commercial success. The variation in number of

Table 2 Morphological and reproductive characters of 4x *T. repens*, 6x *T. ambiguum*, 6x BC₁F₃ and 5x BC₂F₁ hybrid progeny (mean and range) growing in the field at Lincoln, Canterbury. Stolon numbers and nodal rooting are presented relative to Huia white clover = 100. n = number of plants.

	<i>T. repens</i> (n=8)	<i>T. ambiguum</i> (n=8)	6xBC ₁ F ₃ (n=295)	5xBC ₂ F ₁ (n=30)
Morphological characters				
Leaf width (mm)	18.9 (16-21)	26.0 (20-28)	23.6 (13.1-29.0)	21.4 (16.7-26.3)
Stolon diameter (mm)	2.5 (2.2-3.0)	-	4.0 (2.9-5.0)	2.6 (2.0-3.0)
Stolon numbers (% Huia)	100 (5-120)	-	45 (12-65)	104 (70-120)
Nodal rooting (% Huia)	100 (50-125)	-	35 (15-75)	90 (58-125)
Plant diameter (cm)	77 (55-95)	76 (40-95)	36 (15-60)	83 (64-98)
Reproductive characters				
Flower numbers/plant	170 (80-264)	47 (0-160)	116 (12-243)	113 (30-320)
Seeds/flower head (g)	0.10 (0.07-0.14)	0.09 (0.03-0.2)	0.08 (0.02-0.21)	0.02 (0-0.03)
Seed yield/plant (g)	22.7 (17-32.3)	5.3 (0-15)	12.5 (0.6-31.5)	2.0 (0-8.5)

flower heads and fertility of the 6x hybrid material (Table 2) provides scope for improvement through recurrent selection.

Shoot and root growth

Destructively harvested plants showed that 6x hybrids produced 58% of the shoot DM yield (leaf, petiole and stolon) and 78% of the root DM yield compared with the same measurements for white clover (Table 3). The hybrids developed longer taproots and greater large root (>2mm diameter) DM yield compared with white clover plants, where roots were dominated by smaller nodal roots (Table 3). The greater partitioning of growth into the root rather than the shoot by the hybrid has resulted in a lower shoot:root ratio, a feature from the caucasian clover parent that produced about 65% of its growth as root and rhizome. The large, deeper roots of the hybrid may confer greater drought tolerance in dryland pastures compared with white clover. Marshall *et al.* (2001) demonstrated enhanced drought tolerance of hybrids compared with white clover when plants were grown in deep soil bins. At this early stage in the programme, the lower total DM yield of the hybrids compared with white clover was not unexpected, given the new and unselected nature of the hybrid populations. Further hybrids are being developed to

Table 3 Leaf, stolon and root dry matter (DM) yield and taproot length of 4x *T. repens*, 6x *T. ambiguum* and 6x BC₁F₃ hybrid plants (mean ± SEM) after 18 months in the field (n=number of plants).

Plant characteristics	<i>T. repens</i> (n=3)	<i>T. ambiguum</i> (n=3)	6xBC ₁ F ₃ (n=23)
Leaf and petiole DM (g)	56.5 ± 9.3	40.5 ± 13.4	21.8 ± 2.2
Stolon DM (g)	39.9 ± 2.3		34.3 ± 3.4
Large root and rhizome DM (g)	5.9 ± 1.3	54.6 ± 16.2	13.4 ± 1.3
Small root, <2 mm thick DM (g)	16.4 ± 2.9	10.6 ± 3.1	4.0 ± 0.5
Total plant DM (g)	118.8 ± 10.1	105.7 ± 26.2	73.5 ± 6.5
Shoot:root ratio	4.3 ± 0.75	0.62 ± 0.15	3.2 ± 0.22

increase the range of material available for plant breeding.

The seasonal growth of the 6x BC₁F₃ and BC₁F₆ hybrid lines and white and caucasian clover parent species were compared relative to 'Huia' white clover (Figure 2). The mean of the 63 hybrid lines had (P<0.05) less growth than white clover throughout the trial. However, the best hybrid line was similar to white clover except it was lower (P<0.05) in winter/early spring (Figure 2). A promising trend with the best hybrid line was the improving performance in the second year relative to white clover, a trend more like the caucasian clover parent. Caucasian clover showed excellent growth after 1.5 to 2 years, but it must be realised that the evaluation occurred as

spaced plants, and associated grass competition has a major detrimental effect on the establishment success of caucasian clover (Black *et al.* 2002). A key objective in the development of the hybrids was to identify material that can persist and improve in performance over time relative to white clover. The slower establishment of the hybrid was expected given caucasian clover was part of the parentage, but improved long-term persistence and herbage yield of the hybrid clover in a pasture is the ultimate goal. Evaluations of hybrid selections sown with grass in different environments are currently underway.

Figure 2 Herbage growth relative to 'Huia' white clover (—●—), for caucasian clover (—□—), mean (n=63) of the 6x hybrid lines (—▲—), lowest of the 6x hybrid lines¹ (—◆—) and the highest of the 6x hybrid lines² (—■—). Error bars indicate the LSD (P<0.05).

Parentage (the lowest and highest were 6x BC₁F₃ lines):

¹ [(8xH-435 x 'Kopu') x (8xH-435 x 'Kopu')] x [(8xH-435 x 'Prestige') x (8xH-435 x 'Kopu')]

² [(8xH-435 x 'Demand') x (8xH-435 x 'Demand')] x [(8xH-435 x 'Huia') x (8xH-435 x 'Huia')]

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