Cytological and agronomic evaluation of interspecific hybrids between *Trifolium repens* L and *T. occidentale* Coombe.

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Keywords: *Trifolium* interspecific hybrids; *T. repens*; *T. occidentale*; Cytology.

Abstract

Trifolium occidentale is a diploid wild relative of *T. repens* with adaptation to dry, saline coastal habitats. Transfer of drought and salt-tolerant adaptive traits from this potential source of germplasm to *T. repens* could be valuable if interspecific hybridization can be achieved efficiently. To achieve hybridisation, 4x plants of *T. occidentale* were generated through colchicine chromosome doubling. Interspecific 4x F_1 plants were achieved without embryo rescue. F_2 populations and backcross (BC) hybrids to white clover were also efficiently achieved. Although male and female fertility in primary F_1 and F_2 hybrids were lower than in white clover, they were adequate to produce large amounts of seed from small numbers of inflorescences. Thus, early generation pre-breeding interspecific hybrid populations can be readily developed, opening the way for transfer of traits from *T. occidentale* to white clover. For effective introgression (backcross) breeding, it is also essential that interspecific chromosome pairing and recombination occur. In this study, it was apparent that chromosome pairing was occurring not only between *T. occidentale* and *T. repens* subgenomes, but also between the ancestral subgenomes of *T. repens*. Thus, interspecific hybridization has the potential for major genome recombination and opens the way for introgression of traits from *T. occidentale* into white clover.

Eighty hybrid families, comprising backcross one (BC_1), backcross two (BC_2) and their inter-crosses, were evaluated in the field and compared with eight commercial *T. repens* cultivars and nine *T. repens* x *T. uniflorum* backcrosses under three natural summer droughts. Some hybrid families performed as well as, or better than, elite cultivars and had superior recovery after drought periods. Selected plants were inter-crossed to further reshuffle the inter-specific chromosomes for introgression.

Introduction

White clover (*Trifolium repens* L.) is one of the most useful pasture legumes in moist temperate regions of the world due to its rapid growth, high feed quality, efficient nitrogen fixation and high seed production (Williams, 1987). However, it is susceptible to several stresses, including drought (Barbour et al. 1996), viruses (McLaughlin and Pederson 1985, Ragland et al. 1986), fungal diseases, nematodes and root-chewing insects (Yeates et al. 1973, Skipp and Gaynor 1987, Mercer 1989). Resistances to these stresses in white clover are limited and so its wild relatives have been investigated as sources of genetic variation for these traits (Williams 1987, 2014, Hussain et al 2016). One of these wild relatives is *Trifolium occidentale*, a stoloniferous, diploid (2n = 2x = 16) perennial clover that grows in relatively dry coastal habitats, on consolidated dunes and pockets of shallow soil (Coombe 1961). As the species grows naturally in saline, dry habitats, it could be a source of genes that can confer salt and drought tolerance for the improvement of white clover by introgression breeding. The opportunity for this has been increased by the discovery of *T. occidentale* populations with considerable genetic diversity in Spain and Portugal (Williams et al. 2012).

To achieve introgression by conventional breeding methods, it is necessary that interspecific hybridisation can be achieved on a large scale so that hybrid breeding populations can easily be produced and that there is genetic recombination between *T. occidentale* and *T. repens* chromosomes, leading to potential introgression of *T. occidentale* traits. Previous work has shown that interspecific hybridisation was possible if the chromosome number of *T. occidentale* was doubled to produce tetraploid (4x) forms (Chou and Gibson 1968, Gibson and Beinhart 1969, Hussain et al. 2016). Although the hybrids had low initial fertility, it was shown by Hussain et al. (2016) that large breeding populations of F_2 and first backcross (BC_1) plants could be produced without difficulty from small numbers of crosses. Previous evidence of interspecific chromosome pairing was given by Chen and Gibson (1970) who had shown that chromosome homologies existed in the early *T. repens* x *T. occidentale* F_1 hybrids. The likelihood of pairing was also indicated by Ellison et al. (2006), Williams et al. (2012) who showed that white clover was a natural hybrid between ancestral forms of *Trifolium* *pallescens* and *T. occidentale*. Thus, *T. occidentale* was an ancient parent of allotetraploid *T. repens*. The presence of high frequencies of chromosome pairing at metaphase I would signal the likelihood of substantial genetic exchange and therefore the feasibility of using a backcross breeding approach for efficient transfer of traits from *T. occidentale* to *T. repens.* Key objectives of the present research were (i) to artificially double the chromosomes of a range of genetically diverse accessions of *T. occidentale* using colchicine, (ii) to use tetraploid *T. occidentale* plants as pollen parents in crosses with *T. repens* to produce large numbers of F_1 hybrids and (iii) to develop and agronomically evaluate backcrossed and intercrossed progeny which could be used for future introgression breeding.

Materials and Methods

Plant material: The 4x *T. occidentale* plants used in this study were derived by chromosome-doubling (Hussain et al. 2016) of eleven 2x *T. occidentale* lines obtained from the Margot Forde Germplasm Centre (MFGC), Palmerston North, New Zealand. Elite white clover plants from nine commercial cultivars were also obtained from MFGC.

Pollination: Interspecific crosses were conducted by hand on potted plants in an insect proof glasshouse. Crosses between *T. repens* and colchicine treated *T. occidentale* were made only in one direction using *T. repens* as female parents. The same method was used to generate a BC_1 and subsequent backcross generations using elite *T. repens* as female recurrent parent. F_2, BC_1F_2 and BC_2F_2 plants were produced by selfing or inter-crossings of F_1, BC_1 and BC_2 respectively. For certain crosses bumble bees were used in cages to create reciprocal pair cross progeny and the seeds harvested from backcrossing partially self-fertile F_1 and BC_1 hybrids with white clover were placed in F_2+BC_1 and BC_2+ BC_1F_2 groups respectively.

Cytological Observations: Somatic and meiotic chromosome configurations and pollen stainability estimates in F_1s and subsequent hybrid generations were conducted using the procedure outlined by (Hussain and Williams 2016).

Field evaluation: Eighty backcrossed and intercrossed hybrids families were evaluated in a pilot field trial for three years using eight white clover commercial cultivars and nine *T. repens* x *T. uniflorum* backcrosses (BC_2F_1*uni* and BC_2F_2*uni*) as controls (Fig1, table 1). Data were collected on growth yield as a score of 1-10 (1= low growth and 10=high growth). The experimental design was a row x column design (10 rows x 36 columns) with four replicates. Twelve genotypes of each line were sown in a 0.5m x 0.5m plot and 1.5m between rows and columns. Maximum likelihood estimation was used to estimate fixed regression parameters using the 'lmer' function in the 'lme4' package in R. Half-sib families were considered as fixed effects in order to obtain BLUEs (Best Linear Unbiased Estimate). Pairwise difference of means was performed using Fisher's least-significant difference (LSD) test in the 'predictmeans' package in R.

Results and Discussion

Interspecific *T. repens* x 4x *T. occidentale* crosses:

Twenty nine genotypes from 11 different lines of 4x *T. occidentale* were used only as male parents because some *T. occidentale* lines showed self-compatibility. Approximately 140–200 florets were utilised for each cross. Of 52 different interspecific crosses, nine combinations produced fewer than five seeds (averaging 1.7 seeds per 100 florets), and seven produced fewer than 10 seeds (averaging 4.2% seed set). The remaining 36 F_1 crosses yielded 10 to 68 F_1 seeds (averaging 18.6% seed set).

Twenty one F_1 plants (at least one from each of the 14 F_1 families germinated) with pollen stainability of 37–78% were used as male parents in first backcrosses (BC_1) using 6 genotypes from 4 elite white clover cultivars. Approximately 140-200 florets were pollinated in each backcross. Only two backcrosses resulted in less than 10% seed set. The remaining backcrosses produced reasonable numbers of BC_1 seeds (ranging from 11 to 46% seed set, with an average of 21%). BC_1 seed set was moderately correlated with F_1 pollen stainability ($r = 0.76$, $P < 0.01$). Overall, the BC_1 crosses demonstrated wide variation for seed set, with the upper part of the range showing the potential for selection for high fertility. These BC_1 plants were used in further pre-breeding by generation of BC_2 and BC_1F_2 seeds and beyond.

Thirty six F_1 plants were selfed using 2–3 heads per plant. Five produced no seed and the remaining 31 yielded a mean of 8 F_2 seeds per head (range 2–21). Pollen stainability was estimated for 28 F_1 plants and averaged 52% (range 21–78%). Self-seed set was weakly correlated with pollen stainability ($r = 0.48$, $P < 0.01$).

This study investigated the early pre-breeding steps towards using modern *T. occidentale* populations for *T. repens* improvement by interspecific hybridization. Earlier, Gibson and Beinhart (1969) had shown that hybridisation and backcrossing were possible on a small scale, but that work had not led to any breeding populations. The *T. repens* x *T. occidentale* hybrids produced in the present study showed sufficient fertility to enable the production of large breeding populations without embryo rescue from the full range of available *T. occidentale* germplasm for white clover improvement.

Cytological studies: Somatic and meiotic chromosome studies were conducted in F_1, F_2, and BC_1 (Hussain and Williams 2016). The ploidy levels for all plants evaluated remained at tetraploid (4x). Meiotic analysis of pollen mother cells of all hybrid generation showed univalent and multivalent formation at various frequencies (Hussain and Williams 2016) and compared well with those reported by Chen and Gibson (1970). Meiotic chromosome configurations in F_1, and F_2 hybrids strongly indicated the likelihood of recombination not only between *T. repens* and *T. occidentale* chromosomes but also between homoeologous *T. repens* chromosomes.

Table 1. Maximum (Max) and Minimum (Min) mean growth score rang over the three year duration of the trial (SE± 0.402).

Description	Max mean Growth Score	Min mean Growth Score	Lines tested
White clover	6.63	4.80	8
BC_1	7.33	3.83	19
BC_1F_2	3.54	1.93	9
BC_2	6.63	3.33	19
BC_2+BC_1F_2	5.65	3.26	16
BC_2F_2	2.91	2.91	1
F_2+BC_1	4.22	2.44	8
BC_2F_1 *uni*	4.60	4.33	2
BC_2F_2 *uni*	5.66	3.90	7

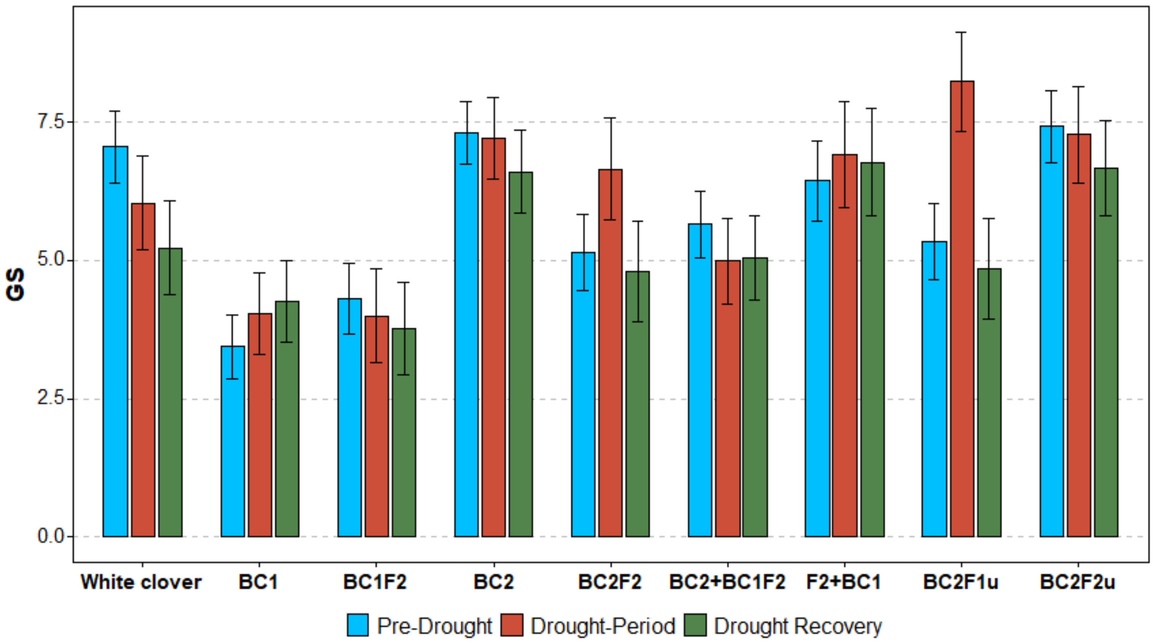

Figure 1. Mean Growth scores of different backcrosses and controls recorded in year 3 during pre-drought, drought and drought recovery periods.

Field evaluation: Mean data recorded on growth scores in the third year during pre-drought, (late

winter to late spring), drought (mid-summer to late summer) and post drought recovery period (mid-autumn to late autumn) are shown in Figure 1. Two *T. repens* x *T. uniflorum* control backcrosses lines (BC_2F_1u) which were previously selected from a drought trial, outperformed significantly ($P<0.05$) the white clover mean growth during drought period (Figure 1). During post drought recovery period, all except one (BC_2F_2) *T. repens* x *T. occidentale* hybrids showed better mean growth recovery than white clover and *T. repens* x *T. uniflorum* hybrids but the difference was non-significant (Figure 1). The individual performance of lines varied greatly particularly in the groups that had high numbers of lines (Table 1). The analyses of the complete data set over the duration of the trial (3 years) showed large variation of mean growth yield across all groups especially the two . larger groups (BC_1 and BC_2) (Table 1). Twelve lines from these two groups were significantly better (SE± 0.402) than the worst performing white clover control cultivar “Huia” (Table 1).
The performance of the interspecific hybrids evaluated in this pilot trial, indicates the potential for plant breeders to select for short term drought and post drought (drought recovery). Dry matter yield in grazing system is extremely important and with ever increasing climatic extremes, drought and drought recovery will be a key focus for forage breeding in the future. Alternative species (*T. occidentale* & *T. uniflorum*) combined with known high yielding species (*T. repens*) could prove a key tool at resolving an ever-increasing problem.

Conclusion

The interspecific hybrids between *T. repens* L. and *T. occidentale* obtained in this study were stoloniferous perennial plants with apparent agronomic merit, warranting further breeding. The fertility and chromosome pairing behaviour fulfil the prerequisites for the successful implementation of a backcross breeding programme to attempt to transfer useful traits (e.g. drought and salt tolerance) from *T. occidentale* to white clover.

Acknowledgement: We acknowledge Grasslands Innovation Ltd. New Zealand for funding the research, and Grace Ehoche (PGG Wrightson seeds Ltd) for statistical analysis

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