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# Improved Drought Stress Tolerance of White Clover through Hybridisation with *Trifolium uniflorum* L.

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# Improved drought stress tolerance of white clover through hybridisation with *Trifolium uniflorum* L.

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**Abstract.** The objective of this study was to determine the effect of hybridisation with *Trifolium uniflorum* L. on drought resistance of *Trifolium repens* L. (white clover). Shoot dry weight of backcross 1 (BC1) hybrids was reduced less by water stress than for backcross 2 (BC2) hybrids and white clover, as were key morphological traits such as leaf area. Under water stress, important differences for the BC1 generation compared to white clover also included lower senescence, higher stolon density, increased root dry weight, and a higher maximum nodal root diameter. Drought decreased the net photosynthetic rate by up to 48% in BC2 and white clover, but there was no significant effect on the BC1 generation. BC1 hybrids were therefore more resistant to water stress than white clover. Smaller effects on stolon morphology suggest BC1 hybrids were better able to maintain cell turgor and growth, and maintenance of photosynthesis under drought may have contributed to smaller reductions in productivity. The findings suggest that BC1 hybrids may be able to maintain higher water uptake during drought than white clover by increased allocation to root biomass.

**Keywords:** *Trifolium repens*, water stress, dry matter production, senescence, morphology, photosynthesis.

## Introduction

The introduction of characteristics to improve limitations to productivity and persistence in white clover (*Trifolium repens* L.) may be achieved through interspecific hybridisation. While white clover is a major temperate legume in mixed grasslands it is limited by a number of factors, including a requirement for high soil moisture (Knowles *et al.* 2003). *Trifolium uniflorum* L. (oneflower clover) is a Mediterranean species, described as being xerophytic (Tela Botanica 2012), although no quantitative studies have been published. If *T. uniflorum* does possess characteristics which contribute to drought resistance, then *T. repens* x *T. uniflorum* hybrids could be expected to perform better under drought than white clover. The objective of this study was to investigate the effect of water stress on growth and morphology of *T. repens* x *T. uniflorum* hybrids compared with white clover cultivars, and the physiological attributes that could be involved in such responses.

## Methods

The experiment was conducted in a rain shelter facility at Lincoln, New Zealand, on a Templeton silt loam soil. Hybrids used in the study were recurrently backcrossed to white clover to produce backcross 1 ($BC_1$) and backcross 2 ($BC_2$) generations. Seven $BC_1$ families, four $BC_2$ families and five white clover cultivars were used. Plants were established from stolon tip cuttings taken from an existing field experiment in July 2009, and transplanted to the field on October 1 2009. A split plot design was used, with each of six replicates containing one plot each of two watering treatments. Watering treatments were imposed from 8 December 2009, with plots in the Watered treatment irrigated weekly to replace potential evapotranspiration, while plots in the Stressed treatment received no irrigation or rainfall.

A subset of related entries (Kopu II $BC_1$, Kopu II $BC_2$, white clover cv. Grasslands Kopu II) was used for photosynthesis measurements on 9 March 2010, after approximately three months of watering treatments. Measurements of the net photosynthetic rate were made between 10:30 am and 1 pm using a LI-6400 infrared gas analyser (LI-Cor Biosciences Inc., Lincoln, Nebraska), on the central trifoliolate leaflet of one 2nd fully expanded leaf (FEL) from each plant. Stolon densities of all plants in the Stressed treatment were scored visually from 1 (high) to 5 (low) on 15 March 2010.

On 17-19 March 2010, stolon morphological parameters were measured on two stolons from every plant using the 2nd FEL, and the internode proximal to the 2nd FEL. At the end of the experiment (23 March 2010), total shoot dry weight (DW) was measured by removing all above ground material, which was oven dried at 80°C for 48 hours. All plants were also scored for senescence at this time, on a visual scale from 1 (minimal/no senescence) to 9 (total death). In the Kopu II subset, total root DW and the root cross-sectional area of the thickest nodal root were measured in 100 mm diameter x 100 mm deep soil cores.

Stolon measurements were analysed using a linear mixed modelling approach in SAS version 9.1 (SAS Institute Inc.) to account for correlations among measurements within the same plant. All other data were analysed via analysis of variance (ANOVA) in Genstat version 11 (VSN International Ltd.). Comparisons are between clover “types” where all plants were measured, and clover “entries” in the Kopu II subset. Significant differences at the 5% level were determined using the means separation methods as stated above. Relationships between stolon morphology and changes in shoot DW were tested using regression and Pearson's correlation in Minitab version 15 (Minitab Inc.).

## Results

### *Dry matter production*

At the end of the experiment, when mean soil moisture in the top 0.2 m was 10% in the Stressed treatment and 30% in the Watered treatment, shoot DWs of both $BC_2$ and white clover in the Watered treatment were larger than $BC_1$ by 26% and 95%, respectively (Fig. 1). However, they did not differ to $BC_1$ in the Stressed treatment due to a significant clover type x watering treatment interaction (P<0.001). Relative to the Watered treatment, shoot DW of the $BC_1$ generation decreased by 47% compared with 68% for $BC_2$ and 69% for white clover. Similar results were seen in the Kopu II subset of plants (data not shown) where there was also a significant clover entry x watering treatment interaction (P=0.020). Shoot DW of Kopu II $BC_1$ did not change under water stress, whereas Kopu II $BC_2$ and Kopu II decreased by 78% and 62%, respectively.

### *Senescence*

Overall, mean senescence scores increased (P<0.001) under water stress (3.05) compared with the Watered treatment (2.01). A significant clover type x watering treatment interaction (P=0.032) indicated a smaller effect of water stress for $BC_1$ than for white clover (Fig. 2). Mean senescence score of the $BC_1$ generation did not change under stress, while that of $BC_2$ and white clover increased relative to the Watered treatment.

### *Stolon morphology*

Internode length, petiole length, leaf lamina area, and specific leaf area all decreased (P<0.001) under water stress, while specific leaf mass increased (P<0.001). Generally, the decreases were smaller for the $BC_1$ generation than for white clover. In particular, leaf lamina area decreased by 486 $mm^2$ for $BC_1$, 681 $mm^2$ for $BC_2$ and 888 $mm^2$ for white clover, which was equivalent to 65%, 73% and 74%, respectively (Fig. 3). Correlation analyses showed that genotypes which had smaller decreases in leaf lamina area had smaller decreases in DW under water stress ($P$=0.013, $R^2$=0.1261). In the Watered treatment, leaf lamina area of white clover was 62% higher than $BC_1$ (P<0.001) and 29% higher than $BC_2$ (P<0.001), and the $BC_2$ generation was 25% higher than the $BC_1$ generation. However, there were no differences among clover types in the Stressed treatment. Mean stolon density score in the Stressed treatment was significantly higher (P<0.05) for $BC_1$ hybrids (4.1) than for $BC_2$ (3.1) and white clover (2.9).

### *Photosynthesis*

Overall, water stress decreased (P=0.001) the net photosynthetic rate ($P_n$) by 34% compared with the Watered treatment (23.5 μmol/$m^2$/s). There were no differences in $P_n$ among clover entries in the Watered treatment, but $P_n$ of Kopu II $BC_1$ was more than 80% higher than Kopu II $BC_2$ and Kopu II in the Stressed treatment (Fig. 4). The $LSD_{0.05}$ comparisons showed that $P_n$ did not change under water stress for Kopu II $BC_1$, but decreased by 48% for Kopu II $BC_2$ and 44% for Kopu II.

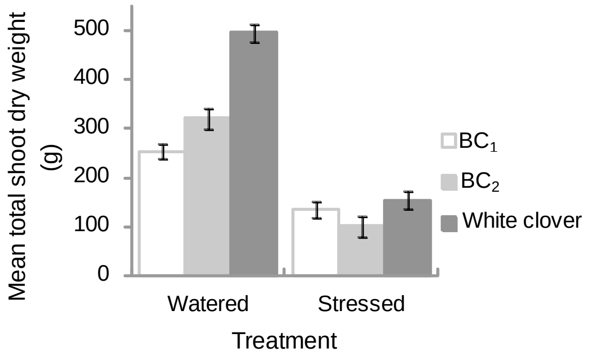


**Figure 1. Mean total shoot dry weight (±SEM) for $BC_1$, $BC_2$ and white clover in the Watered and Stressed treatments.**

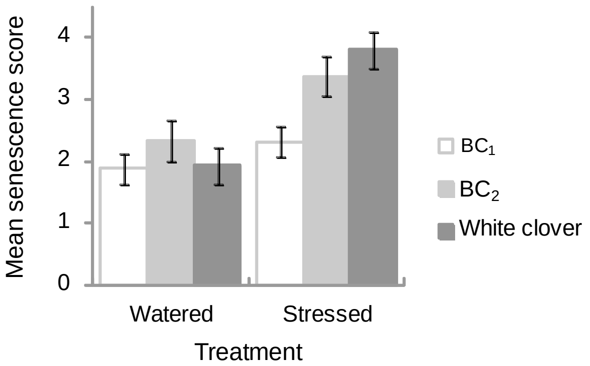


**Figure 2. Mean senescence scores (±SEM) for $BC_1$, $BC_2$ and white clover in the Watered and Stressed treatments.**

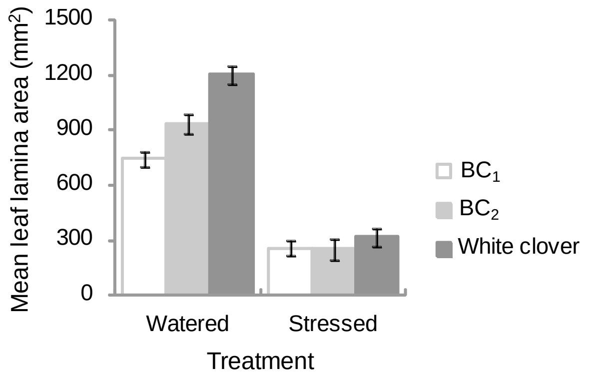


**Figure 3. Mean leaf lamina area (±SEM) for $BC_1$, $BC_2$ and white clover in the Watered and Stressed treatments.**

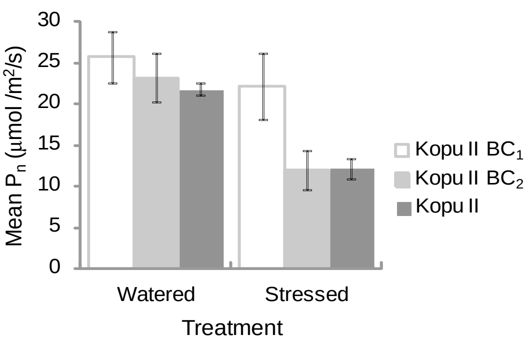


**Figure 4. Mean net photosynthetic rate ($P_n$) (±SEM) of Kopu II $BC_1$, Kopu II $BC_2$ and Kopu II in the Watered and Stressed treatments.**

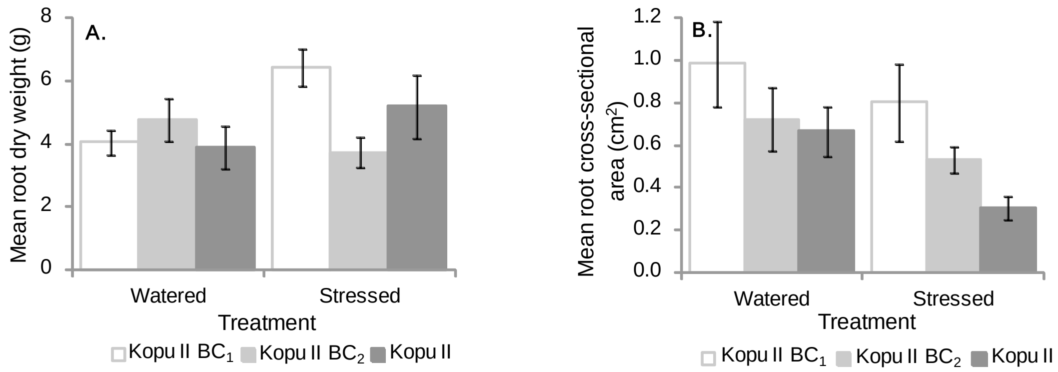


**Figure 5. Mean root dry weight (A) and root cross-sectional area (B) (±SEM) for Kopu II $BC_1$, Kopu II $BC_2$ and Kopu II in the Watered and Stressed treatments.**

*Root measurements*

A clover entry x watering treatment interaction (P=0.037) for root DW reflected a 59% increase in DW in the Stressed treatment for Kopu II $BC_1$, with no change for Kopu II $BC_2$ or Kopu II (Fig. 5A).

In addition, the overall mean root cross-sectional area (*i.e.* root diameter) of Kopu II $BC_1$ (0.896 $cm^2$) was 43% higher than that of Kopu II $BC_2$ (0.627 $cm^2$) and 84% higher than that of Kopu II (0.487 $cm^2$). In the Stressed treatment, mean root cross-sectional area of Kopu II $BC_1$ was also greater than the Kopu II parent (Fig. 5B).

## Discussion

As expected, the *T. repens* x *T. uniflorum* $BC_1$ generation was less affected by water stress than the $BC_2$ generation and white clover. Most importantly, shoot DW of the $BC_1$ generation decreased significantly less than in the other clover types. Turner (1991) concluded that senescence was as important as decreases in growth for decreased biomass production of white clover under drought stress. The lower senescence of the $BC_1$ hybrids is, therefore, likely to have contributed to the smaller decreases in dry matter production. Turner (1990) also found that osmotic adjustment in white clover under drought maintains stolons at the expense of leaf biomass, which would increase survival during these conditions. Lower levels of senescence in the hybrid plants may, therefore, be due to the accumulation of compatible solutes, or protective phenolic compounds (Nichols 2012).

Smaller drought-induced decreases in stolon morphological parameters for $BC_1$ hybrids, particularly leaf lamina area, suggest they were able to maintain turgor, which drives cell expansion and growth. The higher stolon density of the $BC_1$ generation in the Stressed treatment is also likely to have contributed to its DW response and may also contribute to on-farm persistence (MacFarlane *et al.* 1990).

Under water stress, the Kopu II $BC_1$ family was able to maintain photosynthesis, which decreased considerably in Kopu II $BC_2$ and the Kopu II parent. While this would have differential effects on plant growth, it may also reflect differences among the clover types in their responses to water loss. Under drought stress some plants can be termed “water spenders” (Levitt 1980), able to maintain opening of the stomata through higher water uptake. The increased allocation of biomass to roots under water stress may have facilitated such a strategy in Kopu II $BC_1$. In addition, Kopu II $BC_1$ had thicker roots, which penetrate more to depth than fine roots and, therefore, could have increased access to deeper soil moisture.

## Conclusion

Drought resistance of white clover can be improved by interspecific hybridisation. Most importantly, dry matter production of $BC_1$ hybrids was less affected by drought stress than that of white clover. Morphological and physiological responses were observed which are likely to have contributed to the differential effects of drought on plant DW. In more challenging conditions than those in the current experiment an even greater advantage to $BC_1$ hybrids may become apparent. Material now in the early stages of field breeding could lead to commercial cultivars given satisfactory field test results.

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