



# Physiological drought resistance and accumulation of leaf phenolics in white clover interspecific hybrids



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## ARTICLE INFO

### Article history:

Received 1 May 2015

Received in revised form 29 May 2015

Accepted 29 May 2015

Available online 1 June 2015

### Keywords:

Drought

Interspecific hybridisation

*Trifolium repens*

*Trifolium uniflorum*

White clover

## ABSTRACT

This study investigated drought responses in first (BC<sub>1</sub>) and second (BC<sub>2</sub>) backcross generation hybrids of *Trifolium repens* L. × *T. uniflorum* L., and compared these to high-yielding *T. repens* L. (white clover) cultivars. Plant attributes included accumulation of leaf phenolics as well as other biochemical and physiological water stress responses. Measurements were made across broad clover types as well as on a subset of related material. Under drought, net photosynthesis decreased by 44–48% and transpiration rates decreased by 60% in the BC<sub>2</sub> family and in the white clover parent, but were unaltered in the BC<sub>1</sub> family. Drought-induced decreases in leaf water potential were more pronounced in the BC<sub>1</sub> family (−47%) than the BC<sub>2</sub> (−31%) and parental (−28%) material. Quercetin glycoside accumulation generally increased 2–3 fold under drought. Compared with the BC<sub>2</sub> family and with parent plants, the BC<sub>1</sub> family had 2.5–4x higher constitutive kaempferol glycoside levels, and 1.5–2.5x higher kaempferol glycoside accumulation under drought. Constitutive kaempferol glycoside accumulation was related to reduced senescence and to less pronounced decreases in shoot DW under drought stress. Hydroxycinnamic acid accumulation increased by 56–73% under water stress, particularly for the BC<sub>1</sub> generation. These results identify physiological and biochemical traits that help explain drought resistance of *T. repens* × *T. uniflorum* hybrids. The findings suggest merit for the selection of BC<sub>1</sub> families with increased accumulation of protective phenolic compounds in breeding programmes for improved drought resistance.

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## 1. Introduction

White clover (*Trifolium repens* L.) is a key species in temperate grasslands systems, due to its high feed value and contribution of nitrogen from biological N<sub>2</sub> fixation. However its use, productivity and persistence can be restricted by a requirement for high soil moisture (Knowles et al., 2003). Interspecific hybridisation provides a route to overcoming such limitations. Recently, Nichols et al. (2014b) established that growth and morphological characteristics of *T. repens* × *T. uniflorum* L. hybrids, particularly in the backcross 1 (BC<sub>1</sub>) generation, were less affected by water stress than those of white clover. Smaller decreases in shoot DW under water stress in such hybrids were accompanied by smaller decreases in leaf area, lower senescence and higher stolon density. In addition, these BC<sub>1</sub> hybrids show potential for increased

survival of taproots (Nichols et al., 2015), which may enhance persistence, as well as increased resistance to low soil phosphorus levels (Nichols et al., 2014a; Nichols et al., 2014d). Physiological and biochemical factors which contributed to this drought resistance are likely to be introgressed from the *T. uniflorum* parent which occurs in dry habitats in the Mediterranean region.

Plant water status can be assessed by measuring leaf water potential ( $\Psi$ ), which decreases under water stress (Maricle and Adler, 2011). Various studies have recorded differences among plant species, and genotypes within species, in the response of  $\Psi$  to changes in soil moisture (Maricle and Adler, 2011; Santos et al., 2009). The impact of water stress on plants can be monitored by measuring leaf gas exchange, most notably net photosynthesis, stomatal conductance and transpiration. Most plants have increased water use efficiency (WUE) during mild drought due to the non-linear relationship between stomatal conductance and carbon assimilation (Chaves et al., 2003). Barbour et al., (1996) observed no differences in WUE among ten white clover cultivars at a range of soil moisture levels, suggesting there is little variation

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in adaptation of this response in white clover at the population level. This highlights a need to examine opportunities for WUE improvement beyond the species barrier. Physiological WUE at the leaf level can be calculated using measurements of net photosynthesis and transpiration (Condon et al., 2002; Hall et al., 1994). Furthermore, the  $^{13}\text{C}/^{12}\text{C}$  isotopic composition ( $\delta^{13}\text{C}$ ) of plants can be used to calculate  $^{13}\text{C}$  discrimination ( $\Delta$ ) as a measure of WUE in a large number of plants, and it also provides an integrated measurement over time. The relationship between WUE and  $\Delta$  suggests that plants with a higher WUE will discriminate less against  $^{13}\text{C}$  (Farquhar et al., 1982; Farquhar and Richards, 1984), and  $\Delta$  may then be used as a tool to select for high WUE.

Among secondary metabolites, phenolic compounds such as hydroxycinnamic acids and flavonoids have been identified as markers of stress tolerance in plants (Ballizany et al., 2014; Shahidi and Chandrasekara, 2010). Glycosides of the flavonols quercetin and kaempferol are increased by exposure to UV-B radiation (Hofmann et al., 2003) and drought (Ballizany et al., 2012b), and these higher levels are associated with increased stress tolerance. In a full-sib population generated by crossing an elite white clover cultivar with a stress-resistant ecotype, Ballizany et al. (2012a) found that higher quercetin levels under water stress were associated with smaller biomass decreases in intraspecific white clover hybrids. Hofmann and Jahufer (2011) found that higher levels of quercetin in stress-resistant white clover populations were associated with lower levels of constitutive biomass productivity.

Interspecific hybridisation can be used to move beyond intraspecific limitations of stress resistance and biomass productivity (Hofmann and Campbell, 2012), and this has recently been utilised in the development of drought-resistant interspecific *T. repens* × *T. uniflorum* hybrid material (Nichols et al., 2014b). However, little functional information is available for such germplasm. This study examines phenolic compound accumulation in conjunction with other biochemical and physiological traits in such interspecific hybrids, and compares these attributes to the corresponding white clover parent material, to determine their contribution to hybrid drought resistance.

## 2. Materials and methods

The setup for this experiment was that described in Nichols et al. (2014b). Briefly, the experiment was conducted in a rain shelter at Plant & Food Research, Lincoln, New Zealand. This facility automatically moves over the trial area during rainfall events and otherwise exposes plants to ambient field conditions. The site is located on a Templeton silt loam (Udic Ustochrept, USDA soil taxonomy). There were six replicates and two watering treatments, for a total of 12 plots in a 3 × 4 arrangement. Each plot was 3.6 m × 3.95 m in total, with a 400 mm gap between the top and bottom of neighbouring plots and a 1 m gap at the sides.

Sixteen clover entries were used, comprising seven BC<sub>1</sub> families, four BC<sub>2</sub> families and five white clover cultivars (Table 1). Entries were selected to provide a range of white clover cultivars and backcross parentage of differing leaf sizes and known drought responses, as well as BC<sub>1</sub> families which had maintained their dry matter scores over the previous summer in a nearby field experiment (Nichols et al., 2014c). Corresponding BC<sub>2</sub> families were included, where they were available, to provide sequences of related material with decreasing proportions of *T. uniflorum* genes. Additional BC<sub>2</sub> families were selected to increase the pool for this generation. *T. uniflorum* was not examined here, as it propagates poorly from stolon cuttings, which were the basis of the clonal material used in this study to enable the same genetic material to

**Table 1**

Clover entries used in the experiment. The naming convention for BC<sub>1</sub> entries is 'white clover parent × F<sub>1</sub> parent' and for BC<sub>2</sub> families is 'white clover parent × (white clover parent × F<sub>1</sub> parent)'.

Clover type	Description
BC <sub>1</sub>	Crusader × 80-2
BC <sub>1</sub>	Crusader × 900-4
BC <sub>1</sub>	Kopu II × 900-4
BC <sub>1</sub>	Kopu II × 80-2
BC <sub>1</sub>	Sustain × 82-3
BC <sub>1</sub>	Tribute × 900-4
BC <sub>1</sub>	Trophy × 902-6
BC <sub>2</sub>	Crusader × (Crusader × 900-5)
BC <sub>2</sub>	Kopu II × (Kopu II × 902-1)
BC <sub>2</sub>	902-1-OP-4 × Trophy
BC <sub>2</sub>	Durana × (Crusader × 902-1)
White clover	cv. "Crusader"
White clover	cv. Grasslands "Kopu II"
White clover	cv. Grasslands "Sustain"
White clover	cv. Grasslands "Tribute"
White clover	cv. "Trophy"

be compared between watering regimes. For each clover entry, two stolon tip cuttings were taken of six different genotypes from a neighbouring field experiment in July 2009. These were established in 40 × 40 × 120 mm root trainers of sand/peat potting mix, as described by Nichols et al. (2014b), and transplanted into the field on 1 October 2009. Spacing between plants was 660 × 600 mm, with each plant placed in the same relative position (distance and angle) to the surrounding water emitters.

A split plot design was used, with the six replicates corresponding to the six genotypes of each clover entry. Each replicate contained two plots – one of each of two watering treatments. Watering treatments within replicates were assigned at random, as were the positions of the clover entries within each plot. The two clones of each genotype were planted in the same replicate (one in each watering treatment), to enable genotypic effects to be considered. Border plants were used around the outside of every plot to avoid edge effects.

Plots were irrigated using trickle irrigation, with 13 mm lateral lines spaced 220 mm apart. Emitters were spaced 300 mm apart along each lateral line and offset on adjacent laterals to ensure even watering. This system was established in every plot to ensure the physical effects of the irrigation lines on growth were the same across irrigation treatments. Plots which received irrigation represented the Watered treatment and plots which received no irrigation represented the Stressed treatment.

The site was left open to rain for five weeks after planting to aid plant establishment, and operation of the rain shelter began in early November 2009 to begin drying down the soil. Five millimetres of irrigation was applied to all plants on 24 November before the different watering treatments were imposed on 8 December 2009. After that time, the Stressed plots received no further irrigation, and Watered plots were irrigated weekly to replace potential evapotranspiration (PET) +10–20 mm until the end of the experiment (24 March 2010). Watering treatments resulted in significant differences in volumetric soil moisture content, from 0.2 m to 0.95 m depth (Nichols et al., 2014b). In the Watered treatment, soil moisture during the course of the experiment increased at 0.20–0.45 m depth, but did not exceed the field capacity of the soil, and did not change at 0.55–0.85 m depth. Soil moisture decreased at all depths over time in the Stressed treatment, with final readings in the top of the profile being below or close to wilting point. Further detail, including temperature and humidity data at the site during the experimental period are presented in Nichols et al. (2014b).

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