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REGULAR ARTICLE

Genotypic differences in red clover (*Trifolium pratense* L.) response under severe water deficit

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Abstract
Aims In Ontario, Canada, acreage of red clover (*Trifolium pratense* L.) intercropped with winter wheat (*Triticum aestivum* L. em. Thell) has declined, despite well-documented soil and yield benefits. The decline has resulted from increasing prevalence of stand non-uniformity, which has been attributed in part to soil moisture deficits. We examined whether there are genotypic differences in drought response between red clover varieties.
Methods A double-cut (Belle) and a single-cut variety (Altaswede) were grown under four different durations of drought (4, 8, 12 and 16 days below 15% relative soil water content, RSWC). Shoot dry weight, shoot relative water content (RWC), leaf area and crown water content were measured in control, drought and drought + recovery treatments.
Results Belle used significantly more water during soil moisture deficit and had greater leaf area, shoot dry weight and RWC compared to Altaswede. In contrast, Altaswede had significantly higher survival rates than Belle, attributed to maintenance of meristematic tissue viability in the crown where re-growth, after shoot tissue desiccation, can occur.
Conclusions By demonstrating genotypic variation in survival strategies of red clover, traits can be identified for the development of improved varieties. Varieties with higher survival rates during drought will result in more uniform stands and increased utilization of red clover for environmental and yield benefits.

Keywords Cover crop · Drought · Inter-seeding · Red clover · *Trifolium pratense* · Winter wheat

Abbreviations

SC	Single-cut red clover
DC	Double-cut red clover
RSWC	Relative soil water content
RWC	Relative water content
FW	Fresh weight
DW	Dry weight
CON	Control
WS	Water stress
REC	Recovery

Introduction

A common diversified rotation in Ontario, Canada is Corn (*Zea mays* L.) – Soybean (*Glycine max* L. Merr.) – Winter wheat (*Triticum aestivum* L. em. Thell) inter-seeded with red clover (*Trifolium pratense* L) where red clover is frost-seeded onto an existing stand of winter

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wheat. Frost-seeding is a method whereby seed is broadcast onto frozen soils in early spring to take advantage of freeze-thaw cycles which work the seeds into the soil. Red clover is a popular choice among Ontario farmers as it is can be grown in relay cropping with wheat, is easy to establish and manage and is adapted to a wide variety of soil types and conditions. Red clover is adapted to establishment under the wheat canopy due to high shade tolerance as compared to other cover crops (Gist and Mott 1957), can yield up to 5 t/ha aboveground dry biomass, when harvested in either fall or spring, and reduce the most economic rate of nitrogen required for the following corn crop by 44–64 kg N/ha (Gaudin et al. 2013). The use of red clover as an underseeded cover crop to winter wheat is associated with substantial yield increases of all crops included in the cropping system (Meyer-Aurich et al. 2006; Raimbault and Vyn 1991) as well as environmental and soil health benefits including weed control (Mutch et al. 2003; Blaser et al. 2011), improved water infiltration and conservation (Hartwig and Ammon 2002), increased soil stability (Dapaah and Vyn 1998; Raimbault and Vyn 1991), decreased system nitrogen losses (Meyer-Aurich et al. 2006) and increased soil carbon (Ladoni et al. 2016).

Despite substantial benefits, the practice of frost seeding red clover into winter wheat by farmers has dramatically decreased in the last 20 years. The decline in use of red clover is largely due to increasingly non-uniform stands, which limit the ability of farmers to decrease nitrogen application to the subsequent corn crop, thus decreasing the economic incentive to plant red clover. Additionally, not limiting nitrogen application to non-uniform fields of red clover substantially increases soil nitrogen in patches where red clover has survived which resultsin potential nitrogen leaching and nitrous oxide emissions into the atmosphere (Wagner-Riddle et al. 2007).

Various studies have associated low red clover biomass with periods of low precipitation in dry years (Blaser et al. 2006; Mutch et al. 2003; Singer et al. 2006; Queen et al. 2009) and more recent studies suggest that during periods of low precipitation, red clover stand non-uniformity could increase (Loucks 2017; Westra 2015).

The impact of drought episodes on inter-seeded red clover is likely to increase in the near future as moisture extremes in the North American "corn belt" region are expected to increase (Hatfield et al. 2013). Higher incidences of weather extremes such as drought will also increase the value of soil health benefits resulting from the use of red clover as soils with lower water holding capacity are more susceptible to decreases in yield and increased yield variability associated with drought stress (Wang et al. 2016; Gaudin et al. 2015; Munoz et al. 2014).

Two main growth types of red clover, that are most widely grown in Canada, are double-cut, which requires a shorter day length to flower, and a single-cut, which requires a longer day length to flower (Fergus and Hollowell 1960). Single-cut red clover populations are low growing and do not flower or produce stems in the seeding year (Bowley et al. 1984). Low, vegetative first year growth allows single-cut varieties to enter the winter as a rosette, and has been associated with increased winter hardiness and stand persistence in these populations (Choo et al. 1984; Christie and Choo 1991). Single-cut populations have also been shown to display better grazing tolerance (Brummer and Moore 2000). However, because of differences in biomass partitioning, double-cut red clover are more commonly grown as they have greater shoot biomass in the fall and, in general, a higher shoot/root ratio than single-cut red clover (Bruulsema and Christie 1987; Christie et al. 1992; Hejduk and Knot 2010). However, it is unclear whether a shoot/root ratio difference exists between single- and double-cut red clover earlier in development when growing under winter wheat. Differences in biomass production of red clover genotypes have been documented and these differences were observed to be greater during suboptimal growing conditions such as low precipitation in spring (Singer et al. 2006). It is possible that during drought stress, red clover populations with differing growth habits have different strategies for drought tolerance or survival.

The capacity to survive periods of moisture stress could be related to the maintenance of viable meristematic tissue in the red clover crown. The red clover crown is very similar to that of alfalfa (*Medicago sativa* L.), forming from a complex of axillary buds that form as the plant grows (Fergus and Hollowell 1960; Taylor and Quesenberry 1996). After defoliation, or in the spring after over-wintering, most of the re-growth of red clover plants occurs from the crown via basal buds using remobilized carbohydrates and nitrogen from the crown (Black et al. 2009; Li et al. 1996). Wissuwa et al. (1997) observed a crown moisture threshold below which alfalfa plants could not survive. The maintenance of crown tissue viability could be a major contributor to the ability of red clover to survive periods of low soil moisture and re-grow when moisture is no longer limiting.

As past Ontario research does not suggest that poor germination and emergence is the cause of stand non-uniformity of red clover frost seeded into winter wheat, this experiment was designed to investigate the effects of drought on established red clover plants. This study investigates differences in response to drought of two types of red clover with pronounced differences in growth habit, a single-cut (Altaswede) and a double-cut (Belle) red clover variety, during various durations of severe water deficit to determine whether genotypic variation in drought response exists between the two types. Observed genotypic variation between the two types could be used to improve future red clover varieties for growth under winter wheat. We quantified the importance of crown tissue quiescence during water deficit and its importance for red clover capacity to recover from water deficit via new meristematic growth in the surviving crown.

Methods

Plant growth

The experiment was conducted in a growth room from July to September, 2016 at the University of Guelph, Guelph, ON. Growth room conditions were set for a constant day and night air temperature of 23 °C and a relative humidity of 65%. The photoperiod was 16 h and the approximate photosynthetic photon flux density measured at the tops of plants was 200 μmol/m^2/s to mimic light conditions under the wheat canopy. Plant material consisted of two red clover varieties, Belle (Mapleseed, Lindsay, ON), a double-cut variety, and Altaswede (Pickseed Canada Inc., Winnipeg, MB), a single-cut variety. Varieties were selected for their contrasting growth types. Plants were gown in a pre-mixed growth medium containing 2 parts granitic "B sand" (Hutcheson Sand & Mixes, Huntsville ON) and 1 part peat-based PGX soil (Sun Gro Horticulture Canada Ltd., Seba Beach, AB). A total of 2.7 kg was placed into each 2.5-L plastic food container (Airlite Containers, Omaha, Nebraska) without drainage holes.

The experiment was arranged as a completely randomized design with four replications. To minimize variation due to bench position, the pots were rotated daily with one of two alternating rotations: either the front pots were rotated to the back or the far right pots were rotated to the far left. Soil water holding capacity of each pot was calculated before clover planting by subtracting pot and soil dry weight from saturated pot weights. Soil water holding capacity was used to determine the relative soil water content (RSWC) of each pot throughout the experiment.

Seeds were germinated on moistened tissue paper for six days. Uniform seedlings were selected for planting to minimize any differences in seedling vigour among the experimental units. Twenty pre-germinated seedlings were transplanted into each pot and inoculated with *Rhizobium trifolii* on the same day. Pots were watered with a 0.5% solution of fertilizer plus micronutrients (Optimum Cal Mag 12–2-14, Plant-Prod Inc., Brampton, ON) for the first three weeks of growth.

Water use of red clover seedlings during week 1 was considered negligible. Pots were maintained between 50 and 80% RSWC from week 2 until the end of week 3. We assumed that red clover plants would not begin to reduce water use until about 35% RSWC based on previous research on soybean plants utilizing a similar culture system (Hufstetler et al. 2007). To control for soil water evaporation, the mean water lost from three control pots without red clover was measured and subtracted from measurements of daily plant water use in pots with red clover.

Water stress treatments

Preliminary experiments were conducted to determine the appropriate duration of drought such that there was a treatment length where all plants recovered from drought stress and a treatment length where no plants recovered. That time duration was determined to be 14–16 days. 16 days was chosen as the total length of drought stress. Sixteen was divided by 4 to create enough data points such that a dose-response curve could be fit.

Due to environmental differences between the field and growth room, treatments in this experiment attempted to match development stage and drought stress symptomology, rather than soil moisture levels, of red clover grown under winter wheat in field conditions. In similar field experiments, red clover interseeded with winter wheat was found to be, on average, at the 4th trifoliate stage just before wheat harvest. Therefore, drought stress was imposed on red clover in this experiment around the 2nd-3rd trifoliate stage in an attempt to match development stage during growth with winter wheat. Due to the occurrence of red clover non-uniformity, some plants are able to survive under growth with winter wheat and some plants are not. Durations of drought stress were chosen to provide a range of drought stress symptoms such that in one treatment, all plants survived and in another, all plants died.

For the duration of the experiment, all pots in the well-watered control (CON) treatments were watered to 80% RSWC daily. Starting at week 4, a three-week dry down period was imposed on pots in water stress (WS) and water stress with recovery (REC) treatments using a semi-automated pot weighing and watering system described by Walden-Coleman et al. (2013). Also starting week 4, the evaporation control was not used since the red clover canopy in control treatments likely limited soil evaporation to such an extent that the evaporation control would over-estimate the amount of soil surface evaporation. In addition, the evaporation control did not dry as quickly as the pots containing red clover due to plant transpiration. The target weight for pots in WS and REC treatments was calculated based on the heaviest pot for each treatment to ensure that plant dried at the speed of the pot that dried down the slowest each day, thus ensuring the dry down was more uniform across pots. The target weight for pots in CON treatments was 80% RSWC during this three-week dry down period.

Starting at week 7, after the three-week dry down period, all pots in WS and REC treatments reached 15% RSWC and four different time periods below 15% RSWC (4, 8, 12 and 16 days) were imposed (Fig. 1). According to soil water release curves developed for this soil mixture (H.J. Earl, unpublished data), 15% RSWC is the range where soil matric potential declines sharply as the soil dries. Matric potential declines from approximately −0.08 MPa at 20% RSWC to −0.20 MPa at 15% RSWC, then to −0.69 MPa at 10% RSWC (Fig. 1). Estimated permanent wilting point (−1.5 MPa) occurs at approximately 7.5% RSWC (Fig. 1). Following each time period, pots in WS and CON treatments were harvested immediately and pots in REC treatments were returned to 80% RSWC for a one week recovery period before being harvested (Fig. 1). During the one week recovery period for REC treatments, water use by evaporation controls was again subtracted from water use in water stress treatments as the red clover canopy was sparse or absent.

Data collection

Plant water use was calculated as the cumulative daily water use in L/kg soil (with water use in evaporation controls subtracted where applicable). Red clover shoots of the twenty plants per pot were cut 8 mm above the soil surface. Fresh weight (FW) of shoot samples (leaflets and petioles) was taken immediately. When leaves and petioles were wilted, shoot tissue was harvested based on the criteria that tissue was neither beginning to desiccate nor was discoloured. Leaflets were separated from petioles and leaf area in cm^2 of leaflets was then measured using a LI-3100 area meter (Licor, Lincoln, Nebraska, USA). Turgid weight (TW) of shoot samples was taken after 8 h in a zip-lock bag filled with deionized water. Shoot samples were then dried at 80 °C for 4 days, or until a constant weight was achieved, to determine dry weight (DW). Shoot relative water content (RWC) was determined using the equation:

$$RWC = \frac{(FW - DW)}{(TW - DW)} \; x \; 100\% \tag{1}$$

Red clover plant mortality was determined after each drought period and after each recovery period in the same pot. A red clover plant was considered non-viable if: 1) all leaves were desiccated and 2) no new growth was occurring. Plant mortality is expressed as a percentage of the total number of red clover plants that were viable in each pot before the drought period began (n/20 plants × 100). Crown samples were taken according to Striker et al. (2011). The red clover shoot was cut 8 mm above the soil surface and 2 mm below the soil surface to ensure that the 10 mm sample of the crown tissue encompassed the transition zone between the tap root and the shoot. There was no evidence that the transition zone varied in position between the two varieties. A 10 mm sample was deemed large enough to ensure that the crown tissue was harvested in every sample. The crown sample was weighed immediately to determine fresh weight (FW) and then dried at 80 °C for 4 days, or until a constant weight was achieved, to determine dry weight (DW). The equation for crown water content was adapted from Wissuwa et al. (1997) and expressed as:

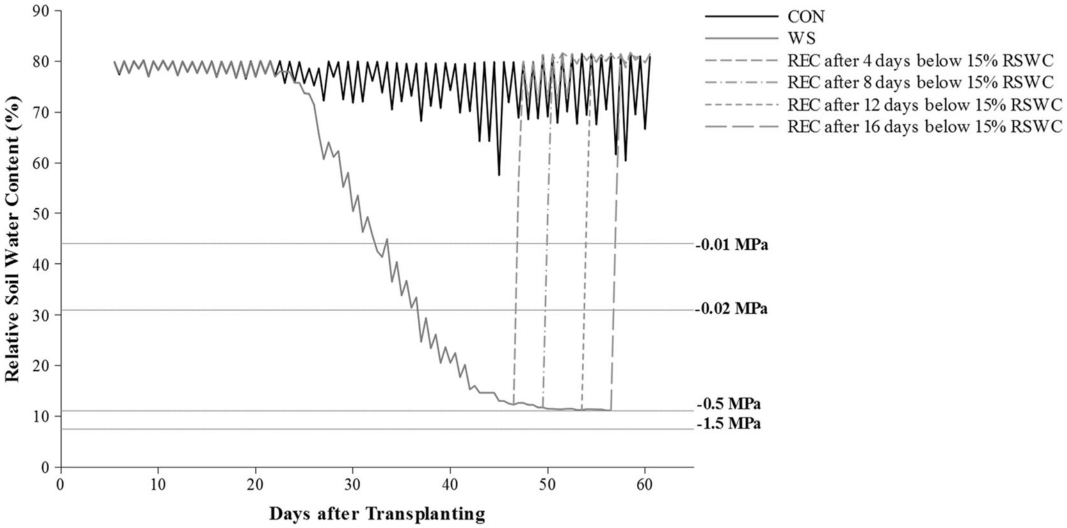

Fig. 1 Daily fluctuation in relative soil water content (RSWC) for pots in well-watered control (CON), water stress (WS) and water-stress with recovery (REC) treatments. Week 1 data are not shown as pots were not weighed. During weeks 2 and 3, pots in all treatments were watered to 80% RSWC. During week 4, pots in both WS and REC treatments were uniformly dried down for 3 weeks until pots reached a RSWC of 15%, at which point a no-watering period of 4, 8, 12 or 16 days was imposed. After each no-watering period, pots in CON and WS treatments were harvested immediately and pots in REC treatments were returned to 80% RSWC for a one-week recovery period and then harvested. Matric potential values according to soil water release curves developed for this soil mixture (H.J. Earl, unpublished data), are shown as horizontal dotted lines, including the value of −1.5 MPa, the apparent "wilting point"

$$Crown\ water\ content = \frac{(FW - DW)}{DW}\ x\ 100\% \qquad (2)$$

Statistical analysis

All data were analyzed using SAS version 9.3 (SAS Institute Inc., Cary, NC). To determine red clover variety and watering regime treatment differences, PROC GLIMMIX was used. Red clover variety (Belle or Altaswede), watering regime (WS, CON or REC) and their interaction were considered fixed effects. A Gaussian distribution with an identity link function were used to analyze shoot dry weight and leaf area and a beta distribution with a logit link was used to analyze shoot RWC using the LaPlace method. Where residual variances were found to be heterogeneous between treatments, a heterogeneous error model was used. AICC fit statistics and residual plots were used to confirm the fit of the heterogeneous error models. Tukey's test for multiple means comparisons was used to compare means, and differences were considered significant at $p < 0.05$. The SLICE statement in SAS was used to determine significances of one factor within levels of another factor when significant interactions occurred. Means and standard errors for RWC were converted to the data scale using the ILINK option.

Two regression analyses using PROC NLMIXED were performed: 1) the relationship between the percentage of viable Belle and Altaswede red clover plants and an increasing number of days below 15% RSWC (4,8,12,16) which was followed by a one week recovery period and 2) the relationship between plant mortality and decreasing crown water content where crown water content was determined directly after each no-watering period and percentage mortality was determined after the one-week recovery period. Data were fit to a dose-response curve, the equation for which was (Seefeldt et al. 1995):

$$y = c + \frac{(\mathrm{D} - \mathrm{C})}{1 + \exp[b(\log(x) - \log(I_{50}))]} \qquad (3)$$

where D is the upper limit, C is the lower limit and b is the rate of change. The value at which 50% or 99% of the red clover plants were still viable was determined from the equation (I_{50} or I_{99}). Values at which 50% or 99% of red clover plants were still viable were compared within the NLMIXED procedure using a t-test.

Results

Shoot dry weight, leaf area and shoot relative water content (RWC)

After 4 days below 15% RSWC, WS significantly reduced shoot dry weight of both red clover varieties by 70.7% compared to CON treatments (Table 1). After a one-week recovery period, red clover varieties significantly increased shoot dry weight by 41.8% compared to WS treatments. Shoot dry weight in REC treatments remained significantly lower than in CON treatments (Table 1). Belle had 15% higher shoot dry weight than Altaswede, however, the interaction between red clover variety and watering regime was not significant for shoot dry weight (Table 2). For both RWC and leaf area, there was a significant interaction between variety and watering regime (Table 2). While no significant differences between Belle and Altaswede were observed within CON and REC treatments, Belle had a 70.0% higher RWC and 202% higher leaf area than Altaswede in WS treatments after 4 days below 15% RSWC (Table 1). Photographs of representative pots of Belle and Altaswede red clover in all three treatments at each of the four different time periods are shown in Fig. 2.

After 8 days below 15% RSWC, there was a significant interaction between red clover variety and watering regime for shoot dry weight (Table 2). While no significant differences between Belle and Altaswede were observed within CON and REC treatments, Belle had 59.8% greater shoot dry weight than Altaswede in WS treatments (Table 1). Leaf area and RWC in WS treatments was significantly reduced by 98.1% and 75.9%, respectively, compared to CON treatments. Leaf area and RWC in REC treatments were still significantly lower than in CON treatments, however, RWC in REC treatments increased by 258% compared to WS treatments (Table 1). There was no difference between red clover varieties and no significant interaction between watering regime and variety for leaf area and RWC (Table 2).

After 12 days below 15% RSWC, no plants were considered viable in WS treatments, as all leaf tissue was desiccated and no new growth was evident, and this treatment was removed from the analysis as there were zero values for all parameters (Table 2). After the one week recovery period, new growth was evident for both Belle and Altaswede (Fig. 2). There was a significant interaction between red clover variety and watering regime for shoot dry weight (Table 2). The interaction was due to the absence of significant differences between Belle and Altaswede - within REC treatments, whereas in CON treatments, Belle had 18.9% higher shoot dry weight than Altaswede (Table 1). There were no significant interactions between red clover variety and watering regime for leaf area and RWC (Table 2) and leaf area and RWC were significantly lower in REC compared to CON treatments (Table 1).

After 16 days below 15% RSWC, no plants were considered viable in WS and REC treatments, as all leaf tissue was desiccated and no new growth was evident, and both treatments were removed from the analysis as there were zero values for all parameters (Table 1). There were no significant differences between Belle and Altaswede varieties within CON treatments for shoot dry weight, RWC and leaf area (Table 2). Belle also used significantly more water per pot than Altaswede during the three-week dry down period as well as during the subsequent 4- and 8-day no-watering periods (Fig. 3). Water use is not presented for the 12- and 16-day no-watering periods as red clover plants were not considered viable in WS treatments after 12 days below 15% RSWC or in either WS and REC treatments after 16 days below 15% RSWC (Fig. 3).

Survival rates

The relationship between percentage survival and days below 15% RSWC is shown in Fig. 4. There was a significant difference between Belle and Altaswede for the I50 value in REC treatments, being 8.3 and 9.5 days below 15% RSWC, respectively, which means that Altaswede plants survived for a longer period below 15% RSWC than did Belle plants (Fig. 4).

Crown water content

The I50 values for crown water content were 57% and 49% for Belle and Altaswede, respectively, and were significantly different between the two varieties (Fig. 5). It appears that crown water content declined more quickly in Altaswede than in Belle (Fig. 5). The I99 was calculated as a measure of the threshold crown water content below which red clover plants cannot survive. The I99 values for crown water content were 44% and 46% for Belle and Altaswede, respectively, and were not significantly different between the two varieties (Fig. 5).

Table 1 The response of shoot dry weight, shoot relative water content (RWC) and leaf area in two red clover varieties. Treatments include a well-watered control (CON), water stress (WS) and water stress with a one week, well-watered, recovery period (REC). WS and REC treatments were subjected to different durations (4, 8, 12 and 16 days) of no-watering periods following a three-week dry down from 80% to 15% relative soil water content (RSWC) while CON treatments were watered daily to 80% RSWC

Treatment	Variety	Shoot dry weight (g pot^{-1})	RWC (%)	Leaf area (cm^2 pot^{-1})
4 days below 15% RSWC				
CON	Belle	8.14 ± 0.16 a	77.4 ± 0.8 a	1139.8 ± 94.1 a
	Altaswede	7.23 ± 0.16 a	75.4 ± 0.9 a	1115.7 ± 94.1 a
WS	Belle	2.66 ± 0.19 a	43.0 ± 1.0 a	226.6 ± 19.3 a
	Altaswede	1.85 ± 0.19 a	25.3 ± 0.9 b	75.1 ± 19.3 b
REC	Belle	3.25 ± 0.25 a	73.7 ± 0.9 a	565.6 ± 34.8 a
	Altaswede	3.13 ± 0.25 a	72.8 ± 0.9 a	568.6 ± 34.8 a
8 days below 15% RSWC				
CON	Belle	8.34 ± 0.28 a	76.2 ± 1.2 a	1206.4 ± 90.9 a
	Altaswede	8.71 ± 0.28 a	74.5 ± 1.2 a	1177.9 ± 90.9 a
WS	Belle	1.55 ± 0.08 a	19.0 ± 1.1 a	32.2 ± 13.6 a
	Altaswede	0.97 ± 0.08 b	17.3 ± 1.1 a	11.9 ± 13.6 a
REC	Belle	0.46 ± 0.19 a	63.7 ± 1.4 a	25.3 ± 17.9 a
	Altaswede	0.87 ± 0.19 a	66.4 ± 1.3 a	84.8 ± 17.9 a
12 days below 15% RSWC [a]				
CON	Belle	8.76 ± 0.30 a	76.8 ± 4.4 a	1270.0 ± 66.9 a
	Altaswede	7.37 ± 0.30 b	75.8 ± 4.5 a	1119.1 ± 66.9 a
WS	Belle	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Altaswede	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
REC	Belle	0.04 ± 0.03 a	58.5 ± 5.2 a	3.0 ± 1.8 a
	Altaswede	0.08 ± 0.03 a	63.3 ± 5.1 a	2.1 ± 1.8 a
16 days below 15% RSWC [a]				
CON	Belle	8.32 ± 0.45 a	75.5 ± 0.33 a	1183.6 ± 56.8 a
	Altaswede	8.01 ± 0.45 a	76.2 ± 0.33 a	1207.4 ± 56.8 a
WS	Belle	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Altaswede	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
REC	Belle	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Altaswede	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Means within columns and treatments followed by different letters are significantly different at $p < 0.05$ according to Tukey's HSD test

[a] Analysis excluded "WS" or "WS" and "REC" treatments as no plants were considered viable

Discussion

The study in this paper was conducted to determine whether there were genotypic differences in drought response between the double-cut variety, Belle, and single-cut variety, Altaswede. The primary difference between the two varieties is the double-cut or single-cut growth type. Growth habits between the two types that could be contributing to the observed differences between the two varieties in terms of drought response will be discussed in detail. The observed higher RWC and leaf area after 4 days below 15% RSWC and higher shoot dry weight after 8 days below RSWC of Belle compared to Altaswede suggests that Belle is more tolerant to drought. This classification is consistent with a recent study which classified red clover genotypes as more drought tolerant if they had a higher growth rate and leaf RWC after drydown from 55% to 14% soil moisture content (Yates et al. 2014). Alfalfa varieties were also considered more drought tolerant if they had

Table 2 Analysis of variance (ANOVA) for shoot dry weight, shoot relative water content (RWC) and leaf area in two red clover varieties. Treatments include a well-watered control (CON), water stress (WS) and water stress with a one week, well-watered, recovery period (REC). WS and REC treatments were subjected to different durations (4, 8, 12 and 16 days) of no-watering periods following a three-week dry down from 80% to 15% relative soil water content (RSWC) while CON treatments were watered daily to 80% RSWC

Source of Variation	d.f.	Shoot dry weight (g pot^{-1})	RWC (%)	Leaf area (cm^2 pot^{-1})
4 days below 15% RSWC				
Treatment	2	**	**	**
Variety	1	**	**	ns
Treatment x Variety	2	ns	**	*
Error	18			
Total	23			
8 days below 15% RSWC				
Treatment	2	**	**	**
Variety	1	ns	ns	ns
Treatment x Variety	2	**	ns	ns
Error	18			
Total	23			
12 days below 15% RSWC[a]				
Treatment	1	**	*	**
Variety	1	**	ns	ns
Treatment x Variety	1	**	ns	ns
Error	12			
Total	15			
16 days below 15% RSWC [b]				
Variety	1	ns	ns	ns
Error	6			
Total	7			

[*] indicates significant differences at $p < 0.05$, [**] indicates significant differences at $p < 0.01$

[a] Analysis excluded "water stress" treatment as no plants were considered viable

[b] Analysis excluded "water stress" and "water stress with recovery" treatments as no plants were considered viable

both a higher leaf RWC and shoot biomass than other cultivars during water deficit (Kang et al. 2011). Belle transpired more water than Altaswede during the three-week dry down period where soil dried from 80% to 15% RSWC and during the subsequent 4 and 8 day no watering periods (Fig. 3).The measured increase in water use of Belle, contrasted to Altaswede, is consistent with higher RWC values after 4 days below 15% RSWC (Table 1) and a visually lower degree of wilting (Fig. 2). Drought tolerance and a lower degree of wilting in more mature alfalfa genotypes was also associated with increased water use during severe soil moisture stress in a study by Annicchiarico et al. (2013). The increase in water use was attributed to either the conservation of water due to lower early root growth or the osmotic adjustment of root and crown tissue via an increased total concentration of water soluble carbohydrates (WSC) which allowed more water to be taken up during severe stress (Annicchiarico et al. 2013).

The observed wilting point of red clover plants, at which water use appears to plateau in Fig. 1, occurs at a surprisingly high matric potential (about −0.5 MPa) as calculated using soil water release curves developed for this soil mixture (H.J. Earl, unpublished data). The apparent discrepancy could simply be due to small inaccuracies in the estimation of RSWC as plant fresh weight could not be accounted for and since matric potential changes very rapidly over a narrow range of RSWC at these low RSWC values. Red clover biomass relative to soil water was small. On the final day of the

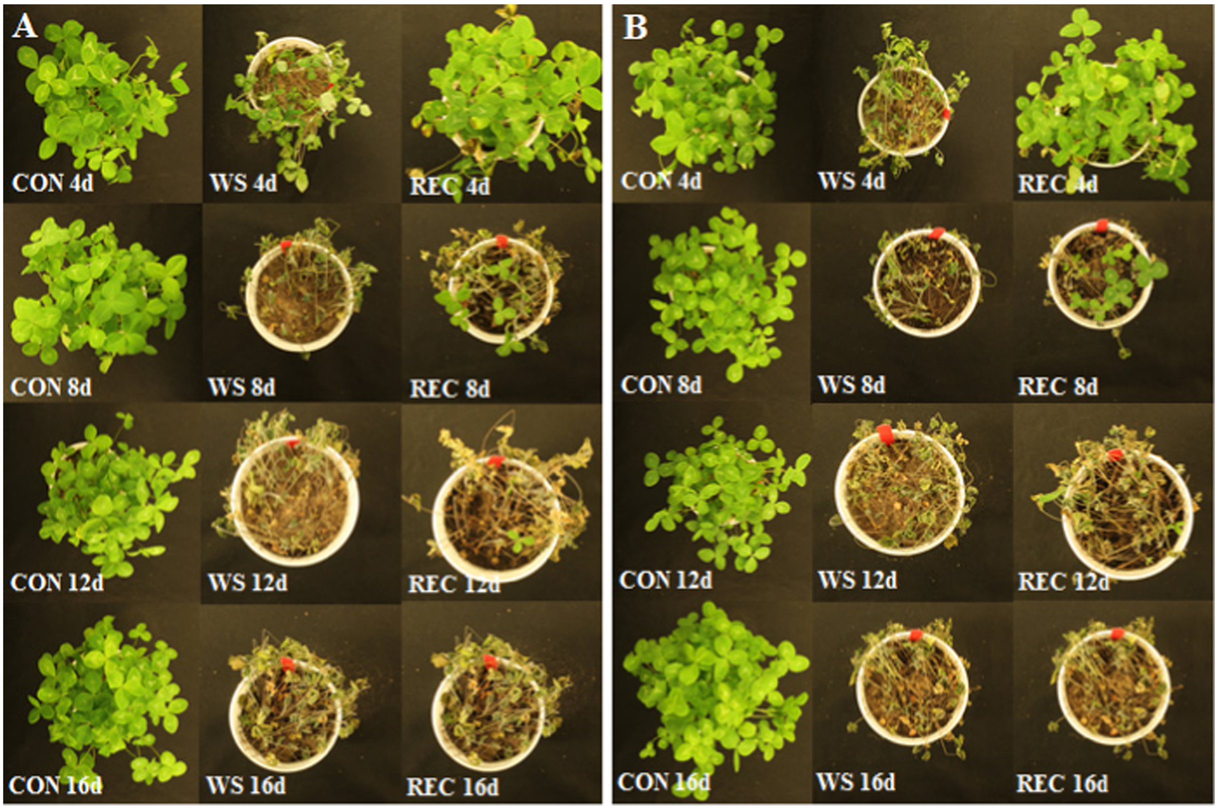

Fig. 2 Photographs of representative pots of Belle (**a**) and Altawede (**b**) red clover varieties in well-watered control (CON), water stress (WS) and water stress with recovery (REC) treatments pictured at 4 days (4d), 8 days (8d), 12 days (12d) and 16 days (16d) below 15% RSWC time periods

experiment when red clover biomass in control treatments was largest, red clover shoot biomass was an average of 5.4%, with a range of 4.8–6.0%, of the weight of the water in the pot at 100% RSWC. Therefore, including estimates of red clover fresh weight in the calculations changes RSWC only slightly, however, it has a large impact on calculated matric potential near the apparent wilting point due to the very non-linear relationship between matric potential and plant water use.

Despite Belle having higher drought tolerance based on the classification of "tolerance" described above, Altaswede had higher drought "survival" than Belle over the progressively longer no-watering periods based on the fact that 50% mortality of Altaswede plants occurred over 24 h later compared to Belle (Fig. 4). The two different drought strategies identified in this paper are therefore tolerance and survival. Altaswede used significantly less water than Belle during the three-week dry down period and subsequent 4 and 8 day no-watering periods (Fig. 3) and this observed lower water use could be due to lower leaf area and therefore less water lost through transpiration compared to Belle (Table 1). Lower water loss in Altaswede could be due to lower stomatal conductance or differences in water use efficiency which could be investigated further in future research. Reduced vegetative growth and/or leaf senescence has been associated with a strategy to withstand water deficit by conserving water through reducing overall transpiration and the remobilization of nutrients to other organs (Erice et al. 2010; Munne-Bosch and Alegre 2004). Limited growth and more rapid leaf senescence during water deficit has been observed for other mature legume stands as a response to drought, however, this type of response is often at the expense of yield (Beebe et al. 2008; Malinowski et al. 2007).

Belle and Altaswede appear to have different strategies, tolerance and survival, respectively, when faced with declining soil moisture. Belle focusses on the maintenance of shoot biomass and water content

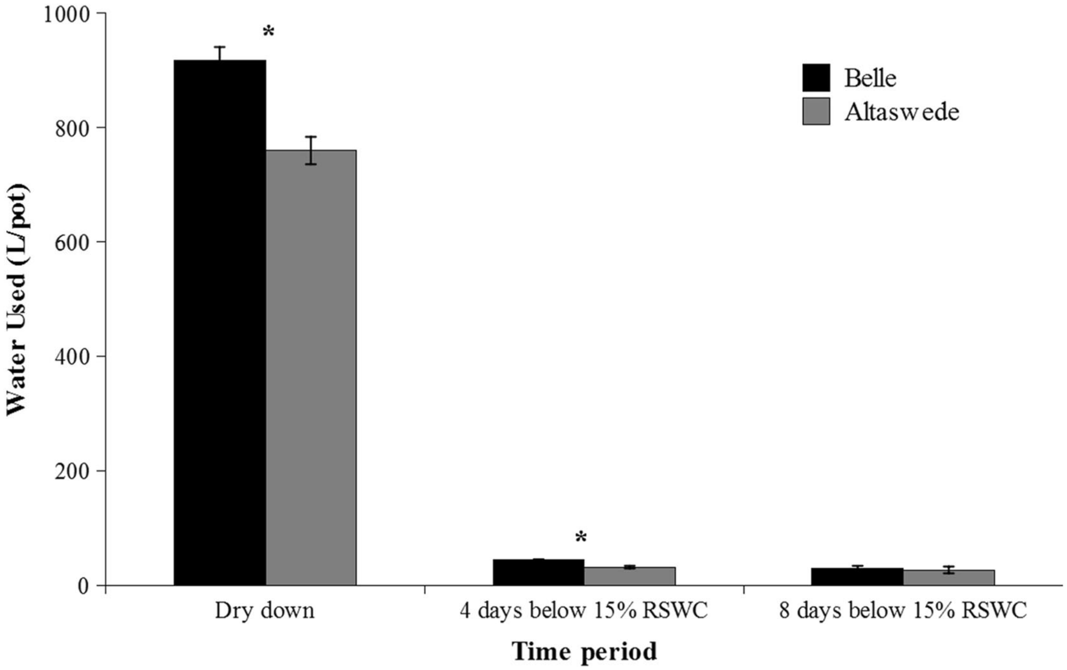

Fig. 3 Mean water use (± SE) of Belle and Altaswede red clover varieties at different time periods including the three-week dry down period where pots were uniformly dried from 80% to 15% RSWC (dry down, N = 32) as well as the subsequent 4 day no-watering period (4 days below 15% RSWC, $N = 32$) and 8-day no watering period (8 days below 15% RSWC, $N = 24$). Asterisks (*) indicate significant differences ($p < 0.05$) between the two varieties at each separate time period

while Altaswede focusses on survival. The observed differences in drought tolerance strategies could be partly due to the differences in growth habit between single- and double-cut red clover. Single-cut red clover populations are lower growing and they do not flower or make stems in the seeding year, instead maintaining low, vegetative growth in the fall, a characteristic which has been linked with winter hardiness and increased stand persistence (Choo et al. 1984; Christie and Choo 1991). A similar characteristic in alfalfa is termed fall dormancy and has not only been associated with greater winter hardiness (Brummer et al. 2000; Weishaar et al. 2005) but increased drought tolerance (Pembleton et al. 2010; Pembleton and Satish 2014). Studies have documented similarities in the response of plants to both drought and cold stress (Tommasini et al. 2008). It is possible that similar mechanisms that respond to decreasing temperature and day length could also respond to declining soil moisture thus facilitating greater survival during drought stress.

The maintenance of viable meristematic tissue in the crown could be the most important mechanism of plant survival both over winter and during periods of abiotic stress (Black et al. 2009; Li et al. 1996). After 12 days below 15% RSWC, all leaf tissue was desiccated and no new growth was evident for either variety of red clover in WS treatments, however, after the one week recovery period, new growth originating from crown tissue was observed for both Belle and Altaswede (Fig. 2). The observed new growth after leaf desiccation suggests that even if leaflet and petiole tissues are desiccated, red clover plants can still survive and re-grow after drought from meristematic tissue in the crown. It's possible that similar mechanisms occur that allow red clover to recover and re-grow after drought stress as those that occur during recovery and re-growth after winter.

After water deficit, crown survival could be largely dependent on crown water content. The crown water content at which 99% plant mortality occurred was 44% and 46%, respectively, for Belle and Altaswede, and was not significantly different between the two varieties (Fig. 5). There appears to be a threshold crown water content at which plant survival and recovery is not possible. Wissuwa et al. (1997) examined the drought response of first year alfalfa stands and estimated that alfalfa ecotypes had a similar threshold for crown survival of about 42% crown water content which is

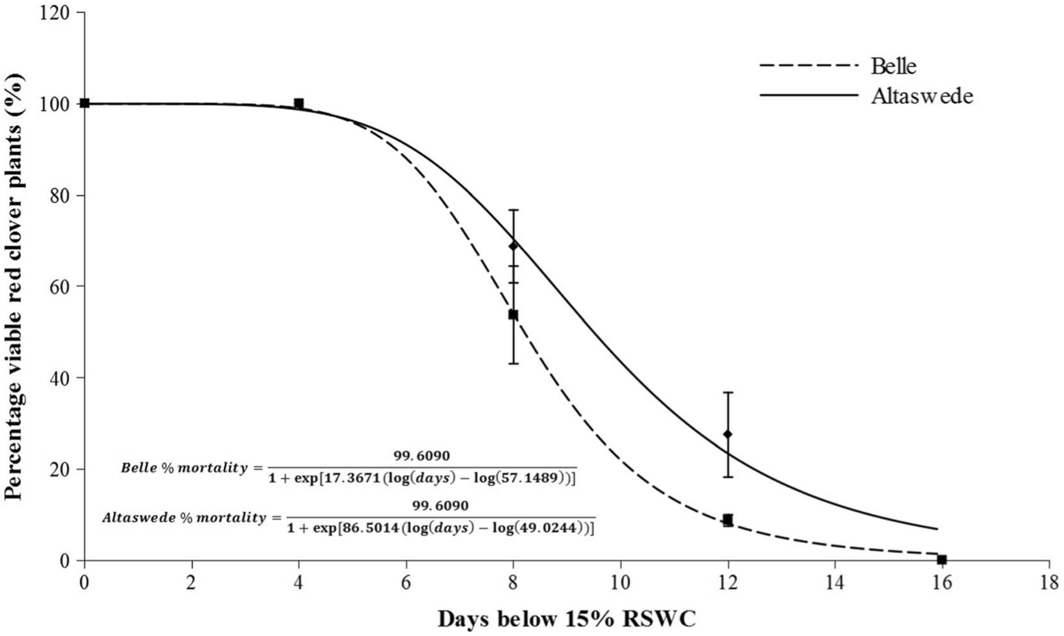

Fig. 4 Dose-response curves showing means (± SE) for survival rates of Belle and Altaswede red clover in pots following a one-week recovery period at 80% RSWC after four different no-watering periods (4,8,12 and 16 days) that directly followed a three week dry down period from 80% to 15% relative soil water content (RSWC). Percentage of viable red clover plants was calculated using the number of viable red clover plants counted after each one-week recovery period. After the pots had reached 15% RSWC, the number of subsequent days without water where 50% of red clover plants survived was called the "I50" value. The I50 values for Belle and Altaswede (± SE) are 8.2 ± 0.23 and 9.5 ± 0.32 days without water, respectively, which are significantly different ($p < 0.05$). $N = 4$

consistent with the results presented in this paper. Wissuwa et al. (1997) were also able to use this value for threshold crown water content to predict mortality in local commercial alfalfa fields the following year; at only one location out of five did they significantly over-estimate actual mortality. Interestingly, the crown water content at which 50% plant mortality occurs is significantly lower for Altaswede than for Belle (Fig. 5) suggesting that Altaswede plants can survive at lower crown water contents. Altaswede also decreased crown water content more quickly than Belle (Fig. 5). It is possible that Altaswede not only decreases growth but reduces physiological activity, perhaps entering into a state of quiescence where only a small amount of water is necessary for survival. Similarly, Volaire (2003) observed that seedlings of a drought resistant perennial cultivar of orchardgrass (*Dactylis glomerata*) survived severe drought stress at a rate 68% higher than cultivars of annual barley (*Hordeum vulgare*). While the water potential of the leaf lamina and the water content of immature tissue found at leaf bases (meristematic tissue) decreased earlier in orchardgrass, the latter was able to stabilize after leaf senescence resulting in higher rates of survival. Annicchiarico et al. (2013) found that a drought tolerant alfalfa variety accumulated high concentrations of water soluble carbohydrates in the root and crown during severe drought stress after defoliation. In this instance, Annicchiarico et al. (2013) suggested that crown reserves also played a role in the osmotic adjustment of crown and root tissue. Correspondingly, Erice et al. (2010) suggested that an observed increase in the root/shoot ratio of drought-stressed alfalfa plants could be a strategy to accumulate reserves for subsequent re-growth during recovery. Because the capacity to use reserves to re-grow after defoliation differs between legume species (Li et al. 1996; Smith 1962), there is room for future research to explore the mechanisms and implications of the accumulation and utilization of crown reserves to survive and re-grow after drought stress. If red clover plants can be selected, in breeding programs, for the ability to conserve crown tissue viability when low soil moisture is detected, greater red clover stand survival could result during years with drought stress.

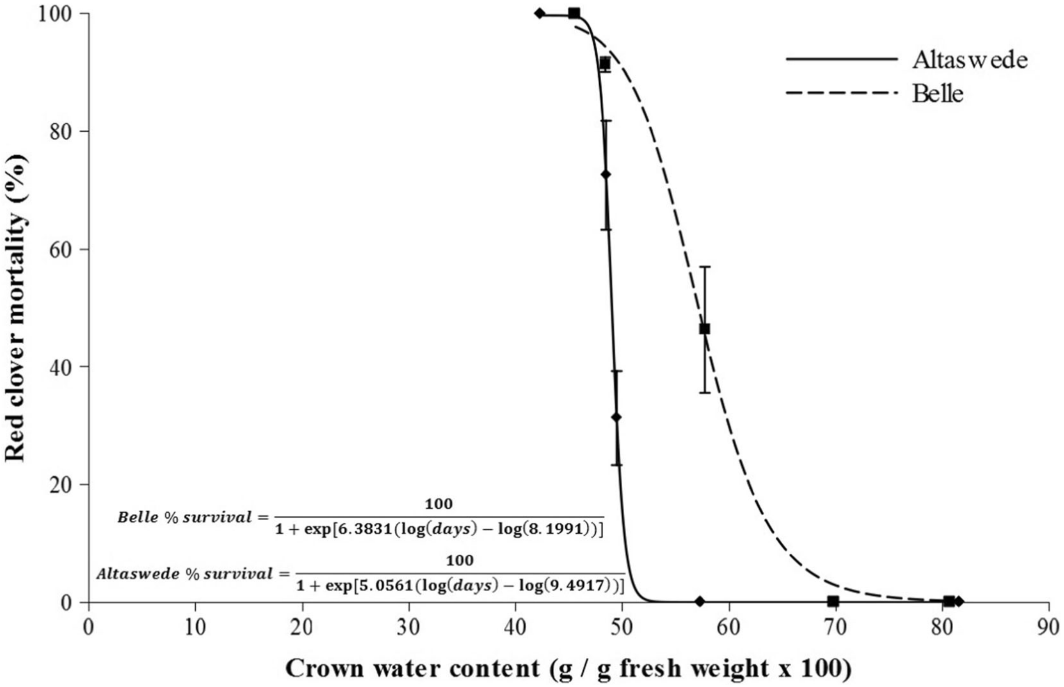

Fig. 5 Dose-response curves showing means (± SE) for Belle and Altaswede red clover plants at decreasing crown water content in pots following a one-week recovery at 80% RSWC period after four different no-watering periods (4,8,12 and 16 days) that directly followed a three-week dry down period from 80% to 15% relative soil water content (RSWC). Crown water content was measured in water stress treatments harvested directly after each no-watering period. Percentage mortality was calculated by counting the number of non-viable red clover plants after each one-week recovery period. The I50 and I99 values are the values of percent crown water content at which 50% and 99% of red clover plants were non-viable after the recovery period. The I50 values for Belle and Altaswede (± SE) are 57% ± 0.74 and 49% ± 0.10, respectively, which are significantly different ($p < 0.05$). The I99 values for Belle and Altaswede (± SE) are 44% ± 3.71 and 46% ± 0.47, respectively, which are not significantly different ($p > 0.05$), $N = 4$

Conclusion

There appear to be two different strategies employed by Belle and Altaswede when faced with declining soil moisture. The observed differences could be associated with differences in growth habit between single-cut and double-cut red clover populations. While Belle is better able to maintain shoot viability and water content during drought stress, Altaswede has higher survival rates. Greater survival of red clover plants under-seeded to winter wheat could be of a greater importance than maintenance of shoot biomass due to farmer concerns about stand non-uniformity. Greater survival during water stress could be due to lower, vegetative growth at the onset of drought and, subsequently, a greater focus on the maintenance of crown and root tissue. Red clover plants that accumulate crown and root reserves and enter a quiescent state in response to low soil moisture could have higher survival rates under winter wheat. Future research should further investigate differences in drought tolerance strategies between single- and double-cut red clover growth types and determine whether a reduction in physiological activity and an increase in crown and root reserves could be a major factor contributing to greater survival of Altaswede or other red clover genotypes. Focussing red clover breeding efforts on higher survival rates instead of biomass production could be important for more successful intercropping systems. Higher survival rates during growth with winter wheat could increase stand uniformity and allow growers to fully realize the benefits associated with uniform stands of red clover.

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