Screening for waterlogging tolerance in subterranean clover (*Trifolium subterraneum* L.)

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Abstract

Subterranean clover (subclover, *Trifolium subterraneum* L.), is the most widely sown annual pasture legume in southern Australia, but winter waterlogging can cause extensive biomass reductions. Determining an efficient and non-destructive screening method for waterlogging tolerance is crucial for subclover breeding programs to identify parents and progeny with tolerance. Tolerance to short (21 days) and extended (35 days) periods of waterlogging, relative to non-waterlogged conditions, was assessed among nine subclover cultivars across the three subclover subspecies (*subterraneum*, *yanninicum* and *brachycalycinum*) in a controlled environment glasshouse. Treatments, imposed after 28 days growth, comprised a free-draining control and waterlogging. A range of measurements was compared with shoot dry weight and shoot relative growth rate. Easily-assessed indicators of short-term waterlogging tolerance were less effect on petiole length, high leaf chlorophyll content and low petiole anthocyanin, while extended waterlogging tolerance was indicated by lower reduction of leaf size and high stomatal conductance.

Keywords

Pasture legumes, leaf size, petiole length, stomatal conductance, anthocyanin pigmentation.

Introduction

Soil waterlogging generates severe hypoxia and subsequent anoxia in the root zone with adverse effects on plant growth and functioning that may lead to premature death of sensitive species (Armstrong 1980). Areas subjected to waterlogging have been estimated as 1-2 million ha in Western Australia (WA) and ~3.8 million ha in Victoria (Setter and Waters 2003), while significant areas in other states are also affected. Annual plants in Mediterranean regions can experience waterlogging from excessive rainfall during the winter-spring growing season and from increasingly intensive and erratic rainfall as a result of climate change (Chapman et al. 2012).

Subterranean clover (subclover, *Trifolium subterraneum* L.) is the most successful annual pasture legume in southern Australia (Ghamkhar et al. 2015) and has a large genetic resource of over 8800 genotypes (Nichols et al. 2013). Therefore, broad evaluation of genetic variation of subclover genotypes in response to waterlogging stress is crucial to enable breeding cultivars that are more tolerant. In this study we aimed to determine an efficient and non-destructive screening tool for waterlogging based on simple selection criteria convenient for either glasshouse or field studies.

Methods

Nine cultivars representing the three subclover subspecies were used: ssp. *subterraneum* -Mt. Barker (B), Denmark (D), and Seaton Park (SP); ssp. *brachycalycinum* -Clare (C), Antas (A) and Rosedale (R); and ssp. *yanninicum* -Trikkala (T), Yarloop (Y) and Meteora (M). Cultivars were selected based on: previous published studies on waterlogging tolerance (Rogers and West 1993; Francis and Devitt 1969; Reed et al. 1985; Gibberd et al. 2001); diversity in flowering time (Nichols et al. 2013), and widespread use in sown pastures.

The experimental design was a randomised block with four replicates. Seeds were scarified and germinated in Petri dishes (38 h at 15 °C) on 12 August, 2019. Germinated seedlings were transplanted into plastic pots (200 mm diameter) filled with 5.3 kg of soil (1:1 river sand and loam; pH 6.4) in a controlled temperature glasshouse (20/15 °C) and *Rhizobium leguminosarum* bv. *trifolii* strain WSM1325 was applied by watering can. The water content (w w^{-1}) at field capacity (FC) was 13.7 %. Each pot (three plants) was an experimental unit. The treatments were: (i) a free-drained control (watered to 80 % FC); and (ii) waterlogged (water maintained 10 mm above the soil surface from 28 days after sowing). Three harvests were conducted: (i) an initial harvest (at the commencement of the waterlogging treatment); (ii) Harvest I - 21 days after treatment (DAT); and (iii) Harvest II - 35 DAT.

For each harvest, plants were removed from the soil by washing on the sieve and oven-dried for 72 h at 60 °C for shoot dry weights (DW). Shoot relative growth rate (RGR) was calculated using the standard equation of Hunt (1982). Traits and their measurement techniques were: leaf size (third trifoliate leaf), by comparison with the photographic plates and formulae of Williams et al. (1964); petiole length of the same leaves, by a ruler; stomatal conductance (adaxial), by a leaf porometer (ICT International Model: SC-1); chlorophyll content of the youngest fully-opened leaves, by a Konica Minolta SPAD-502 Plus; and petiole anthocyanin pigmentation, by using 1-4 scores for increasing expression.

Data are graphed as mean ± standard error (SE) using SigmaPlot 14 (Systat Software, Inc.) and were analysed in *R* software (version 3.6.3). The effects of cultivars, treatments and their interactions on response variables were assessed using two-way ANOVA. Pearson correlation coefficients and their significance levels were calculated between shoot DW/RGR and measured traits.

Results

Both shoot DW and RGR had significant treatment × cultivar interactions at Harvests I (21 DAT) and II (35 DAT) (both $P<0.001$; Fig.1). Waterlogging had a severe effect on shoot DW of all subclover cultivars at both Harvest I and Harvest II (30-70 % and 21-63 % of controls, respectively). All traits had a significant treatment effect (Table 1).

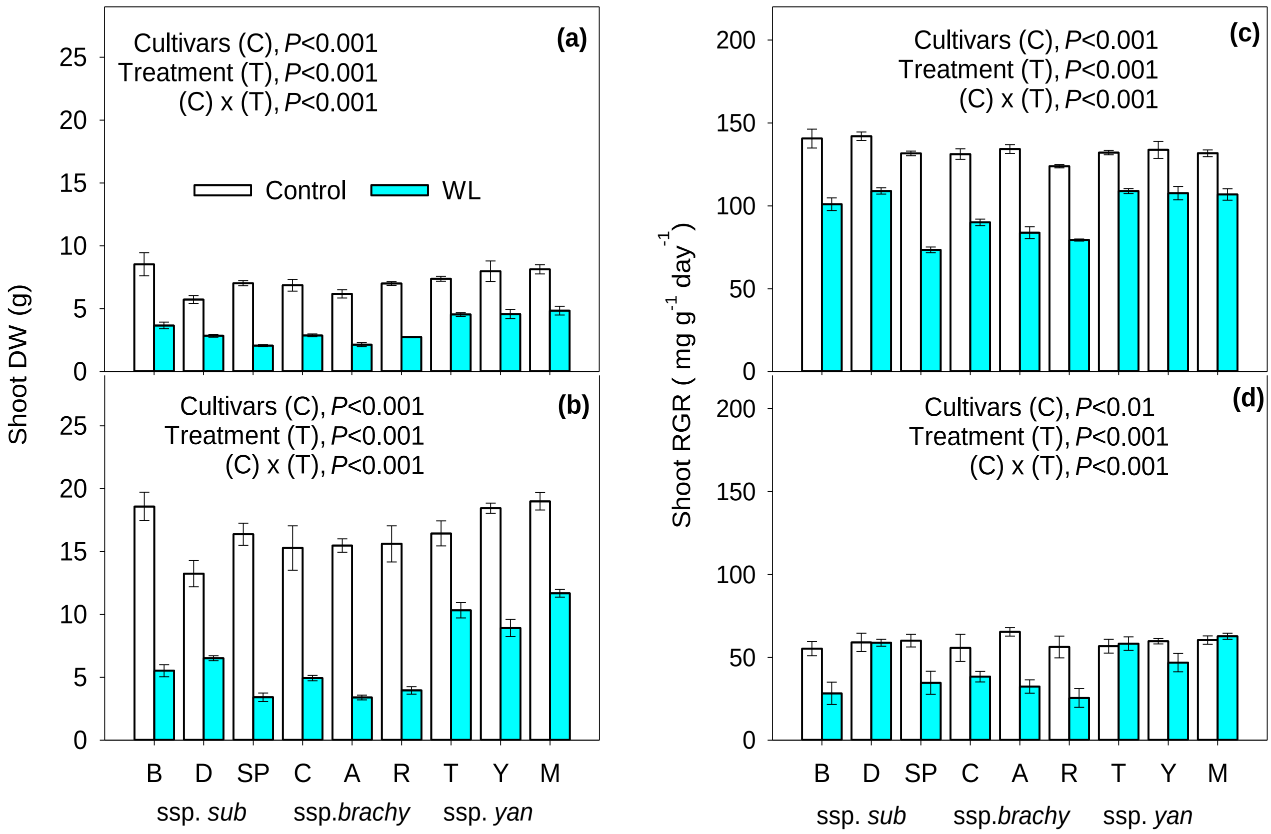

Fig. 1. Shoot dry weight (DW) (a, b) and relative growth rate (RGR) (c, d) for nine cultivars from three subspecies of subclover at 21 days (a, c) and 35 days (b, d) after treatment (mean ± SE, n=4). Treatments were imposed after 28 days of growth: control (free-draining) and waterlogged (WL). Results of two-way ANOVA are given in each panel. Subspecies: ssp. *subterraneum* (*sub*); ssp. *brachycalycinum* (*brachy*); and ssp. *yanninicum* (*yan*). Cultivars: Mt. Barker (B); Denmark (D); Seaton Park (SP); Clare (C); Antas (A); Rosedale (R); Trikkala (T); Yarloop (Y); and Meteora (M).

Relationships between shoot growth (shoot DW and RGR) and measured traits were analysed to explore their potential for contributing to waterlogging tolerance (Table 2).

At Harvest I, there were high positive correlations between shoot DW and petiole length, and between shoot RGR and leaf chlorophyll content, while petiole anthocyanin pigmentation exhibited highly negative correlation with shoot RGR (Table 2). At Harvest II, leaf size had strong positive correlations with both shoot DW and RGR, while stomatal conductance had high positive correlation with shoot RGR (Table 2).

Table 1. Mean squares and significance level of two-way ANOVA for nine subclover cultivars for responses of traits measured at Harvest I (21 days after treatment, DAT) and Harvest II (35 DAT) (mean ± SE, n=4). Treatments were imposed after 28 days of growth: control (free-draining) and waterlogged.

Traits	Harvest I (21 DAT)			Harvest II (35 DAT)		
	Cultivar (C)	Treatment (T)	C × T	Cultivar (C)	Treatment (T)	C × T
Shoot DW	7***	265***	1***	38***	1,791***	15***
Shoot RGR	610***	25,876***	294***	443***	4,520***	404***
Leaf size	20***	521***	20***	46***	1,253***	22***
Petiole length	33***	804***	19***	38***	2,075***	11**
Stomatal conductance	13,919	1,294,360***	12,019	14,860***	3,528,053***	24,144**
Anthocyanin pigmentation	1***	27***	1***	2***	17***	2***
Chlorophyll content	278***	1,766***	46 n.s.	156***	276*	176***

n.s., *, P<0.05; **, P<0.01; ***, P<0.001.

Table 2. Pearson correlation coefficients (r) and significance for traits with shoot dry weight (DW) and shoot relative growth rate (RGR) of nine subclover cultivars grown under waterlogged conditions at Harvest I and II. The waterlogging treatment was imposed after 28 days of growth.

Traits	Harvest I (21 DAT)		Harvest II (35 DAT)	
	Shoot DW	Shoot RGR	Shoot DW	Shoot RGR
Leaf size	0.79*	0.77*	0.95***	0.82**
Petiole length	0.90**	n.s.	0.69*	n.s.
Stomatal conductance	n.s.	n.s.	n.s.	0.89**
Anthocyanin pigmentation	n.s.	-0.84**	-0.72*	-0.68*
Chlorophyll content	0.67*	0.81**	n.s.	n.s.

n.s., P<0.05; P<0.01; P<0.001.

Conclusion

The most accurate method to assess waterlogging tolerance is shoot biomass (shoot DW/RGR) relative to control plants grown in free-draining conditions. However, for screening purposes, biomass analysis is destructive, as it does not allow plants to continue growth for future measurements, and is time-consuming and labour-intensive. Alternatively, sensitive indicators for waterlogging stress, such as leaf size, petiole length, stomatal conductance, leaf chlorophyll content and petiole anthocyanin pigmentation are relatively simple, inexpensive and do not damage the plants. These are readily measurable screening traits that would be a useful suite for selection tools to screen a large number of subclover genotypes in plant breeding programs and could be combined with visual biomass ratings, particularly under field conditions. However, a limitation for stomatal conductance could be the time required, and the rating scales for anthocyanin pigmentation may lack precision, due to uneven ratings between assessors. Analyses of root traits is in progress (Enkhbat et al. 2021) and will provide a greater understanding of waterlogging tolerance mechanisms. However, such traits are unlikely to be suited to wide-scale screening programs in subclover.

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