Variation in waterlogging response among ecotypes of *Trifolium subterraneum* ssp. *yanninicum* and their relationships with site of origin

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Research Article

Keywords: pasture legumes, genetic resources, seed size, cotyledon, eco-geographic variables, flowering time

Posted Date: September 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-896192/v1>

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Abstract

Background and Aims

In the annual pasture legume *Trifolium subterraneum*, ssp. *yanninicum* exhibits higher waterlogging tolerance than ssp. *brachycalycinum* and ssp. *subterraneum*. This study investigates waterlogging tolerance within ssp. *yanninicum* ecotypes and explores correlations with seedling phenotypic traits and site of origin eco-geographic variables.

Methods

Twenty eight diverse ssp. *yanninicum* ecotypes collected from the Mediterranean region and four cultivars were grown in a controlled environment glasshouse. After 14 days of growth seedling traits were measured. After 21 days of growth, free-drained (control) and waterlogged treatments were imposed for 28 days. Eco-geographic variables were generated from 'WorldClim' using collection site locations.

Results

Under waterlogging, shoot relative growth rate (RGR) ranged from 87–108% and root RGR ranged from 80–116% of controls. Waterlogging reduced shoot dry weight (DW) in four of 32 genotypes, while root DW was reduced in 13 genotypes. Leaf size was maintained, or even increased, under waterlogging in 31 genotypes. However, petiole length was more affected by waterlogging and has value as a waterlogging tolerance indicator. Waterlogging tolerance was not significantly correlated with seedling DW, flowering time or precipitation at the site of origin, while shoot growth under waterlogging had a positive correlation with summer temperatures at origin.

Conclusions

Genotypes of ssp. *yanninicum* tolerated transient waterlogging and greater tolerance was observed among ecotypes, rather than cultivars. An easy-to-measure indicator of tolerance was found in petiole length reduction. This study highlights untapped genotypic variability for breeders to improve the productivity and persistence of ssp. *yanninicum* under waterlogging.

Introduction

Soil waterlogging, a widespread abiotic stress worldwide, reduces crop production by 20–50% or more, depending on plant growth stage and duration and intensity of waterlogging (Bertholdsson 2013; Solaiman et al. 2007). Although Australia is one of the drier continents, soil subject to transient waterlogging covers a large area of farmland in southern Australia; estimated to be as much as 1 to 2 million ha in Western Australia (WA), and around 3.8 million ha in Victoria, as well as significant areas in other states (Malik et al. 2002; Setter and Waters 2003). The annual cost in terms of crop production lost in Australia has been estimated as AU\$90 million (Hamilton et al. 2000). While rainfall has decreased for the cropping regions of southern Australia over the past few decades and annual rainfall is projected to further decline, the intensity and frequency of heavy rainfall events are projected to increase with climate change (Chapman et al. 2012; Jarvis et al. 2010).

Annual plants in Mediterranean regions can experience waterlogging from excessive rainfall during the growing season (Real et al. 2008). In Australia, transient waterlogging occurs in poorly drained duplex soils (i.e. texture-contrast soils where a loamy clay dominates in the B horizon) which occupy 40 and 60% of agricultural land area in Victoria and WA, respectively (Tennant et al. 1992).

Soil waterlogging, also known soil flooding, occurs in the root-zone and is defined as the excess water saturation of soil pores affecting oxygen concentration and leading to hypoxia (low O₂ concentration) and eventually to anoxia (absence of O₂) (Armstrong 1980). Under waterlogging, the movement of gases, such as O₂ and CO₂, through water-filled soil pores and within roots into cells of plant organs dramatically decreases (gas diffusion is 10 000 times slower in water than in air) (Bailey-Serres and Voesenek 2008; Colmer 2003; Voesenek and Sasidharan 2013). Oxygen deficiency inhibits aerobic respiration, resulting in a shortage of carbohydrates and energy that leads to an 'energy crisis' and disrupts photosynthesis and the uptake of nutrients from soil (Colmer and Greenway 2011; Pang et al. 2004). In addition, waterlogging reduces the availability in soil of some essential nutrients (e.g. N), but other ions such as manganese (Mn²⁺), iron (Fe²⁺), sulphur (S²⁻) and carboxylic acids can accumulate to toxic levels due to microbial anaerobic metabolism (Ponnamperuma 1984). Thus, soil waterlogging adversely affects plant growth and function and damage symptoms such as wilting, premature leaf senescence, growth cessation and premature death can result (Marschner 1995; Stoddard et al. 2006).

Subterranean clover (*Trifolium subterraneum* L., commonly known as subclover), originates from the Mediterranean region and Western Europe (Nichols et al. 1996) and is the most widespread pasture legume sown in southern Australia; a region with a Mediterranean-type climate and soils inherently low in nitrogen (N) (Nichols et al. 2013). Subclover has been sown over 29 million ha in southern Australia. Subclover has three subspecies used in agriculture in Australia: ssp. *subterraneum*, ssp. *brachycalycinum*, and ssp. *yanninicum*, each adapted to different soil types (Katznelson and Morley 1965).

Subspecies *yanninicum* was first commercialised for its high productivity on soils prone to flooding, particularly those with a high clay content (Francis and Devitt 1969; Reed et al. 1985). It is relatively infrequent in the wild, most often distributed in high rainfall coastal areas with mild winter temperatures (Ghamkhar et al. 2015). Among the Australian genetic resource collection of 8,812 subclover genotypes from 2,993 sites, ssp. *yanninicum* comprises only 2.2% of all genotypes (Nichols et al. 2013). Cultivars of ssp. *yanninicum* are important commercially and they are mostly grown in areas with high-medium annual rainfall (450–1200 mm), particularly on soils prone to waterlogging (Nichols et al. 2013). However, current cultivars of ssp. *yanninicum* were developed from a narrow genetic base (Nichols et al. 2013). There is, therefore, a need to explore the diversity among ecotypes and to identify traits for use in future breeding of ssp. *yanninicum* to improve productivity and resilience to waterlogging and broaden the genetic base of cultivars.

Several studies have examined waterlogging tolerance among these three subspecies of subclover and found ssp. *yanninicum* to generally exhibit a higher tolerance to waterlogging. Evidence for this comes from agronomic trials (Craig 1992; Peak and Morley 1973; Reed et al. 1985; Sandral et al. 2003), ecological surveys (Cocks 1994; Francis 1976; Katznelson and Morley 1965; Piano 1984) and glasshouse studies (Devitt and Francis 1972; Enkhbat et al. 2021b; Francis and Devitt 1969; Francis et al. 1974; Gibberd and Cocks 1997; Marshall and Millington 1967). However, genotypic variation in waterlogging tolerance among ecotypes of ssp. *yanninicum* has not been previously examined, nor its relationship with site of origin.

Exploration of waterlogging tolerance within species can yield significant benefit if tolerant genotypes are identified (Bailey-Serres and Voesenek 2008; Malik et al. 2015; Rogers et al. 2011). For example, Rogers et al. (2011) examined diversity in waterlogging tolerance among 23 genotypes of messina (*Melilotus siculus* (Turra) Vitman ex B.D. Jacks.), an annual forage legume with high tolerance to waterlogging, and identified several genotypes with greater shoot growth in a deoxygenated, stagnant treatment than in the aerated control. Similarly, Justin and Armstrong (1987) found that shoot growth in the majority of 91 wetland species was unaffected by, or increased under, waterlogging. Gibberd et al. (2001) explored waterlogging tolerance among 20 *Trifolium* species/subspecies and found nine genotypes without reduction in shoot growth under waterlogging, and two genotypes with increased growth, compared to drained controls. A review of shoot growth in wheat (*Triticum aestivum* L.) during waterlogging found that tolerant varieties consistently showed about twice the shoot growth of intolerant varieties (Setter and Waters 2003). Waterlogging tolerance has been found to vary among genotypes of many other crop and pasture legumes, including: barley (*Hordeum vulgare* L.) (Setter et al. 1999), chickpea (*Cicer arietinum* L.) (Palta et al. 2010), faba bean (*Vicia faba* L.) (Solaiman et al. 2007), lentil (*Lens culinaris* Medic.) and pea (*Pisum sativum* L.) (Malik et al. 2015), narrow leaf lupin (*Lupinus angustifolius* L.) (Davies et al. 2000) and lotus species (*Lotus* sp.) (Real et al. 2008).

Some phenotypic characteristics have a demonstrated relationship to waterlogging tolerance (Setter and Waters 2003). Under field conditions in a Mediterranean-type climate, where waterlogging can be experienced in late autumn/early winter through to early spring, long season wheat cultivars show a high yield advantage over short season cultivars (Gardner and Flood 1993; McDonald and Gardner 1987). Large-seeded pea and lentil genotypes demonstrated higher waterlogging tolerance than small-seeded genotypes, due to greater carbohydrate reserves and high early seedling vigour (Malik et al. 2015). Kabuli-chickpea, with larger seed and more vigorous early growth than desi chickpea, is better adapted to transient waterlogging (Palta et al. 2010; Siddique et al. 2000). Sultana et al. (2013) found large- (100 seed weigh ≥ 10 g) and dark-seeded, long season genotypes of pigeon pea (*Cajanus cajan* L.) had greater waterlogging tolerance at the seedling stage than light-coloured, small-seeded (100 seed weigh ≤ 10 g), short season genotypes. The advantage of the long season genotype reflects capacity for early sowing and establishment enabling them to avoid waterlogging during the intolerant stages of germination and emergence, as well as their flowering time being late enough to avoid spring waterlogging damage (Gardner and Flood 1993; McDonald and Gardner 1987).

The most common approach to define waterlogging tolerance is to assess factors related to the maintenance of growth rates and grain yield (e.g. shoot and root biomass, petiole and leaf growth) under waterlogging, relative to non-waterlogged conditions (Setter and Waters 2003). Morphological responses, such as shoot and root biomass production, formation of lateral roots and altered root distribution, are also crucial determinants of waterlogging tolerance. Root functioning and development in waterlogged soil is important to maintain stable plant growth (Yamauchi et al. 2018). For instance, an increase in the number of newly-formed adventitious roots (emerging above and along the soil surface during waterlogging) provides an internal path for oxygen movement (Yamauchi et al. 2018). Furthermore, waterlogging results in chlorophyll degradation in leaves due to nitrogen inhibition (Malik et al. 2015) and reduced uptake of nutrients (e.g. K^+) into tissues (Colmer and Greenway 2011). Hence, examining waterlogging tolerance by assessing biomass production and chlorophyll content are recognized methodologies to identify genotypes with a higher tolerance to, and better performance under, waterlogged conditions.

Ecotypes from different geographical locations are a primary source of genetic diversity (Abdi et al. 2020; Ghamkhar et al. 2007). Measurements of their phenotypic traits provides an understanding of their adaptation, distribution and production potential (Hill 1996) and their prospective use in plant breeding and selection programs (Erskine et al. 1989; Piano et al. 1996). Enkhbat et al. (2021a) found large variation in morphological traits and flowering time corresponding to eco-geographic variables among 90 ecotypes of ssp. *yanninicum* in a common garden environment. Use of ssp. *yanninicum* from a diversity of origins, combined with the exploration of associations of stress tolerance with eco-geographic variables from their collection site, is an efficient approach to identify superior waterlogging tolerance. The overall objective of this study was, therefore, to investigate diversity for waterlogging tolerance in ssp. *yanninicum*. We tested three main hypotheses: (i) variation for waterlogging tolerance exists within ssp. *yanninicum*; (ii) such variability is related to phenotypic and growth trait differences; and (iii) ecotypic variability for waterlogging tolerance is related to eco-geographic variables at their sites of origin.

Materials And Methods

Plant materials

Seeds of 32 genotypes of ssp. *yanninicum* were obtained from the Australian Pastures Genebank (APG) in 2019 and grown at the University of Western Australia Shenton Park Field Station to obtain fresh seed and for confirmation of subspecies identity (Enkhbat et al. 2021a). Genotypes of ssp. *yanninicum* consisted of: (i) 28 ecotypes (Treatment names: A-AB) collected from the wild in the Mediterranean basin, selected on the basis of diversity of geographic coordinates (longitude, latitude and altitude; referred to as passport data) and flowering time, as determined by (Enkhbat et al. 2021a); and (ii) four cultivars: Yarloop, Larisa, Meteora and Trikkala (Table 1). Included among these were four ecotypes (Treatments E, G, H and Q) with black-coloured seeds (as opposed to the usual amber/cream colour) identified by Enkhbat et al. (2021a). Cultivars Meteora and Larisa are ecotypes collected with passport data from Greece. However, cv. Yarloop is a naturalized strain from WA (Nichols et al. 1996; 2013; Oram 1989) and cv. Trikkala is derived from cross breeding and so neither have passport data (Nichols et al. 2013; PBR 2021). The geographic origins of the ecotypes is shown in Supplementary data Fig. S1. Additionally, the waterlogging-susceptible cultivars Antas (ssp. *brachycalycinum*) and Seaton Park (ssp. *subterraneum*) (Enkhbat et al. 2021b) and the waterlogging-tolerant *T. michelianum* cv. Frontier (Reed et al. 1985; Real et al. 2008; Rogers et al. 2009, 2011; Teakle et al. 2012; Striker et al. 2015; Kidd et al. 2020) were included as benchmarks for

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Table 1
List of 35 genotypes used in this study.

List of 55 genotypes used in this study.							
Genotype	Treatment	Classification	Seed colour	Country	Passport data		
	Name				of origin	Latitude	Longitude
ssp. <i>yanninicum</i>					(°N)	(°E)	(m a.s.l.)
Yarloop		Cultivar	Amber	Australia			
Larisa		Cultivar	Amber	Greece	39.58	21.99	90
Meteora		Cultivar	Amber	Greece	39.58	21.96	100
Trikkala		Cultivar	Amber				
CIZ001YANN-A	A	Ecotype	Amber	Turkey	38.64	27.28	25
CIZ011YANN-B	B	Ecotype	Amber	Turkey	38.65	27.29	20
CPI 039314YB	C	Ecotype	Amber	Greece	39.49	22.14	92
CPI 039315YC	D	Ecotype	Amber	Greece	39.49	22.11	89
CPI 056904C	E	Ecotype	Black	Tunisia	37.03	9.06	157
CPI 083937D	F	Ecotype	Amber	Greece	38.98	21.27	725
CPI 083957Q	G	Ecotype	Black	Greece	39.62	20.89	470
CPI 083967L	H	Ecotype	Black	Greece	39.72	20.78	475
CPI 089797	I	Ecotype	Amber	Italy	40.49	8.51	310
CPI 089804E	J	Ecotype	Amber	Italy	40.63	8.87	220
CPI 103925A	K	Ecotype	Amber	Spain	37.4	-6.22	50
CPI 103933	L	Ecotype	Amber	Spain	37.63	-6.65	360
DGG041-B	M	Ecotype	Amber	Greece	39.68	19.8	90
DGG044WHITE-B	N	Ecotype	Amber	Greece	39.65	19.82	80
DGG046-A	O	Ecotype	Amber	Greece	39.67	19.78	75
DGG047-C	P	Ecotype	Amber	Greece	39.67	19.78	75
DGG048-C	Q	Ecotype	Black	Greece	39.66	19.75	130
EP042WHITE-C	R	Ecotype	Amber	Italy	40.21	8.79	370
EP053WHITE-C	S	Ecotype	Amber	Italy	40.29	8.78	660
EP055WHITE-C	T	Ecotype	Amber	Italy	40.44	8.71	420
EP059WHITE-C	U	Ecotype	Amber	Italy	40.46	8.73	425
EP060WHITE-A	V	Ecotype	Amber	Italy	40.45	8.73	415
EP079WHITE-C	W	Ecotype	Amber	Italy	40.91	9.45	38
EP082WHITE	X	Ecotype	Amber	Italy	40.9	9.49	25
EP084WHITE	Y	Ecotype	Amber	Italy	40.81	9.66	20
EP091WHITE-B	Z	Ecotype	Amber	Italy	40.52	9.62	75
LO0460	AA	Ecotype	Amber	Spain	38.85	-5.6	398
LO0751	AB	Ecotype	Amber	Spain	39.1	-5.33	370
ssp. <i>brachycalycinum</i>							
Antas		Cultivar					
ssp. <i>subterraneum</i>							
Seaton Park		Cultivar					
<i>T. michelianum</i>							
Frontier		Cultivar					
Ecotypes collected from the wild are shown with their country of origin and 'passport data' associated with their sites of collection							

Climatic data

Climatic variables for collection sites of the ecotypes were extracted from the ‘WorldClim’ (Version 2) data base at 2.5 arc-minutes spatial resolution (Booth 2018; Ghamkhar et al. 2015; Hijmans et al. 2005; Trevor et al. 2014) and used to map a spatial model in the *R* software package, as described in Enkhbat et al. (2021b). The 19 imported bioclimatic variables for all 30 sites are shown in Supplementary data Table S1, while a summary of the eco-geographic variables for 29 ecotypes is provided in Table 2. Ecotype E was the only ecotype collected from Tunisia (Supplementary data Fig. S1), a site with exceptionally high annual and winter precipitation (Table 1, Supplementary data Table S1), which greatly affected trait correlations with climatic variables and was, therefore, excluded from these analyses.

Table 2
Codes and definition of bioclimatic (BIOCLIM) variables and summary of eco-geographic (passport data and BIOCLIM) variables for 29 ssp. *yanninicum* ecotypes.

Definition of eco-geographic variables	Code	Summary of variables		
		Min	Mean	Max
Latitude (°N)		37.4	39.7	40.9
Longitude (°E)		-6.7	13.1	27.3
Altitude (m)		20.0	230.8	725.0
Annual mean temperature (°C)	BIO1	11.9	15.2	18.0
Mean diurnal range (°C, mean of monthly max-min temp)	BIO2	7.2	9.6	12.1
Isothermality [(mdr/amt) × 100]	BIO3	3.2	3.5	3.8
Temperature seasonality (standard deviation × 100)	BIO4	516.9	604.3	736.1
Maximum temperature of the warmest month (°C)	BIO5	27.3	30.5	35.3
Minimum temperature of the coldest month (°C)	BIO6	-0.8	3.6	5.8
Temperature annual range (°C)	BIO7	21.8	27.0	33.7
Mean temperature of the wettest quarter (°C)	BIO8	5.2	10.1	13.2
Mean temperature of the driest quarter (°C)	BIO9	20.2	23.1	25.6
Mean temperature of the warmest quarter (°C)	BIO10	20.2	23.2	25.8
Mean temperature of the coldest quarter (°C)	BIO11	3.9	7.9	10.7
Annual precipitation (mm)	BIO12	470.0	794.8	1182.0
Precipitation of the wettest month (mm)	BIO13	61.0	126.4	198.0
Precipitation of the driest month (mm)	BIO14	1.0	9.8	29.0
Precipitation seasonality (coefficient of variation)	BIO15	41.0	56.5	76.0
Precipitation of the wettest quarter (mm)	BIO16	164.0	342.7	554.0
Precipitation of the driest quarter (mm)	BIO17	18.0	50.3	100.0
Precipitation of the warmest quarter (mm)	BIO18	24.0	56.7	100.0
Precipitation of the coldest quarter (mm)	BIO19	155.0	316.6	496.0

Plant growth

The experiment was conducted in a naturally-lit glasshouse set at 20/15 °C day/night at The University of Western Australia (31° 98'S, 115° 50'E) from June to August 2020. Plants were grown in sterilised, free-draining plastic pots (2.8 L) containing 3.2 kg of steam-pasteurised soil which had been dried at 40 °C. Weedstop® matting (Gale Pacific Limited) was placed in the bottom of each pot to prevent soil loss. The soil was a 1:1 mix of washed river sand and loam with a pH of 6.2 (CaCl₂). The water content (w w⁻¹) at field capacity (FC) was 15.5%. Soil analyses, performed by CSBP Laboratories (Bibra Lake, Western Australia), are presented in Supplementary data Table S2. Two days prior to transplanting seeds, pots were watered to 60% of FC and all essential nutrients were applied (mg kg⁻¹ soil⁻¹): P 20.5, K 88.7, S 34.2, Ca 41.0, Cl 72.5, Mg 3.95, Mn 3.26, Zn 2.05, Cu 0.51, B 0.12, Co 0.11, and Mo 0.08 according to Enkhbat et al. (2021b). These nutrients were reapplied in the same amounts at 14 days after sowing (DAS).

Seeds were germinated as described by Enkhbat et al. (2021b) with seed scarification followed by germination in Petri dishes (day of sowing) for 36–39 hours in a darkened temperature-controlled room at 15 °C. Six seeds with newly-emerged radicles were transplanted (radicle placed downwards) to 10 mm depth and covered with soil. All pots were watered to 80% FC with 40 mL of *Rhizobium leguminosarum* bv. *trifolii* strain WSM1325 (Group C) inoculum (mixture of 2.8 g of rhizobia in 1 L of water). Plants were grown for 21 days under free-draining conditions and were re-watered to 80% FC every 2–3 days. Pots were re-arranged weekly to minimise positional effects. Plants were randomly thinned to three per pot at 14 days after sowing (DAS) with the thinned plants used to

Experimental design and treatments

The experiment had a factorial randomised block design with two factors, two treatments and 35 genotypes in four replications. Each pot was an experimental unit. The two treatments consisted of: (i) a free-drained (control) treatment which was watered to 80% of FC for duration of the experiment; and (ii) a waterlogged treatment which commenced after 21 DAS and was maintained for 28 days. To impose the waterlogged treatment, pots were placed inside the same sized pots, sealed by plastic bags, and the water level was maintained 10 mm above the soil surface by daily watering.

Harvests

Two harvests were undertaken: initial harvest at 21 DAS and final harvest at 49 DAS. For each harvest, soil was gently washed with flowing water from the roots of all three plants in the pot onto a sieve (3 mm mesh).

Measurements

Seed and seedling phenotypic traits

Seed size Four replicates of 50 random seeds per genotype were counted using a Condator seed counter (Preuffer, Germany) and mean seed weight calculated.

Hypocotyl length and cotyledon size/weight Hypocotyl length and the size and weight of an individual cotyledon of each genotype were measured in 12 seedlings of each genotype (three seedlings from each pot) at 14 DAS. Intact seedlings were gently pulled from the soil. Hypocotyl length was measured by ruler from the junction of the cotyledons until the joint with the radicle (Supplementary data Fig. S2). Cotyledon size was estimated by comparison with the photographic plates and conversion formulae in Williams et al. (1964). Cotyledon weight was determined by cutting cotyledons at the junction with the shoot and oven-drying at 60°C for three days before weighing.

Leaf size and petiole length At the initial harvest (21 DAS), leaf size (three leaflets combined) and petiole length of the first trifoliate leaf in each pot were measured for each ssp. *yanninicum* genotype. Leaf size was estimated by comparison with the photographic leaf size standards for subclover of Williams et al. (1964) and using their conversion formulae. Petiole length of the same leaves was measured by ruler.

Days to first flowering Data for days to first flowering (DFF, the number of days from sowing to the appearance of the first open flower) for the 32 genotypes of ssp. *yanninicum* are from Enkhbat et al. (2021a).

Traits under waterlogging

Shoot and root RGRs Harvested plants were separated into shoots and roots at both harvests and oven-dried at 60 °C for four days and weighed. Shoot and root relative growth rates (RGR) were calculated using the standard equation of Hunt (1982):

$$RGR = (\ln DW2 - \ln DW1) / (t2 - t1)$$

where: DW2 = dry weight at final harvest (g); DW1 = dry weight at initial harvest (g), and t2-t1 = number of days between harvests (28 days).

Leaf size and petiole length At the final harvest (49 DAS), leaf size and petiole length of the fourth trifoliate leaf of each plant were measured using the same methods as the initial harvest for all subclover genotypes. Because *T. michelianum* has a different leaf shape to subclover, leaf size of cv. Frontier was measured using a portable leaf area meter (LI 3000; LI-COR Biosciences, Lincoln, USA).

Chlorophyll content At 44 DAS, relative changes in chlorophyll content (SPAD unit) of the youngest fully-opened leaf of all plants were measured by a chlorophyll meter (Konica Minolta SPAD-502 Plus, Osaka, Japan).

Surface root proliferation Relative differences in the amount of surface roots found growing above the soil surface under waterlogged conditions were assessed at 49 DAS (final harvest) for each genotype using a 1–10 visual rating scale, where 1 was the lowest root density and 10 was the highest density.

Statistical analyses

Data are graphed as mean $\pm$ standard error ($\pm$ SE) using SigmaPlot 14 (Systat Software, Inc.). Genotypes were ordered in the graphs based on their tolerance, defined as maintenance of shoot growths relative to controls, and this order was consistent in all other graphs. Among genotypes of ssp. *yanninicum* data were analysed by two-way ANOVA, with genotype and treatment as main factors, in R software (version 3.6.3). Least significant difference (LSD) Fisher's protected tests were used to test treatment effects for each genotype.

One-way ANOVA was used to compare genotypic variation of ssp. *yanninicum* mean seed weight and traits measured at 14 DAS (cotyledon size/weight and hypocotyl length) and at 21 DAS (leaf size and petiole length of the first trifoliate leaf, shoot and root DWs). Broad-sense heritability (H^2) was also estimated for these traits across the genotypes of ssp. *yanninicum*, according to Falconer (1989). It was also used to assess genotypic variation for surface root growth under waterlogging in all genotypes.

Pearson correlation coefficients and their levels of significance were calculated between all traits measured at 14 and 21 DAS and traits under waterlogged conditions at 28 days after treatment (DAT) for genotypes of ssp. *yanninicum*. They were also calculated between plant traits and eco-geographic (passport data and BIOCLIM) variables of collection sites.

Results

Variation for phenotypic traits

Significant genotype differences ($0.01 < P < 0.001$) were found among the 32 ssp. *yanninicum* genotypes for all seed and seedling traits (Table 3). The mean values for these traits are shown in Supplementary data Table S3.

Table 3

The mean, range, significance level (P -value) and broad-sense heritability (H^2) among genotypes of phenotypic seedling traits prior to imposition of treatments for 32 genotypes of ssp. *yanninicum* ($n = 4$).

Trait	Mean	Minimum	Maximum	P -value	H^2 (%)
Mean seed weight (mg)	8.61	5.60	13.60	***	94
<i>At 14 DAS</i>					
Cotyledon size (cm ²)	0.45	0.34	0.57	***	47
Cotyledon DW (mg)	1.71	1.12	2.75	***	66
Hypocotyl length (mm)	9.13	6.75	13.29	***	58
<i>At 21 DAS</i>					
Leaf size (cm ²)	2.53	1.69	3.60	***	64
Petiole length (cm)	3.62	1.92	5.70	***	71
Shoot DW (g)	0.07	0.05	0.11	***	56
Root DW (g)	0.04	0.02	0.05	**	24
<i>Days to flowering</i>					
DFF (days)	123	100	149	***	94
* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.					
Days to first flowering (DFF, the number of days from sowing to first open flower) are from Enkhbat et al. (2021a), recorded from an early May sowing at Shenton Park, Western Australia (31°57'S, 115°5'E).					

Plant growth under waterlogging

Waterlogging tolerance was assessed as the maintenance of plant growth under waterlogged conditions, relative to plants grown in free-draining conditions (control).

Shoot and root RGR

There was a significant treatment $\times$ genotype interaction for root RGR ($P < 0.001$) but not for shoot RGR at 28 DAT (Fig. 1). Shoot RGRs under waterlogging ranged from 87–108% of controls, while root RGRs ranged from 80–116% of controls (Supplementary data Table S4).

Waterlogging reduced shoot RGRs in four out of 32 ssp. *yanninicum* genotypes and root RGRs in 13 genotypes, compared to their controls (Fig. 1; Supplementary data Table S4). Among the genotypes of ssp. *yanninicum*, ecotype D showed a superior tolerance to waterlogging with both shoot ($P < 0.05$) and root ($P < 0.01$) RGRs were significantly higher than its controls (105% and 113% of controls, respectively). In contrast, both shoot and root RGRs of cv. Seaton Park ssp. *subterraneum* (82% and 73% of controls, respectively) and cv. Antas ssp. *brachycalycinum* (87% and 85%) were severely decreased by waterlogging compared to their respective controls (Fig. 1; Supplementary data Table S4). For cv. Frontier *T. michelianum* both shoot and root DWs were unaffected by waterlogging and did not differ from control treatment (99% and 97% of controls, respectively).

Shoot and root DW

There was a significant treatment $\times$ genotype interaction for both shoot ($P < 0.05$; Fig. 2a) and root ($P < 0.001$, Fig. 2b) DWs among the 32 ssp. *yanninicum* genotypes at 28 DAT. Marked variation was observed under waterlogging for both shoot (66–120% of controls) and root (58–137%) DWs (Supplementary data Table S4).

Waterlogging reduced shoot DWs in only four out of 32 genotypes compared to controls, but reduced root DWs in 13 genotypes. Among *yanninicum* genotypes, ecotype D showed superior tolerance to waterlogging with both shoot ($P < 0.05$) and root ($P < 0.01$) DWs significantly higher than its controls (115% and 133% of controls, respectively) (Fig. 2). In contrast, both shoot and root DWs of cv. Seaton Park ssp. *subterraneum* (58% and 49% of controls, respectively) and cv. Antas ssp. *brachycalycinum* (64% and 68%) were severely decreased, compared to their respective controls. For cv. Frontier *T. michelianum*, both shoot and root DWs were unaffected by waterlogging and did not differ from control treatment (92% and 87% of controls, respectively).

Leaf size and petiole length

For leaf size, there was a significant treatment $\times$ genotype interaction ($P < 0.01$; Fig. 3a) among genotypes of ssp. *yanninicum*. Leaf size under waterlogging ranged from 76–130% of controls (Supplementary data Table S4). Waterlogging reduced leaf size in only one ecotype, compared to its control ($P < 0.01$) (Fig. 3a; Supplementary data Table S4). In contrast, five ecotypes had significantly larger leaves than their respective controls. Leaf sizes for both cv. Seaton Park ssp. *subterraneum* (49%) and cv. Antas ssp. *brachycalycinum* (67%) were severely reduced by waterlogging, whereas leaf size in cv. Frontier *T. michelianum* (94%) was unchanged by the waterlogging treatment.

For petiole length, there was a significant treatment $\times$ genotype interaction ($P < 0.001$; Fig. 3b) among genotypes of ssp. *yanninicum*. Petiole length ranged from 63–105% of controls (Supplementary data Table S4). Waterlogging reduced petiole length in 13 out of 32 genotypes compared to controls. Petiole length in the remaining 19 genotypes was unaffected by waterlogging and did not differ from control treatment. Petiole lengths for cv. Seaton Park ssp. *subterraneum* (58%), cv. Antas ssp. *brachycalycinum* (77%) and cv. Frontier *T. michelianum* (77%) were significantly reduced compared to their respective controls.

Surface root proliferation

There was a significant genotype difference ($P < 0.001$) for surface root density under waterlogging (Fig. 4; Supplementary data Table S4). At 28 DAT under waterlogging, surface roots were observed in all genotypes, irrespective of their tolerance. Notably, the highest surface roots occurred in two of the black-seeded ecotypes: H (score 10.0) and G (score 9.4). The density of surface roots in the other genotypes ranged from scores of 1–7.

Chlorophyll content

There was a significant treatment $\times$ genotype interaction ($P < 0.001$; Fig. 5) for leaf chlorophyll content among genotypes of ssp. *yanninicum*. Leaf chlorophyll content under waterlogging was reduced significantly ($0.01 < P < 0.001$) for all genotypes compared to their controls (Supplementary data Table S4).

Relationships between waterlogging tolerance with phenotypic traits

For ssp. *yanninicum*, correlation analyses were conducted for all measured traits (Table 4). Among all possible correlations, of most interest was the identification of traits that correlated with growth (shoot and root RGRs) under waterlogging. Second in importance were traits that correlated with leaf size and petiole length.

Table 4

Pearson correlation coefficients (r) and significance for traits among 32 ssp. *yanninicum* genotypes (4 cultivars and 28 ecotypes). Mean seed weight of 50 seeds was measured at the day of sowing. Other traits were measured at 14 and 21 days after sowing (DAS) prior to waterlogging being imposed and at 28 days after treatment (DAT) under waterlogging. Waterlogging treatment (water level was kept 10 mm above the soil surface) was imposed after 21 days of growth.

	Traits	1	2	3	4	5	6	7	8	9	10	11	12	13	14
<i>At 28 DAT (waterlogged treatment)</i>															
1	Shoot RGR (% of control)														
2	Root RGR (% of control)	0.86***													
3	Leaf size (% of control)	0.55**	n.s.												
4	Petiole length (% of control)	0.59***	0.57***	0.49**											
5	Chlorophyll content (% of control)	n.s.	n.s.	n.s.	n.s.										
6	Surface roots (1–10 score)	n.s.	n.s.	n.s.	n.s.	n.s.									
<i>Flowering and seed weight</i>															
7	DFF (days)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.								
8	Mean seed weight (mg)	n.s.	n.s.	n.s.	-0.38*	n.s.	n.s.	n.s.							
<i>At 14 DAS</i>															
9	Cotyledon size (cm ²)	-0.40*	n.s.	-0.53**	-0.52**	n.s.	n.s.	n.s.	0.67***						
10	Cotyledon DW (mg)	n.s.	n.s.	-0.39*	-0.45**	n.s.	n.s.	n.s.	0.92***	0.76***					
11	Hypocotyl length (mm)	n.s.	n.s.	n.s.	-0.39*	n.s.	n.s.	n.s.	n.s.	0.41*	0.35*				
<i>At 21 DAS</i>															
12	Leaf size (cm ²)	n.s.	n.s.	-0.59***	-0.53**	n.s.	n.s.	n.s.	0.63***	0.76***	0.69***	0.37*			
13	Petiole length (cm)	-0.38*	-0.35*	-0.48**	n.s.	n.s.	0.52**	n.s.	0.44*	0.55**	0.49**	n.s.	0.52**		
14	Shoot DW (g)	n.s.	n.s.	n.s.	-0.36*	n.s.	n.s.	n.s.	0.92***	0.73***	0.90***	n.s.	0.68***	0.63***	
15	Root DW (g)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.62***	0.41*	0.40*	n.s.	0.39*	0.43*	0.66

n.s. not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Strength of correlation is shown by increasing intensity of green for positive and by increasing intensity of blue for negative.

Days to first flowering (DFF, the number of days from sowing to first open flower) is from Enkhbat et al. (2021a), recorded from an early May sowing at Shent Park, Western Australia (31°57'S, 115°5'E).

Strong positive correlations were found between shoot and root RGRs under waterlogging. Under waterlogging, petiole length had strong positive correlations to both shoot and root RGRs, while leaf size had a high positive correlation with only shoot RGR. Surprisingly, neither chlorophyll content nor surface root proliferation were significantly correlated to shoot and root RGRs under waterlogging.

Seedling phenotypic traits under free-draining conditions had negative correlations with traits measured under waterlogging (28 DAT). In other words, genotypes of ssp. *yanninicum* with larger seeds (bigger cotyledons) and vigorous seedling biomass under free-draining condition with larger leaves and longer petioles (21 DAS) showed a low RGR under waterlogging. DFF did not correlate significantly with any other trait (Table 4).

Relationships of variation for waterlogging tolerance with eco-geographic variables for ssp. *yanninicum* ecotypes

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Pearson correlation coefficients between measured traits and eco-geographic variables are shown in Table 5 for passport data and eight standard BIOCLIM precipitation variables (BIO12-BIO19) and in Table 6 for 11 standard BIOCLIM temperature variables (BIO1-BIO11). A total of 29 ecotypes of ssp. *yanninicum* were used to examine correlations of plant response to waterlogging with eco-geographic variables from their collection sites. Transient waterlogging is strongly affected by much localized topography while heat or drought stress damage can be over quite a wide geographic area (Khazaei et al. 2013). For this reason the broad geographic/ecological approach offered by the analysis of collection site agro-variables is less useful in the prediction/identification of transient waterlogging than heat and drought stress.

Table 5

Pearson correlation coefficients (r) and significance between mean values of traits and 11 eco-geographic variables (passport data and BIOCLIM precipitation variables BIO12-BIO19) for 29 ssp. *yanninicum* ecotypes with known passport data. Mean seed weight of 50 seeds was measured prior sowing. Other traits were measured at 14 and 21 days after sowing (DAS) and at 28 days after treatment (DAT) under waterlogged conditions. The waterlogging treatment (water level was kept 10 mm above the soil surface) was imposed after 21 days of growth. Codes and definitions of BIOCLIM variables are in Supplementary data Table 2.

Traits	Lat	Lon	Alt	BIO12	BIO13	BIO14	BIO15	BIO16	BIO17	BIO18	BIO19
	(°N)	(°E)	(m)	(mm)	(mm)	(mm)		(mm)	(mm)	(mm)	(mm)
<i>At 28 DAT</i>											
Shoot RGR (% of control)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Root RGR (% of control)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Leaf size (% of control)	n.s.	n.s.	n.s.	-0.41*	-0.41*	n.s.	n.s.	-0.41*	n.s.	n.s.	-0.43*
Petiole length (% of control)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Chlorophyll content (% of control)	n.s.	n.s.	n.s.	n.s.	n.s.	-0.43*	n.s.	n.s.	-0.43*	-0.43*	n.s.
Surface roots (1–10 score)	n.s.	n.s.	n.s.	n.s.	n.s.	0.59**	n.s.	n.s.	0.46*	n.s.	n.s.
<i>Seed weight</i>											
Mean seed weight (mg)	n.s.	n.s.	n.s.	0.56**	0.57**	n.s.	n.s.	0.56**	n.s.	n.s.	0.54**
<i>At 14 DAS</i>											
Cotyledon size (cm ²)	n.s.	n.s.	n.s.	0.54**	0.50**	n.s.	n.s.	0.50**	n.s.	n.s.	0.47*
Cotyledon DW (mg)	n.s.	n.s.	n.s.	0.57**	0.55**	n.s.	n.s.	0.55**	n.s.	n.s.	0.52**
Hypocotyl length (mm)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>At 21 DAS</i>											
Leaf size (cm ²)	n.s.	n.s.	n.s.	0.58***	0.55**	n.s.	n.s.	0.54**	n.s.	n.s.	0.54**
Petiole length (cm)	n.s.	0.55**	n.s.	0.57**	0.52**	0.59***	n.s.	0.52**	0.54**	0.49**	0.52**
Shoot DW (g)	n.s.	n.s.	n.s.	0.59***	0.57**	n.s.	n.s.	0.57**	n.s.	n.s.	0.54**
Root DW (g)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>n.s.</i> not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.											
Strength of correlation is shown by increasing intensity of green for positive and by increasing intensity of blue for negative.											

Table 6

Pearson correlation coefficients (r) and significance between mean values of traits and 11 eco-geographic variables (BIOCLIM temperature variables BIO1-BIO11) for 29 ssp. *yanninicum* with known passport data. Mean seed weight of 50 seeds was measured at the day of sowing. Other traits were measured at 14 and 21 days after sowing (DAS) and at 28 days after treatment (DAT) under waterlogged condition. Waterlogging treatment (water level was kept 10 mm above the soil surface) was imposed after 21 days of growth. Codes and definitions of BIOCLIM variables are in Supplementary data Table 2.

Traits	BIO1	BIO2	BIO3	BIO4	BIO5	BIO6	BIO7	BIO8	BIO9	BIO10	BIO11
	(°C)	(°C)			(°C)	(°C)	(°C)	(°C)	(°C)	(°C)	(°C)
<i>At 28 DAT</i>											
Shoot RGR (% of control)	n.s.	n.s.	n.s.	n.s.	0.42*	n.s.	n.s.	n.s.	0.43*	0.44*	n.s.
Root RGR (% of control)	n.s.	n.s.	n.s.	n.s.	0.39*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Leaf size (% of control)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Petiole length (% of control)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Chlorophyll content (% of control)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Surface roots (1–10 score)	-0.45*	n.s.	n.s.	n.s.	n.s.	-0.46*	n.s.	-0.57**	n.s.	n.s.	-0.47*
<i>Seed weight</i>											
Mean seed weight (mg)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>At 14 DAS</i>											
Cotyledon size (cm ²)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Cotyledon DW (mg)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Hypocotyl length (mm)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>At 21 DAS</i>											
Leaf size (cm ²)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Petiole length (cm)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.39*	-0.39*	n.s.
Shoot DW (g)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Root DW (g)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>n.s.</i> not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.											
Strength of correlation is shown by increasing intensity of green for positive and by increasing intensity of blue for negative.											

Geographic coordinates did not correlate significantly with any traits measured both under waterlogging and under control treatments and seed weight (Tables 5 and 6). Only petiole length at 21 DAS had a strong positive correlation with longitude, showing genotypes from eastern end of the botanical range tended to have long petioles at seedling stage.

Precipitation variables at the site of origin did not correlate with shoot or root RGRs under waterlogging except leaf size (Table 5). Smaller leaves under waterlogging (at 28 DAT) had to be associated with higher annual precipitation (BIO12) and winter precipitation (BIO13, BIO16 and BIO19). In contrast, annual (BIO12) and winter precipitation (BIO13, BIO16 and BIO19) showed positive correlations with several traits measured under free-draining conditions. Notably, growth of petioles at 21 DAS positively correlated with the majority of precipitation variables including annual, summer and winter precipitation (BIO12, BIO13, BIO14, BIO16, BIO17, BIO18 and BIO19) (Table 5).

Temperature variables at site of origin correlated with RGR under waterlogging (Table 6). Notably, warmer temperatures at site of origin correlated with increased growth under waterlogging of ssp. *yanninicum*, showing positive correlations between shoot/root RGRs and maximum temperature of the warmest month (BIO5), mean temperature of the driest (BIO9) and warmest quarter (BIO10). Surface root proliferation under waterlogging negatively correlated with winter temperature variables (BIO6, BIO8 and BIO11).

Discussion

This study evaluated variation for waterlogging tolerance among a diverse range of ssp. *yanninicum* genotypes by assessing the maintenance of plant growth (shoot and root RGRs) relative to free-drained conditions. Variation for waterlogging tolerance was evident, with several ecotypes displaying high tolerance with equivalent or greater growth under waterlogging than in free-drained controls. It is noteworthy that the highest tolerance to waterlogging was observed among wild ecotypes rather than commercialized cultivars. The variation in waterlogging tolerance among ssp. *yanninicum* and associated traits that contributed to waterlogging tolerance or explained sensitivity to waterlogging are now discussed in more detail.

Plant growth under waterlogging

Root growth was more affected by waterlogging than shoot growth in ssp. *yanninicum*, supporting the assertions of Colmer and Voesenek (2009), Herzog et al. (2016), Nakai et al. (2009) and Striker (2012a) that soil waterlogging causes a direct effect on the root system reducing its growth and function (Bailey-Serres and Voesenek 2008; Colmer and Voesenek 2009; Malik et al. 2002). A high positive correlation between root and shoot growth rate under waterlogging in the present study demonstrated the primary role of root systems to waterlogging tolerance (Armstrong et al. 1983; Gibberd and Cocks 1997; Gibberd et al. 2001; Rogers and West 1993; Yamauchi et al. 2018). Moreover, all genotypes developed surface roots in response to waterlogging, in a similar finding to Francis and Devitt (1969) and Enkhbat et al. (2021b). The ability of plants to alter their root distribution patterns along and above the soil surface is a common response to waterlogging to obtain oxygen (Voesenek et al. 1999). Root proliferation near to soil surface in the present study supported findings of Mano et al. (2006) for maize (*Zea mays* L.), Erskine et al. (1994) and Solaiman et al. (2007) for lentil, Malik et al. (2015) for lentil, grasspea (*Lathyrus sativa* L.) and pea, and Cannell and Belford (1980) for canola (*Brassica napus* L.).

Leaf size and petiole length

Leaf growth among genotypes of ssp. *yanninicum* was the least affected by waterlogging, while petiole growth was more sensitive. Francis and Devitt (1969) also found no reduction in leaf size among 25 genotypes of ssp. *yanninicum* under waterlogging. Strong positive correlations between leaf size and waterlogging tolerance were also found in the three subspecies of subclover (Enkhbat et al. 2021b) and in *Brassica* species (Ashraf and Mehmood 1990). This could be explained as ssp. *yanninicum* invests more assimilates to increase leaf biomass proportion rather than petiole growth in response to soil waterlogging. A high proportion of leaf biomass and larger leaves can provide increased uptake of atmospheric oxygen and greater exchange of gases (Colmer 2003; Striker et al. 2005; Striker and Colmer 2017). Additionally, reduction in petiole length under waterlogging can be used as a visual indicator of the strength of waterlogging stress in ssp. *yanninicum*.

Chlorophyll content

A significant reduction in chlorophyll content, with characteristic yellowing of the leaves, was observed in all genotypes of ssp. *yanninicum* as found among subclover subspecies (Enkhbat et al. 2021b), *Brassica* species (Ashraf and Mehmood 1990), *Salix* species (Talbot et al. 1987) and barley (Pang et al. 2004). Leaf chlorophyll degradation is a common symptom of waterlogging stress (Smethurst and Shabala 2003). Huang Bingru et al. (1994); Malik et al. (2002); Striker and Colmer (2016) have all reported reductions in leaf chlorophyll content caused by inhibition of N, and damaged photosynthetic apparatus, especially when waterlogging extends for several weeks.

Relationships of variation for waterlogging tolerance with phenotypic traits

This study supports previous findings that subclover seedling biomass, leaf size and petiole length are a reflection of cotyledon weight/size, which depends on seed weight (Black 1956; Black 1957; Donald 1951; Gladstones 1967). This could be explained by the significance of large cotyledons (i.e. large seeds) that can deliver an initial advantage for growth rates of subsequent seedling which allows individual plants to be superior in terms of capture of light, space and soil resources and subsequent biomass accumulation, including the production of larger leaves and longer petioles (Black 1960; Black 1957; Brougham 1958; Davidson and Donald 1958; Gladstones 1967). However, in present study, early seedling vigour of ssp. *yanninicum* was irrelevant for tolerance to waterlogging. Palta et al. (2010) also found that vigorous early growth of seedlings is unrelated for tolerance to waterlogging in chickpea. This can be explained as genotypes with lower initial shoot biomass need to improve their growth rates above the soil surface to respond soil waterlogging. As discussed earlier, an increased proportion of aerial organs enables higher oxygen uptake and greater exchange of gases under waterlogging (Colmer 2003; Striker et al. 2005; Striker and Colmer 2017). It also could be a genotype with high initial shoot biomass saves reserves (energy and carbohydrates) for later use, since resources already accumulated in biomass (aerial organs) might be enough to respond waterlogging stress. Striker (2012b) highlighted that plant growth responses under waterlogging do not fully define tolerance to the stress. Little or absence of growth under waterlogging can save reserves and deliver benefits for rapid growth following alleviation of waterlogging stress (Setter and Waters 2003; Striker 2012b). Enkhbat et al (2021) found a high growth of ssp. *yanninicum* under waterlogging had a negative effect on both shoot and root RGRs at the subsequent recovery stage compared than the two other subclover subspecies with lower growth under waterlogging but had higher recovery than ssp. *yanninicum*. Further research is, therefore, required to investigate performance of ssp. *yanninicum* after alleviation of waterlogging stress comparing their growth under waterlogging to evaluate tolerance to waterlogging of ssp. *yanninicum*.

Relationships of variation for waterlogging tolerance with eco-geographic variables

Waterlogging tolerance of ssp. *yanninicum* was not correlated with any of geographic coordinates and this can be associated with the restricted distribution of ssp. *yanninicum*. The natural habitat of ssp. *yanninicum* is reported to be low altitude coastal areas (often flat meadows) with high mean annual rainfall (> 500 mm) and mild winter temperatures (Katznelson 1966; Francis 1976; Ghamkhar et al. 2015). The distribution of ssp. *yanninicum* in a lower profiles of the landscape (below 1400 m a.s.l), which tends to collect more water, could be a consequence of ssp. *yanninicum* to maintain inherent features to confer adaptation to waterlogging than the two other subclover subspecies that can be found in elevation up to 2190 m a.s.l for ssp. *brachycalycinum* and 2940 m a.s.l for ssp. *subterraneum* (Ghamkhar et al. 2015).

Annual and winter rainfall at the site of origin were highly correlated with seed size and initial seedling growth of ssp. *yanninicum*. This supports Pecetti and Piano (2002) and Piano et al. (1996), who found that individual seed weight of subclover increases with increasing annual rainfall at the site of origin. As discussed earlier, if seed weight is high, subsequent seedling growth (winter biomass) and associated traits such as cotyledon size/weight, leaf size and petiole length tend to be high. But, waterlogging tolerance of ssp. *yanninicum* was not correlated with rainfall at the site of origin, which could be associated with their restricted distribution with uniform climatic regions, as discussed earlier. Additionally, warmer temperatures at the collection site favoured waterlogging tolerance in ssp. *yanninicum*.

Plant Breeding Implication

This study has identified promising material for plant breeders as new parents for crossing to enhance waterlogging tolerance and increase the genetic base in this globally important annual legume. The observed results in this study could be different in their natural environments (Villemereuil et al. 2015) due to this experiment being conducted under highly controlled experimental conditions. Environmental factors, such as low temperatures or nutrient deficiencies, are likely to influence plant growth compared to glasshouse conditions. Further studies should be conducted under waterlogged field conditions to validate results delivered from the glasshouse experiment.

This study focused on selection criteria for waterlogging tolerance of ssp. *yanninicum* on morphological responses, such as shoot and root biomass production, leaf and petiole growth and formation of surface roots. The approach could be enhanced by future research to focus on other key responses to waterlogging mechanisms such as shoot physiological responses, changes in root anatomical and morphological responses.

Finally, this study only assessed growth of ssp. *yanninicum* under waterlogging. Recovery ability following alleviation of waterlogging stress is crucial for plants to gain shoot biomass and to evaluate tolerance to waterlogging (Striker 2012b), particularly in self-regenerating pastures for seed production (Striker and Colmer 2017). Recovery ability in the three subspecies of subclover studied in Enkhbat et al. (2021b), that both of shoot and root growth after 21 days recovery was lower in ssp. *yanninicum* than ssp. *subterraneum*. Response to post-waterlogging stress within genotypes of ssp. *yanninicum* is, therefore, essential to identify genotypes with better performance after waterlogging.

Declarations

Acknowledgements

This research was conducted as part of the Annual Legume Breeding Australia (ALBA) Joint Venture between PGG Wrightson Seeds and UWA. The project is under the International Collaborative Program in Agricultural Sciences between Nagoya University and UWA. G.E. is supported by an Australian Government Research Training Program (RTP) scholarship and a Science Industry PhD Fellowship from the Government of Western Australia. We gratefully acknowledge Robert Creasy, Evonne Walker, Bill Piasini, Elise Robinson and Robert Jeffery for technical help.

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Figures

Figure 1

Relative growth rates (RGR) of (a) shoot and (b) root for 32 ssp. yanninicum genotypes, consisting of 4 cultivars (hatched) and 28 ecotypes, and ssp. brachycalycinum cv. Antas, ssp. subterraneum cv. Seaton Park and T. michelianum cv. Frontier after 28 days of treatment (mean $\pm$ SE $n=4$). Treatments were imposed after 21 days of growth: free-drained (control) and waterlogged (WL, water level kept 10 mm above the soil surface). Results of two-way ANOVA are given in each panel. The single cultivars of ssp. brachycalycinum, ssp. subterraneum and T. michelianum are not included in the statistical analyses. The significant differences ($P < 0.05$; Fisher LSD test) between control and waterlogged treatments of each genotype are shown: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (black indicates the control treatment was higher and red indicates that the waterlogged treatment was higher)

Figure 2

Dry weights (DW) of (a) shoot and (b) root for 32 *ssp. yanninicum* genotypes, consisting of 4 cultivars (hatched) and 28 ecotypes, and *ssp. subterraneum* cv. Seaton Park, *ssp. brachycalycinum* cv. Antas and *T. michelianum* cv. Frontier after 28 days of treatment (mean $\pm$ SE $n=4$). Treatments were imposed after 21 days of growth: free-drained (control) and waterlogged (WL, water level kept 10 mm above the soil surface). Results of two-way ANOVA are given in each panel. The single cultivars of *ssp. brachycalycinum*, *ssp. subterraneum* and *T. michelianum* are not included in the statistical analyses. The significant differences ($P < 0.05$; Fisher LSD test) between control and waterlogged treatments of each genotype are shown: * $P<0.05$; ** $P<0.01$; *** $P<0.001$ (black indicates the control treatment was higher and red indicates that the waterlogged treatment was higher)

Figure 3

(a) leaf size and (b) petiole length for 32 *ssp. yannanicum* genotypes, consisting of 4 cultivars (hatched) and 28 ecotypes, and *ssp. brachycalycinum* cv. Antas, *ssp. subterraneum* cv. Seaton Park and *T. michelianum* cv. Frontier after 28 days of treatment (mean $\pm$ SE $n=4$). Treatments were imposed after 21 days of growth: free-drained (control) and waterlogged (WL, water level kept 10 mm above the soil surface). Results of two-way ANOVA are given in each panel. The single cultivars of *ssp. brachycalycinum*, *ssp. subterraneum* and *T. michelianum* are not included in the statistical analyses. The significant differences ($P < 0.05$; Fisher LSD test) between control and waterlogged treatments of each genotype are shown: * $P<0.05$; ** $P<0.01$; *** $P<0.001$ (black indicates the control treatment was higher and red indicates that the waterlogged treatment was higher). Note the y-axis scale change in the panels

Figure 4

Surface root proliferation after 28 days of waterlogging for 32 ssp. yanninicum genotypes, consisting of 4 cultivars (hatched) and 28 ecotypes, and ssp. brachycalycinum cv. Antas, ssp. subterraneum cv. Seaton Park and T. michelianum cv. Frontier grown under waterlogging (mean $\pm$ SE n=4). The waterlogging treatment was imposed after 21 days of growth (water level kept 10 mm above the soil surface). Surface roots were scored on a 1-10 scale (where 1 is the lowest growth and 10 is the most vigorous growth)

Figure 5

Chlorophyll content for 32 *ssp. yannanicum* genotypes, consisting of 4 cultivars (hatched) and 28 ecotypes, and *ssp. brachycalycinum* cv. Antas, *ssp. subterraneum* cv. Seaton Park and *T. michelianum* cv. Frontier after 28 days of treatment (mean $\pm$ SE $n=4$). Treatments were imposed after 21 days of growth: free-drained (control) and waterlogged (WL, water level kept 10 mm above the soil surface). Results of two-way ANOVA are given in the panel. The single cultivars of *ssp. brachycalycinum*, *ssp. subterraneum* and *T. michelianum* are not included in the statistical analyses

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