Petiole length reduction is an indicator of waterlogging stress for *Trifolium subterraneeum* ssp. *yanninicum*

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Abstract

**Background and aims** The pasture legume *Trifolium subterraneeum* ssp. *yanninicum* exhibits waterlogging tolerance. This study investigates diversity for waterlogging tolerance within ssp. *yanninicum*. We tested the hypotheses that (1) variation for waterlogging tolerance exists within ssp. *yanninicum* and (2) is related to phenotypic and growth trait differences, which (3) reflect eco-geographic variables at site of origin.

**Methods** Twenty-eight diverse ssp. *yanninicum* ecotypes collected from the Mediterranean region and four cultivars were grown in a controlled environment glasshouse. Seedling traits were measured at 14 and 21 days after sowing. Waterlogged and free-draining (control) treatments were then imposed for 28 days.

Relative distance and multivariate plasticity indices were calculated.

**Results** Under waterlogging, shoot (87–108% of controls) and root (80–116% of controls) relative growth rates (RGRs) differed significantly among ssp. *yanninicum*. Waterlogging tolerance, as assessed by shoot RGR, had strong positive correlations with root RGR (*r* = 0.86; *P* < 0.001), petiole length (*r* = 0.59; *P* < 0.001) and leaf size (*r* = 0.55; *P* < 0.01) under waterlogging. The proportion of biomass as leaf increased under waterlogging, due to leaf size being maintained (mean 102% of controls), but petiole length decreased (mean 84% of controls). Petiole length was the most plastic trait. Seed size, seedling traits, maturity duration and eco-geographic variables at site of origin were not related to waterlogging tolerance.

**Conclusions** Wide variation in waterlogging tolerance exists within ssp. *yanninicum*. Petiole length reduction, an easy-to-measure and non-destructive indicator, could be used as a preliminary selection tool when screening large numbers of ssp. *yanninicum* for waterlogging tolerance in a breeding program.

**Keywords** Pasture legumes · Genetic resources · Phenotypic plasticity · Heritability · Eco-geographic variables
Introduction

Soil waterlogging, a widespread abiotic stress worldwide, can reduce crop production by 20–50% or more, depending on plant growth stage and duration and intensity of waterlogging (Bertholdsson 2013; Solaiman et al. 2007). Soil waterlogging occurs in the root-zone and is defined as the excess water saturation of soil pores affecting oxygen concentration, 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 through roots into cells of plant organs dramatically decreases (gas diffusion is 10 000 times slower in water than in air) (Bailey-Serres and Voese 2008; Colmer 2003; Voese and Sais 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 (Ponnampemura 1984). Thus, soil waterlogging adversely affects plant growth and function and can result in wilting, premature leaf senescence, and growth cessation, and premature death (Marschner 1995; Stoddard et al. 2006).

The most common approach to define waterlogging tolerance is to assess factors related to the maintenance of shoot growth and accumulated yield under waterlogging, relative to non-waterlogged conditions (Setter and Waters 2003). A given genotype copes with spatial and temporal environmental heterogeneity by producing a range of phenotypes (Grassein et al. 2010). This phenotypic variability is produced by two complementary mechanisms: genetic variability and phenotypic plasticity (Grassein et al. 2010). Genetic variation is modified during evolution by natural selection, with genotypes expressing the most favoured phenotypes being selected; this leads to development of diverse ecotypes in response to diverse environments (Erskine et al. 1989; Grassein et al. 2010; West-Eberhard 1989). Conversely, phenotypic plasticity is the ability of a genotype to be altered by environmental influences and is a more flexible and quicker response to changes in growing conditions (Grassein et al. 2010; Whitman et al. 2009). Hence, both genotypic variation and phenotypic plasticity in trait variation is important for a given genotype to deal with ambient conditions.

Mediterranean-climate zones are characterized by mild, wet winters and hot dry summers, with large intra- and inter-annual variability (Porqueddu et al. 2016). The five regions of the world with Mediterranean-type climates are the Mediterranean basin itself, southern Australia, the southern tip of South Africa, California and central Chile (Porqueddu et al. 2016). Plants in Mediterranean regions can experience ‘transient waterlogging’, which is waterlogging caused by excessive rainfall during the winter growing season, with the duration dependent on the amount of rain, evapotranspiration and soil structure (Malik et al. 2002). Although the upper soil levels dry out in subsequent spring and summer months, plants can suffer prolonged adverse effects from waterlogging after the water recedes (Colmer and Flowers 2008; Malik et al. 2002).

In southern Australia soil subject to transient waterlogging covers a large area of farmland with an estimated annual cost in terms of crop production lost of AU$90 million (Hamilton et al. 2000). While rainfall has decreased for this region 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, which is likely to increase the incidence of waterlogging (Chapman et al. 2012; Jarvis et al. 2010). Therefore, selection of crop and pasture plants with tolerance to waterlogging is paramount for maintaining productivity.

Waterlogging tolerance varies among genotypes of many crop and pasture legumes. 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). 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 an aerated control. Gibberd et al. (2001) explored waterlogging tolerance among 20 Trifolium species/subspecies.
and found nine genotypes had no reduction in shoot growth under waterlogging, and two genotypes had increased growth, compared to drained controls. Exploring waterlogging tolerance within species, therefore, could yield significant benefits for breeding programs by identifying genotypes with greater tolerance (Bailey-Serres and Voesenek 2008; Malik et al. 2015; Rogers et al. 2011).

Subterranean clover (Trifolium subterraneum L., commonly known as subclover), originates from the Mediterranean region and Western Europe (Nichols et al. 2013). It has been introduced to southern Australia, where it is the most widespread pasture legume, having been sown over 29 million ha (Nichols et al. 2013). Subclover forms the basis of many permanent or semi-permanent pastures in irrigated and high rainfall areas, while in low and medium rainfall areas it is commonly grown in rotation with cereal crops. It is highly valued in the livestock and grains industries as a source of high-quality forage and for its ability to fix atmospheric nitrogen (N) (Nichols et al. 2013). Subclover consists of three subspecies: ssp. subterraneum; ssp. brachycalyceum; and ssp. yanninicum (Katznelson and Morley 1965). Each subspecies is used in Australian agriculture and each has different soil preference. In particular, ssp. yanninicum exhibits a higher tolerance to waterlogging and is mostly grown in medium-high rainfall areas (450–1200 mm mean annual rainfall) with soils prone to waterlogging (Francis and Devitt 1969; Nichols et al. 2013; Reed et al. 1985). Evidence for the superior waterlogging tolerance of ssp. yanninicum 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).

The Australian Pastures Genebank (APG) contains the world’s largest subclover collection, with more than 9,000 subclover ecotypes collected from around 3,000 sites in its native habitat in the Mediterranean basin and surrounding areas (Smith et al. 2021). Of these, ssp. yanninicum is the rarest of the three subspecies, comprising only 2.2% of all subclover ecotypes; this reflects ssp. yanninicum being confined to latitudes 38.5–40.5 °N and hence having been collected in the wild from only: Greece, Italy, Spain, Turkey and Tunisia (Nichols et al. 2013). Its typical native habitat is coastal, or areas with mild winter temperatures, on soils prone to winter waterlogging where mean annual precipitation is >450 mm (commonly >750 mm) (Ghamkhar et al. 2015).

Nichols et al. (2013) reported the current cultivars of ssp. yanninicum were developed from a narrow genetic base. There is, therefore, a need to explore the diversity among ecotypes and to identify phenotypic traits for use in future breeding to improve productivity and resilience of ssp. yanninicum and broaden its genetic base. Enkhbat et al. (2021a) found large variation for 10 morphological traits, flowering time and leaf isoflavone content among 108 ecotypes of ssp. yanninicum. Furthermore, all traits (except stem diameter and leaf mark crescent size) were correlated with at least one of 22 eco-geographic variables from collection sites. However, neither ecotypic variation for waterlogging tolerance within ssp. yanninicum, nor its relationship with site of origin parameters, have been previously examined.

Genetic variation in subclover exists for highly heritable and readily measurable traits such as flowering time (maturity) and seed size which are considered crucial traits for adaptation of annual legumes. The optimum flowering time in subclover differs among environments to facilitate seed set and burr burial prior to onset of the dry summer, while maximising the period of vegetative growth (Piano 1984). Seeds tend to be smaller in hot and dry environments enabling them to be germinate successfully with less water at the start of the season and to be mature rapidly at the end of the season (Piano et al. 1996). The following studies in other species have demonstrated a relationship between these traits and waterlogging tolerance. Under field conditions in a Mediterranean-type climate, long-season wheat cultivars show a 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) also found large- 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, 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). Exploring relationships between waterlogging tolerance and readily measurable, highly heritable traits in subclover could provide a simple selection criterion for preliminary screening for waterlogging tolerance.

The survival of a single genotype when experiencing abiotic stress (e.g. waterlogging) is highly related of its ability to quickly express of alternative phenotypes, referred as ‘phenotypic plasticity’ (Pennacchi et al. 2021). *Trifolium* species can adjust their root phenotype to develop lateral roots emerging along and above the soil surface as a response to waterlogging (Enkhbat et al. 2021b; Gibberd and Cocks 1997; Rogers and West 1993). Production of large number of adventitious and young lateral roots enables enhanced porosity (a gas volume per unit of root volume) and improved transport of oxygen, and other gases, within plants (from aerial shoots to submerged roots) throughout newly formed aerenchyma (Armstrong 1980; Drew et al. 1979; Trought and Drew 1980). Aerenchyma, a plastic response to waterlogging, provides large interconnected gas channels improving internal aeration capacity (Colmer and Flowers 2008). But, measuring aerenchyma (i.e. root porosity) is unlikely to be suitable for large number of genotypes as it requires time and fast processing of fresh roots. Instead, the assessment of newly formed adventitious roots and lateral root biomass could be a simple and convenient approach.

Growth of leaves and petioles in subclover is sensitive to waterlogging and shows a highly plastic response (Enkhbat et al. 2021b). This is reflects the aerial part of plant, as the interface between the plant and above-ground physical environment, playing an important role in carbon and water metabolism under waterlogging (Colmer 2003; Pennacchi et al. 2021; Striker et al. 2005). Furthermore, species that often experience prolonged but shallow waterlogging events possess traits, such as hyponastic growth (towards a vertical position) and petiole elongation, that contributed to waterlogging tolerance (Herzog and Pedersen 2014). Striker (2012a) emphasized that hyponastic growth is a common response of species with high tolerance to transient waterlogging. Although subclover has a prostrate growth habit, hyponastic growth and inherently long petioles of ssp. *yanninicum* under waterlogging could be morphological features that confer specific adaptation to waterlogging (Enkhbat et al. 2021b; Francis et al. 1974). Hence, evaluating waterlogging tolerance among ssp. *yanninicum* by assessing phenotypic changes induced by the environment is crucial to identify genotypes with a higher tolerance to, and better performance under, waterlogged conditions.

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) this variation is related to phenotypic and growth trait differences; and (iii) these trait differences are related to eco-geographic variables at site 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. These comprised: (i) 28 ‘ecotypes’ (coded A-AB), originating from wild populations collected in their native Mediterranean basin habitat; and (ii) four cultivars: Yarloop, Larisa, Meteora and Trikkala (Table 1; Supplementary data Fig. S1). In this study, ‘genotype’ is a collective term for both ecotypes and cultivars. As *T. subterraneum* is a self-pollinated, and therefore true-breeding, species (Nichols et al. 2013), the plants of individual genotypes are identical. The subspecies identity of each genotype was confirmed on the basis of distinguishing morphological features of ssp. *yanninicum* described by Katznelson and Morley (1965) and Nichols et al. (2013). Further details of this process are provided in Enkhbat et al. (2021a).

The ecotypes were selected on the basis of diversity for (i) collection site ‘passport data’, which includes latitude, longitude, altitude and other collection site information; and (ii) flowering
time, as determined by (Enkhbat et al. 2021a). Among these were four ecotypes (E, G, H and Q) with black-coloured seeds (as opposed to the usual amber/cream colour) described in Enkhbat et al.
(2021a). Cultivars Larisa and Meteora are themselves ecotypes, originally collected from Greece as CPI 039313Y and CPI 039327YB, respectively (Nichols et al. 2013; Oram 1989) and have associated passport data. However, cv. Yarloop is a naturalized strain originally found growing in the Yarloop district of Western Australia (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). 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 (Nichols et al. 2007) were included as benchmarks for waterlogging susceptibility and tolerance, respectively. This gave a total of 35 genotypes (Table 1).

### 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 (Ghamkhar et al. 2015; Hijmans et al. 2005) on the basis of the passport data. This information was used to map a spatial model in the R software package, as described in Enkhbat et al. (2021b). Nineteen bioclimatic (BIOCLIM) variables (Supplementary data Table S1) were imported for all 30 sites. These are a standard set of eco-geographic variables that have been used in previous studies (Abdi et al. 2020; Enkhbat et al. 2021a; Ghamkhar et al. 2015). BIOCLIM variables for 29 ecotypes (including cv. Larisa and Meteora) were calculated (Table 2, Supplementary data Table S1). 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

### Table 2 Definition of bioclimatic (BIOCLIM) variables, BIOCLIM codes and summary of eco-geographic (passport data and BIOCLIM) variables for 29 ssp. yanninicum ecotypes

| Definition of eco-geographic variables | BIOCLIM Code | Summary of variables |
|----------------------------------------|---------------|----------------------|
| Latitude (°N)                          |               | Min 37.4 39.7 40.9   |
| Longitude (°E)                         |               | Mean 13.1 15.2 18.0 |
| Altitude (m)                           |               | Max 230.8 273.0 725.0|
| 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 |
trait correlations with climatic variables. It was, therefore, excluded from these analyses.

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 in 0.01 M CaCl₂ (Soil pH measurement in CaCl₂ is usually preferred over H₂O, as it is less affected by soil electrolyte concentration and provides a more consistent measurement (Minasny et al. 2011)). The water content (w w⁻¹) at field capacity (FC) was 15.5%. Soil analyses were performed by CSBP Laboratories, Bibra Lake, Western Australia (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). Prior to sowing, seeds were scarified with a Kimseed Venables Seed Scarifier (Kimberley Seeds, Western Australia). Seeds were imbibed in Petri dishes containing double layers of moistened Whatman No 1 filter paper for 36–39 h in a darkened temperature-controlled room at 15 °C (referred here as the day of sowing). Six seeds with newly-emerged radicles were transplanted into each pot (radicle placed downwards) at 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 rearranged weekly to minimise positional effects. Plants were randomly thinned to three per pot at 14 DAS with the removed plants used to measure seedling phenotypic traits.

Experimental design and treatments

The experiment had a factorial randomised block design with two factors (treatment and genotype), comprising two treatments and 35 genotypes in four replications. Each pot was an experimental unit. The two treatments consisted of: (i) a free-draining (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: an initial harvest at 21 DAS and a 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). At both harvests, plants were separated into shoots and roots 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):

$$\text{RGR} = \frac{(\ln \text{DW}_2 - \ln \text{DW}_1)}{(t_2 - t_1)}$$

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

Measurements

Seed measurements

Mean seed weight Four replicates of 50 random seeds per genotype were counted using a Condator seed counter (Preuffer, Germany) and weighed using a balance to calculate mean seed weight.

Measurements at 14 DAS

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). 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.

**Measurements at 21 DAS (Initial harvest)**

**Leaf size and petiole length** Leaf size (three leaflets combined) and petiole length of the first trifoliate leaf of each plant 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 conversation formulae. Petiole length of the same leaves was measured by ruler.

**Measurements at 49 DAS (Final harvest)**

**Leaf size and petiole length** 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 concentration** Relative changes in chlorophyll concentration were measured by a Soil Plant Analysis Development (SPAD) chlorophyll meter (Konica Minolta SPAD-502 Plus, Osaka, Japan), which produces relative SPAD meter values that are proportional to the amount of chlorophyll present in leaf (Ling et al. 2010). SPAD values of the youngest fully-opened leaf of all plants were determined at 44 DAS. Specific leaf weight (SLW) for 16 randomly selected genotypes was estimated, according to Nichols et al. (2009), to determine consistency of leaf thickness (a factor affecting correlation strength between SPAD value and chlorophyll concentration) among genotypes under control and waterlogged treatments.

**Surface root proliferation** Relative differences in the amount of surface roots growing above the soil surface under waterlogged conditions were assessed for each pot using a 1–10 visual rating scale, where 1 was the lowest and 10 was the most vigorous growth.

**Flowering time**

**Days to first flowering** Data for days to first flowering (DFF), measured as the number of days from sowing to the appearance of the first open flower, for the 32 ssp. *yanninicum* genotypes are from Enkhbat et al. (2021a) recorded at Shenton Park, Western Australia (31°57’S, 115°5’E) from a sowing date of 21 May 2019.

**Phenotypic plasticity**

Phenotypic plasticity was evaluated for traits measured at 49 DAS by a quantitative estimation of the phenotypic change induced by the environment, using (i) relative distance plasticity index (RDPI) and (ii) the multivariate plasticity index (MVPi).

**The relative distance plasticity index (RDPI)**

The relative distance plasticity index (RDPI) provides unbiased estimation of phenotypic variation and strong statistical power to test plasticity (Valladares et al. 2006). RDPI was used to quantify and compare levels of phenotypic plasticity for individual traits, based on phenotypic distances among all pairs of replicates of each genotype grown in control and waterlogged treatments. RDPI ranges from 0 (no plasticity) to 1 (maximal plasticity) and was calculated according to Valladares et al. (2006):

\[
RDPI = \frac{\sum |X_c - X_{wl}|}{n} \frac{1}{(X_c + X_{wl})}
\]

where \(X_c\) and \(X_{wl}\) are the phenotypic values for a particular trait of a single genotype in the control and waterlogged treatments, respectively, and \(n\) is the total number of distances.

**Multivariate plasticity index (MVPi)**

Multivariate plasticity index (MVPi) is a multivariate-based plasticity index, used to assess general plasticity; genotypes with higher MVPi values have higher phenotypic plasticity (Pennacchi et al. 2021). The MVPi of each genotype was calculated as the average of total
observed Euclidian distances (ED) for all treatment combinations (total of 16 treatment combinations: 2 treatments each with 4 replicates) where:

$$\text{MVPI} = \frac{(ED_{1:1} + ED_{1:2} + \cdots + ED_{1:m} + ED_{2:1} + \cdots + ED_{2:n} + ED_{n:1} + \cdots + ED_{n:m})}{(n \cdot m)}$$

and $n$ and $m$ are the number of replicates for each combination of treatments.

**Statistical analyses**

Data were analysed using R software (version 3.6.3) and graphed as mean ± standard error (±SE) using SigmaPlot 14 (Systat Software, Inc.). Genotypes were ordered in the graphs based on their tolerance, defined as maintenance of shoot RGR relative to controls, and this order was used for consistency in all other graphs. Among genotypes of ssp. *yaninicum*, data were analysed by two-way ANOVA, with genotype and treatment as main factors. The assumptions of equal group variance and the normality of residual distribution were verified by Bartlett’s and Shapiro-Wilk tests, respectively. A robust ANOVA test with the robust covariance matrix was conducted when heteroskedasticity was a problem. Dependent variables were log-transformed when errors were not normally distributed. As the experiment was a factorial design in a randomised block with four replicates, a two-factor model with a nested structure (randomised genotypes and replicates) for errors, was used by the ‘lme’ function in the ‘nlme’ package in R. Least significant difference (LSD) Fisher’s protected tests were used to test treatment effects for each genotype and to compare the means of phenotypic plasticity index values with the package ‘agricolae’.

One-way ANOVA was used to compare genotypic variation of ssp. *yaninicum* mean seed weight, days to first flowering and seedling traits measured at 14 and 21 DAS. It was also used to assess genotypic differences for surface root proliferation under waterlogging and for RDPI and MVPI plasticity indices, in addition to trait differences between amber and black-seeded ssp. *yaninicum* genotypes. The assumption of constant variance of one-way ANOVA were satisfied, but non-parametric tests (e.g. Kruskal-Wallis) were used when data were not normally distributed. Broad-sense heritability ($H^2$), the proportion of variation in a given trait attributable to genotypic variation, was estimated according to Falconer (1989) for mean seed weight, DFF and seedling traits measured at 14 and 21 DAS across the ssp. *yaninicum* genotypes.

Multivariate analysis was conducted using hierarchical clustering analysis on principal components (HCPC) to perform clustering, and used complementarities between clustering and principal component analysis (PCA), to describe genotype similarities and features of the data set (Husson et al. 2010; Lê et al. 2008). To classify genotypes of ssp. *yaninicum*, a hierarchical clustering and partitioning on the first two principal components was first performed using the HCPC function of the ‘FactoMiner’ package in R on the outputs ‘res.pca’ of the PCA function. The number of clusters was chosen at the suggested level of tree cut based on the gain in inertia. Second, a description of each cluster group was achieved by the ‘desc.var’ object in the HCPC function which provided ‘v-test’ values indicating the average of a variable in the cluster (mean in category) as lower or greater than the average of the variable for the whole data set (overall mean). All variables in the data set measured at 14 and 21 DAS (traits under free-draining) and 49 DAS (traits under waterlogging) were used to describe clustered groups. A variable correlation plot of the first two principal components (PC1 and PC2) was produced to visualize the interrelationships of the variables and to sort them by importance for waterlogging tolerance. Finally, a PCA biplot was produced to visualize the interrelationship of eco-geographic variables of collection sites (passport data and BIOCLIM) in clustered groups.

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 49 DAS among ssp. *yaninicum* genotypes. They were also calculated between plant traits and eco-geographic (passport data and BIOCLIM) variables of collection sites.

**Results**

Variation for seed and seedling traits measured at 14 and 21 DAS

Broad variation with highly significant genotype differences was observed for mean seed weight,
DFF and all seedling traits measured at 14 and 21 DAS (0.01 < \( P < 0.001 \)) (Table 3; Supplementary data Table S3). Mean seed weight was highly heritable (\( H^2 = 94\% \)) and ranged from 8.6 to 13.6 mg. All seedling traits measured at 14 and 21 DAS also showed high heritability \( H^2 \geq 47\% \), except root DW at 21 DAS. At 14 DAS, cotyledon size ranged from 0.5 to 0.6 cm\(^2\), cotyledon DW from 1.7 to 2.8 mg and hypocotyl length from 9.1 to 13.3 mm, while at 21 DAS leaf size ranged from 2.5 to 3.6 cm\(^2\), petiole length from 3.6 to 5.7 cm, shoot DW from 74.9 to 111.2 mg and root DW from 37.6 to 52.2 mg (Table 3).

Variation between genotypes with amber and black-coloured seeds

The only significant differences between amber and black-seeded genotypes were for leaf size and petiole length at 21 DAS (both \( P < 0.05 \)) and surface root proliferation at 49 DAS (\( P < 0.01 \)) under waterlogging (Supplementary data Table S4). Surface root proliferation in black-seeded genotypes was significantly higher than amber-seeded genotypes, largely attributable to Ecotypes G and H (Supplementary data Table S4).

### Table 3

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

| Trait                | Mean  | Minimum | Maximum | \( H^2 \) (%) | \( P \)-value |
|----------------------|-------|---------|---------|---------------|---------------|
| **Mean seed weight (mg)** | 8.61  | 5.60    | 13.60   | 94            | ***           |
| **At 14 DAS**        |       |         |         |               |               |
| 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            | ***           |
| **At 21 DAS**        |       |         |         |               |               |
| Leaf size (cm\(^2\)) | 2.53  | 1.69    | 3.60    | 64            | ***           |
| Petiole length (cm)  | 3.62  | 1.92    | 5.70    | 71            | ***           |
| Shoot DW (mg)        | 74.92 | 49.45   | 111.18  | 56            | ***           |
| Root DW (mg)         | 37.57 | 24.28   | 52.23   | 24            | ***           |
| **Days to flowering**| 123   | 100     | 149     | 94            | ***           |
| DFF (days)           |       |         |         |               | ***           |

For all traits, one-way ANOVA showed a significant effect of genotype (\( P < 0.001 \)). 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 in 2019 at Shenton Park, Western Australia (31°57’S, 115°5’E).

There was a significant treatment \( \times \) genotype interaction for both shoot and root RGRs (both \( P < 0.05 \)) for 32 ssp. *yanninicum* genotypes (Fig. 1). Shoot RGRs under waterlogging ranged from 87 to 108% of controls, while root RGRs ranged from 80 to 116% of controls (Supplementary data Table S5).

Waterlogging reduced RGR compared to controls for four for shoot RGR and 13 genotypes for root RGR (Fig. 1; Supplementary data Table S5). Several genotypes showed higher RGRs of either shoots and roots under waterlogging, but only Ecotype D showed significantly higher RGR of both shoots (\( P < 0.05 \)) and roots (\( P < 0.01 \)) among all other genotypes. In contrast, both shoot and root RGRs of ssp. *subterraneum* cv. Seaton Park (82% and 73% of controls, respectively) and *brachycalyction* cv. Antas ssp. (87% and 85%, respectively) were severely decreased by waterlogging compared to their respective controls (Fig. 1; Supplementary data Table S5). For *T. michelianum* cv. Frontier, both shoot and root RGRs were unaffected by waterlogging (99% and 97% of controls, respectively).
Shoot and root DW

There was a significant treatment × genotype interaction for both shoot ($P<0.05$; Fig. 2a) and root ($P<0.001$, Fig. 2b) DWs among the *A. yanninicum* genotypes. Marked variation was observed under waterlogging for both shoot (66–120% of controls) and root (58–137%) DWs (Supplementary data Table S5).
Waterlogging significantly reduced shoot DWs in only four genotypes compared to controls, but reduced root DWs in 13 genotypes. Ecotype D again showed superior tolerance to waterlogging with both shoot ($P < 0.05$) and root ($P < 0.01$) DWs significantly higher under waterlogging (115% and 133% of controls, respectively) (Fig. 2). In contrast, both shoot and root DWs of ssp. *yanninicum* genotypes, consisting of 4 cultivars (hatched) and 28 ecotypes, along with ssp. *subterraneum* cv. Seaton Park, ssp. *brachycalycinum* cv. Antas and *T. michelianum* cv. Frontier after 28 days of treatment (mean ±SE n=4). Treatments were imposed at 21 days after sowing: free-draining (control) and waterlogged (WL, water level kept 10 mm above the soil surface). Two-way ANOVA results are given in each panel. The single cultivars of ssp. *subterraneum*, ssp. *brachycalycinum*, and *T. michelianum* are not included in the statistical analyses. The significant differences ($P < 0.05$; Fisher LSD test) between control and WL 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 WL treatment was higher). Genotypes are ordered from negative to positive impact of WL on shoot relative growth rate (see Fig. 1)
**Leaf size and petiole length**

For leaf size, there was a significant treatment × genotype interaction \((P < 0.001; \text{Fig. 3a})\) among genotypes of *ssp. yanninicum*. Under waterlogging, leaf size ranged from 76 to 130% of controls (Supplementary data Table S5). Waterlogging significantly reduced leaf size in only one genotype (Ecotype E), compared to its control \((P < 0.01)\) (Fig. 3a; Supplementary data Table S5). In contrast, five ecotypes had significantly larger leaves than their respective controls. Leaf sizes for both *ssp. subterraneum* cv. Seaton Park (49% of control) and *brachycalycinum* cv. Antas (67% of control) were severely reduced by waterlogging, whereas

Fig. 3 (a) Leaf size and (b) petiole length for 32 *ssp. yanninicum* genotypes, consisting of 4 cultivars (hatched) and 28 ecotypes, along with *ssp. subterraneum* cv. Seaton Park, *ssp. brachycalycinum* cv. Antas and *T. michelianum* cv. Frontier after 28 days of treatment (mean ±SE \(n=4\)). Treatments were imposed at 21 days after sowing: free-draining (control) and waterlogged (WL, water level kept 10 mm above the soil surface). Two-way ANOVA results are given in each panel. The single cultivars of *ssp. subterraneum*, *ssp. brachycalycinum*, and *T. michelianum* are not included in the statistical analyses. The significant differences \((P < 0.05; \text{Fisher LSD test})\) between control and WL 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 WL treatment was higher). Genotypes are ordered from negative to positive impact of WL on shoot relative growth rate (see Fig. 1). Note the y-axis scale change between the panels.
leaf size in *T. michelianum* cv. Frontier (94% of control) was unchanged by the waterlogged treatment.

For petiole length, there was a significant treatment × genotype interaction (*P* < 0.01; Fig. 3b) among ssp. *yanninicum* genotypes. Petiole length ranged from 63 to 105% of controls (Supplementary data Table S5). Waterlogging reduced petiole length in 13 out of 32 genotypes compared to controls including cultivars Meteora, Yarloop and Trikkala. Petiole length in the remaining 19 ssp. *yanninicum* genotypes was unaffected by waterlogging and did not differ from their controls. Petiole lengths for ssp. *subterraneum* cv. Seaton Park (58%), ssp. *brachycalycinum* cv. Antas (77%) and *T. michelianum* cv. Frontier (77%) were significantly reduced compared to their respective controls. Furthermore, a visual observation indicated a hyponastic growth response of all ssp. *yanninicum* under waterlogged conditions (Fig. 4), but this effect was not quantified.

**Surface root proliferation**

There was a significant genotype difference (*P* < 0.001) for surface root density under waterlogging among genotypes of ssp. *yanninicum* (Fig. 5; Supplementary data Table S5). Surface roots were observed in all genotypes, irrespective of their waterlogging tolerance. The highest surface root proliferation scores occurred in two of the black-seeded ecotypes: H (score 10.0) and G (score 9.4). The proliferation scores of surface roots of the other genotypes ranged from 1 to 7.

**Chlorophyll concentration**

There was a significant treatment × genotype interaction (*P* < 0.001; Fig. 6) for SPAD values among genotypes of ssp. *yanninicum*. SPAD values under waterlogging were reduced significantly (0.01 < *P* < 0.001) for all genotypes compared to their controls (Supplementary data Table S5). SLW in 16 genotypes (except Ecotype K) remained constant between control and waterlogged treatments (Supplementary data Table S6).

**Multivariate analysis: hierarchical clustering on principal components (HCPC) and interrelationships of traits**

The output of the HCPC function produced by the two first principal components (PC) suggested partitioning of the ssp. *yanninicum* genotypes into three cluster groups: Group I (cv. Larisa, cv. Trikkala and Ecotypes A, D, O, P, S and AB), Group II (cv. Meteora and Ecotypes B, C, F, H, I, J, K, L, T, U, V, W, X, Y, Z and AA) and Group III (cv. Yarloop and Ecotypes E, G, M, N, Q and R) (Fig. 7). This clustering explains 58% of the total variance in the dataset. A dendrogram of hierarchical clustering (showing the optimal level of division suggested by the HCPC function in a solid black line), contribution of variables to PC1 and PC2 and scree plot for the dimensionality of the data are shown in Supplementary data Fig. S4.

Descriptions of each cluster group with characterized variables (the output of the v-test) are given in Table 4. Genotypes in Group I had the highest

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**Fig. 4** Ecotype T at 49 days after sowing (DAS) showing (a) prostrate growth under free-draining condition (control) and (b) hyponastic growth under waterlogged (WL) condition. The WL treatment was imposed at 21 DAS and the water level was kept 10 mm above the soil surface

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Fig. 5  Surface root proliferation after 28 days of waterlogging for 32 ssp. *yanninicum* genotypes, consisting of 4 cultivars (hatched) and 28 ecotypes, along with ssp. *subterraneum* cv. Seaton Park, ssp. *brachycalycinum* cv. Antas and *T. michelianum* cv. Frontier grown under waterlogging (WL, mean ± SE n = 4). The WL treatment was imposed at 21 days after sowing (water level kept 10 mm above the soil surface). Surface roots were scored on a 1–10 visual rating scale with 1 the lowest and 10 the most vigorous growth. The one-way ANOVA result is given in the panel. Genotypes are ordered from negative to positive impact of WL on shoot relative growth rate (see Fig. 1).

Fig. 6  SPAD values for 32 ssp. *yanninicum* genotypes, consisting of 4 cultivars (hatched) and 28 ecotypes, along with ssp. *subterraneum* cv. Seaton Park, ssp. *brachycalycinum* cv. Antas and *T. michelianum* cv. Frontier after 28 days of treatment (mean ± SE n = 4). Treatments were imposed at 21 days after sowing: free-draining (control) and waterlogged (WL, water level kept 10 mm above the soil surface). Two-way ANOVA results are given in the panel. The single cultivars of ssp. *subterraneum*, ssp. *brachycalycinum*, and *T. michelianum* are not included in the statistical analyses. Genotypes are ordered from negative to positive impact of WL on shoot relative growth rate (see Fig. 1).
waterlogging tolerance, with above average means for both shoot and root RGRs and DWs (Fig. 7; Table 4). Notably, Ecotype AB in Group I is distinctive from the other genotypes in Group I. Group II is characterized by below average means for seed weight and seedling traits measured at 14 and 21 DAS, but had average means for waterlogging traits (49 DAS). Group III is characterized by higher than average mean seed weights and seedling traits (14 and 21 DAS), but lower than average means for waterlogging traits (49 DAS).

Among all measured traits, shoot and root RGRs had the strongest positive correlation ($r = 0.86; P < 0.001$) (Fig. 8; Supplementary data Table S7). Under waterlogging, petiole length had strong positive correlations to both shoot ($r = 0.59; P < 0.001$) and root ($r = 0.57; P < 0.001$) RGRs, while leaf size had a high positive correlation with shoot RGR ($r = 0.55; P < 0.01$). Surprisingly, neither SPAD value nor surface root proliferation were significantly correlated with shoot and root RGRs under waterlogging.

None of the seedling traits measured at 14 and 21 DAS contributed to waterlogging tolerance, that is, they did not show a significant positive correlation with any of the waterlogging traits measured at 49 DAS (Fig. 8; Supplementary data Table S7). On the contrary, seedling traits had negative correlations with waterlogging traits. Thus, genotypes of ssp. yunninicum with larger seeds (and hence, bigger cotyledons) and vigorous seedlings under free-draining conditions with larger leaves and longer petioles at 21 DAS showed a low RGR under waterlogging (Fig. 8; Supplementary data Table S7). Furthermore, DFF, SPAD value and surface root proliferation had low contributions to the first two PCs (Fig. 8). Therefore, DFF, all seedling traits measured at 14 and 21 DAS, SPAD value and surface root proliferation at 49 DAS were excluded from analyses of phenotypic plasticity to assess waterlogging tolerance among ssp. yunninicum genotypes. Furthermore, shoot and root DWs were also excluded, as the calculation of both shoot and root RGRs were based on dry weights. Hence, traits used for phenotypic plasticity analyses were shoot and root RGRs at 49 DAS, leaf size and petiole length at 49 DAS.

**Phenotypic plasticity in response to waterlogging**

The relative distance plasticity index (RDPI) Shoot RGR, leaf size and petiole length, but not root RGR, expressed significant differences ($0.01 < P < 0.001$) in RDPI among 32 ssp. yunninicum genotypes (Fig. 9). The highest level of plasticity was observed for petiole length, followed by leaf size.

Among all genotypes, the RDPI values in Group III (in particular Ecotypes E, R and N) and Ecotypes AB and S in Group I, consistently showed high plasticity for shoot and root RGRs, leaf size and petiole length (Supplementary data Table S8). Ecotype AB was more plastic than the other genotypes in Group I, especially for shoot RGR, leaf size and petiole length (Supplementary data Table S8).

**Multivariate plasticity index** MVPi values calculated for the combination of shoot and root RGRs, leaf size and petiole length measured at 49 DAS varied significantly among ssp. yunninicum genotypes (Fig. 10 and Supplementary data Table S8). Genotypes in Group III (except Ecotype M) tended to have high MVPi values (higher phenotypic plasticity), while genotypes in Group I (except Ecotypes AB and S) and Group II (except Ecotypes AA and U) tended to have with low MVPI values. Ecotypes in Group III (except Ecotype M) and Ecotype AB in Group I showed similarly large plasticity, but the direction of response to waterlogging was opposite (positive for Group I and negative for Group III) (Table 4).
Table 4  Description of cluster groups: output of v-test

| Variables                                    | Unit         | Mean in cluster | Overall mean | P-value |
|----------------------------------------------|--------------|-----------------|--------------|---------|
| Cluster group I                              |              |                 |              |         |
| Shoot dry weight (49 DAS)                    | % of control | 109.0           | 93.4         | ***     | +       |
| Root relative growth rate (49 DAS)           | % of control | 102.6           | 93.7         | ***     | +       |
| Root dry weight (49 DAS)                     | % of control | 105.7           | 85.1         | ***     | +       |
| Shoot relative growth rate (49 DAS)          | % of control | 103.1           | 97.7         | ***     | +       |
| Mean seed weight                             | mg           | 9.9             | 8.6          | n.s.    | +       |
| Cluster group II                             |              |                 |              |         |
| Hypocotyl length (14 DAS)                    | mm           | 8.5             | 9.1          | ***     | -       |
| Petiole length (21 DAS)                      | cm           | 3.3             | 3.6          | ***     | -       |
| Root dry weight (21 DAS)                     | mg           | 34.0            | 37.6         | ***     | -       |
| Leaf size (21 DAS)                           | cm²          | 2.3             | 2.5          | ***     | -       |
| Cotyledon dry weight (14 DAS)                | mg           | 1.5             | 1.7          | ***     | -       |
| Cotyledon size (14 DAS)                      | cm²          | 0.4             | 0.5          | ***     | -       |
| Shoot dry weight (21 DAS)                    | mg           | 63.5            | 74.9         | ***     | -       |
| Mean seed weight                             | mg           | 7.2             | 8.6          | -       | -       |
| Cluster group III                            |              |                 |              |         |
| Cotyledon size (14 DAS)                      | cm²          | 0.5             | 0.5          | ***     | +       |
| Leaf size (21 DAS)                           | cm²          | 3.0             | 2.5          | ***     | +       |
| Petiole length (21 DAS)                      | cm           | 4.5             | 3.6          | ***     | +       |
| Shoot dry weight (21 DAS)                    | mg           | 94.3            | 74.9         | ***     | +       |
| Cotyledon dry weight (14 DAS)                | mg           | 2.1             | 1.7          | ***     | +       |
| Mean seed weight                             | mg           | 10.7            | 8.6          | ***     | +       |
| Root dry weight (21 DAS)                     | mg           | 44.0            | 37.6         | ***     | +       |
| Leaf size (49 DAS)                           | % of control | 94.2            | 104.8        | ***     | -       |
| Root dry weight (49 DAS)                     | % of control | 68.9            | 85.1         | ***     | -       |
| Root relative growth rate (49 DAS)           | % of control | 86.2            | 93.7         | ***     | -       |
| Petiole length (49 DAS)                      | % of control | 73.9            | 84.7         | ***     | -       |
| Shoot dry weight (49 DAS)                    | % of control | 78.7            | 93.4         | ***     | -       |
| Shoot relative growth rate (49 DAS)          | % of control | 91.9            | 97.7         | ***     | -       |

Treatments were imposed after 21 days after sowing (DAS): free-draining (control) and waterlogged (WL, water level kept 10 mm above the soil surface). The significant differences between mean of the cluster group and the overall mean are shown; ***P < 0.001. Symbol (+) indicates a higher and (–) indicates a lower mean of the cluster group than the overall mean. Traits used to describe cluster groups were measured at 14 and 21 DAS under free-draining conditions and at 49 DAS under control and WL conditions.

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

The relationships between ecotypes and 22 eco-geographic variables are shown in Fig. 11. The first two PCs in the biplot explain 65% of the total variance in the dataset. There was no distinct partitioning for cluster Groups I, II and III in terms of eco-geographic variables at the site of origin (Fig. 11). Neither shoot nor root RGRs correlated with any eco-geographic variables (Fig. 11; Supplementary data Table S9 and Fig S5), except weak positive correlations of shoot RGR with temperature variables: BIO5 ($r = 0.42; P < 0.05$), BIO9 ($r = 0.43; P < 0.05$) and BIO10 ($r = 0.44; P < 0.05$). These were associated with only a small number of genotypes in Group I (including AB, D and A) with high tolerance to waterlogging (Fig. 11).
Discussion

High variation for waterlogging tolerance, as assessed by the maintenance of shoot RGR relative to free-draining conditions, was evident among ssp. *yanninicum* genotypes, supporting the first hypothesis of this study. It is noteworthy that the highest tolerance to waterlogging was observed among wild ecotypes rather than commercialized cultivars, with several ecotypes displaying similar or greater growth under waterlogging than free-draining conditions. Ecotypes D (CPI 039315 YC) and AB (LO0751) showed a superior waterlogging tolerance among genotypes. The high tolerance in Ecotype AB is attributable to its large plastic response, demonstrating its enhanced fitness to waterlogged conditions. Overall, many genotypes of ssp. *yanninicum* tolerated transient waterlogging that had relatively stable growth with low MVPi values, a general plasticity, showing little distance between waterlogged and free-draining conditions, particularly genotypes in cluster Groups I and II. In contrast, high MVPi values for genotypes in cluster Group III demonstrated that these genotypes were vulnerable to waterlogging display large phenotypic changes in response to waterlogging stress. The study identified genetic resources with a range of ecotypes that could be used as parents in plant breeding programs to improve tolerance to waterlogging in subclover.

Relationships of variation for waterlogging tolerance with phenotypic traits

The second hypothesis, that the variation in waterlogging tolerance is related to phenotypic and growth trait differences, was supported. High genetic variation existed among ssp. *yanninicum* genotypes in seed and seedling traits measured at 14 and 21 DAS as well as maturity duration. However, these highly heritable, readily measurable traits (e.g. seed size, seed colour, seedling traits and maturity duration) did not contribute to waterlogging tolerance. Palta et al. (2010) also found that vigorous early growth of seedlings is unrelated to tolerance to waterlogging in chickpea. This may reflect genotypes with lower initial shoot biomass needing to improve their growth rates above the soil surface in order to respond to soil waterlogging. An increased proportion of aerial organs enables higher oxygen uptake and greater exchange of...
The multivariate plasticity index (MVPi) calculated for the combination of shoot relative growth rate (RGR), root RGR, leaf size and petiole length at 49 days after sowing (DAS) for 32 ssp. yanninicum genotypes, consisting of 4 cultivars and 28 ecotypes (mean±SE, n=4). Treatments were imposed at 21 DAS: free-draining (control) and waterlogged (WL, water level kept 10 mm above the soil surface). Cluster groups are indicated by colour: Group I (red), Group II (green) and Group III (blue). Different letters indicate significant differences among genotypes (P<0.05; Fisher LSD test) and result of one-way ANOVA is given in panel. Genotypes are ordered from negative to positive impact of WL on shoot relative growth rate (see Fig. 1).

PCA biplot of eco-geographic variables and for 29 ssp. yanninicum genotypes, consisting of two cultivars (Trikkala and Yarloop excluded) and 27 ecotypes (Ecotype E excluded). Cluster groups are indicated by colour: group I (red), group II (green) and group III (blue). The eigenvectors (length indicates relative importance) are: 1–19, BIO1-BIO19 (See Table 2 for a code definitions of BOCLIM variables); 20, latitude; 21, longitude; 22, altitude.
explained sensitivity to waterlogging are now discussed in more detail.

**Shoot and root growth**

Root growth was more affected by waterlogging than shoot growth in ssp. *yanninicum*. This supports the assertions of Colmer and Voesenek (2009), Herzog et al. (2016), Nakai et al. (2009) and Striker (2012a) that soil waterlogging directly effects root system growth and function. 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). All genotypes developed surface roots in response to waterlogging, as also found by Francis and Devitt (1969) and Enkhbat et al. (2021b). Enkhbat et al. (2021b) demonstrated that under waterlogging subclover forms lateral roots positioned near the better-aerated soil surface with improved porosity. Root proliferation near to soil surface demonstrates that roots are highly plastic and this plasticity strength enables ssp. *yanninicum* to cope with waterlogging by enhancing genotype fitness.

**Leaf size and petiole length**

Leaf growth among genotypes of ssp. *yanninicum* was little affected by waterlogging, while petiole length was often reduced. Francis and Devitt (1969) found no reduction in leaf size among 25 genotypes of ssp. *yanninicum* under waterlogging. The relative stability of leaf growth might be mediated through substantial plasticity of physiological characters (Schlichting 1986) as demonstrated in this study by a significant reduction in SPAD value, with characteristic yellowing of the leaves and reduction of SPAD values. Observed high positive correlations between both leaf and petiole growth with shoot growth under waterlogging could be explained as a snorkelling effect (part of the shoot protruding into the air), by enhancing internal aeration whereby the air-exposed tissues conduct oxygen from the atmosphere to oxygen-deprived below-ground tissues (Herzog and Pedersen 2014). Reduction of petiole length under waterlogging is a relatively simple, inexpensive and non-destructive measurement, can be used as an indicator of waterlogging stress in ssp. *yanninicum* and can be used as preliminary selection tool to screen large number of genotypes for waterlogging tolerance in plant breeding programs.

**Relationships of variation for waterlogging tolerance with eco-geographic variables**

Variation in waterlogging tolerance of ssp. *yanninicum* showed no relationship with any of the eco-geographic variables. Thus, the third hypothesis was not supported. This result could be attributed to the restricted distribution of ssp. *yanninicum* as its natural habitat is reported to be low altitude (below 1400 m a.s.l) coastal areas (often flat meadows) with high mean annual rainfall (>450 mm) and mild winter temperatures (Francis 1976; Ghamkhar et al. 2015). The distribution of ssp. *yanninicum* in lower profiles of the landscape, which tend to collect more water, could be a reason for ssp. *yanninicum* having inherent features conferring adaptation to waterlogging compared to ssp. *brachycalyicum* and ssp. *subterraneum*, which are found in elevations up to 2190 and 2940 m a.s.l, respectively (Ghamkhar et al. 2015).

**Plant breeding implications**

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. However, as results could differ in field environments (Villemereuil et al. 2015), field studies under waterlogged conditions are required to validate our results. Our approach could be strengthened with focus on other key responses to waterlogging such as shoot physiological responses, and changes in root anatomical and morphological responses. Finally, exploring post-waterlogging stress among genotypes of ssp. *yanninicum* is crucial to identify genotypes with better performance after waterlogging subsides.

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