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Grass pea (*Lathyrus sativus*) interesting panoply of mechanisms to cope with contrasting water stress conditions – a controlled study of sub populational differences in a worldwide collection of accessions

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A R T I C L E I N F O

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A B S T R A C T

The grain legume *Lathyrus sativus* L. (grass pea) is of high economic importance for food and feed in Asian and African developing countries and is part of cultural heritage of marginal areas in the Mediterranean region. Its outstanding robustness under adverse environmental conditions, when compared with other legume species, raises particular interest under the current climate change scenario. The aim of this work was to study the range, extent and mechanisms underlying this species' reported tolerance to water stress. To achieve this, a worldwide representative collection of 194 grass pea accessions was subjected to specific well-watered, water deficit and waterlogging conditions. These conditions were defined previously to elicit differential physiologic responses among the collection on 21 days old plants as continuously kept at 95% field capacity, at 5 days without irrigation reaching 55% field capacity, and at 14 days of partial submergence, respectively. The assessment encompassed a comprehensive set of 20 traits, including gas exchange, chlorophyll a fluorescence, leaf photosynthetic pigments, leaf water status and biomass partitioning. This detailed phenotypic analysis demonstrated that leaf relative water content and gas-exchange related traits are particularly useful to differentiate accessions' response to water treatments. We discovered that accessions with light and large seeds mostly of Mediterranean and East European origins produce plants with high total dry biomass, a trait that is mostly genetically determined and less affected by the water treatment. On the other hand, it is mainly dark and small seeded accessions that show advantageous physiological responses to water deficit and/or waterlogging, according to pigment contents, root to shoot ratio and water use efficiency traits. This integrative screening also allowed the identification of accessions displaying contrasting levels of tolerance and susceptibility to each stress (five tolerant and five susceptible to each stress), plus one multi-tolerant (PI283546) and one multi-susceptible (PTLS1001) accession. A comprehensive and deep understanding of grass pea's water stress tolerance mechanisms is the first step for a more efficient exploitation of this grain legume as a model crop for multiple abiotic stress resistance studies and opens doors for further progresses on crop breeding efforts towards securing food and feed supplies.

1. Introduction

Within the current climate change scenario, several agricultural regions are more prone to severe drought and extreme flood events (FAO, 2017). Identifying crop sources of abiotic stress tolerance and associated mechanisms and genetics, is therefore a priority for more efficient multi-stress tolerance breeding efforts (Chaves and Oliveira, 2004; Duc et al., 2015; Fukao et al., 2019).

Lathyrus sativus L. (grass pea) is a highly climate resilient (Gonçalves et al., 2022; Lambein et al., 2019) and an economically important annual feed and food grain legume in both Asian and African developing countries, with a growing interest in the Mediterranean region (Dixit

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et al., 2016; Vaz Patto et al., 2015). Its robustness under adverse conditions, in particular to both water deficit and waterlogging stresses, salinity and temperature extremes (Araújo et al., 2015; Dixit et al., 2016; Malik et al., 2015; Tokarz et al., 2020; Vaz Patto et al., 2006; Wiraguna et al., 2020) has been linked with its content on β-Oxalyldiaminopropionic acid (β-ODAP), a naturally occurring amino acid derivative (Jiao et al., 2011; Xing et al., 2001). In addition, improved performance of the antioxidant machinery and osmotic adjustment mechanisms have been pointed out as a possible basis for the exceptional stress resilience of grass pea (Jiang et al., 2013; Malik et al., 2015; Piwowarczyk et al., 2017; Talukdar, 2012; Tokarz et al., 2020; Zhou et al., 2016).

Both cold-season and warm-season grain legumes production may happen under rain-fed conditions (Daryanto et al., 2015). Water stress episodes, depending on its duration and timing along the growing season, impact the vitality, productivity and yield of the plant. Extended periods of drought, intermittent rainfall, as well as transient partial or total submergence can severely reduce grain legumes potential yields, especially if happening during germination and pod filling phases (Araújo et al., 2015; Blessing et al., 2018; Daryanto et al., 2015; Malik et al., 2015; Wiraguna et al., 2021; Zaman et al., 2019).

Optimizing water use under variable environmental conditions involves a complex interaction between plant morphology, physiology, and biochemistry. Ultimately, instantaneous water use efficiency (WUA), calculated as the carbon assimilation rate (A) over transpiration rate (E) can provide a good indicator of the plant water relations at any given moment and is not uncommon that breeding programs aim at varieties with stable WUE (Araújo et al., 2015), since this trait directly impacts pod yield (Vadez and Ratnakumar, 2016).

Legumes, like all plants in general, primarily sense water scarcity in the roots and, by abscisic acid mediation, stomatal closure is triggered as one of the first responses (Wilkinson and Davies, 2002). This compromises gas exchange and photosynthesis (Basu et al., 2016; Dubois and Inzé, 2020; Takahashi and Shinozaki, 2019). This was the case in 8-week-old *Medicago truncatula* plants subjected to a short 4–5 days period of suspended irrigation (Nunes et al., 2009), and in vegetative stage common bean (*Phaseolus vulgaris* L.) plants after 24 days of no-watering (Mathobo et al., 2017) or at 40% pot soil-field capacity water deficit (Leitão et al., 2021a).

Leaf morphologic adaptations to water deficit such as leaf movement, rolling and shedding, and decrease in leaf number and size, have been commonly observed among legumes as well as other crops (Karamanos, 1978; Khatun et al., 2021; Laxa et al., 2019; O'Toole and Cruz, 1980; Puglielli et al., 2019; Silva Lobato et al., 2016). These adaptations contribute to decreasing transpiration rate and, therefore, improving instantaneous WUE and maintaining leaf relative water content (Chaves et al., 2003). Abscisic acid also induces modifications in the root systems to increase surface for water uptake, namely root densification, which is a common drought avoidance strategy (Eziz et al., 2017), present in some legumes (Nunes et al., 2008), including grass pea (Piwowarczyk et al., 2017).

Waterlogging conditions primarily cause hypoxia stress at the root level, imbalances on oxygen levels and entrapment (hence accumulation) of ethylene in the roots as first and main triggers of the overall plant response (Sasidharan and Voesenek, 2015). A switch from aerobic respiration to fermentation occurs, leading to hampered growth and development of aerial parts (Jia et al., 2021; Jiang et al., 2013; Karamanos, 1978; Palta et al., 2010; Pan et al., 2019) and eventually to plant death (Sasidharan and Voesenek, 2015).

Specifically at the leaf level, stomatal closure and consequent decrease of gas-exchange rate are frequently observed in legumes under waterlogging conditions (Bansal and Srivastava, 2015; Garcia et al., 2020; Posso et al., 2018), as well as in non-legume species (Bradford and Hsiao, 1982; Gil et al., 2009; Rodríguez-Gamir et al., 2011). Photosynthetic impairments often come along with damage at photosystem level (Maricle and Caudle, 2012), and the transpiration decrease further impairs water conductivity, leading to deficiencies in plant absorbance of essential nutrients (Akhtar and Nazir, 2013; Ashraf, 2012).

Solaiman et al. (2007) and Malik et al. (2015) carried out two similar studies comparing the response of several legume seedlings subject to transient waterlogging plus recovery / reaeration periods, and highlighted contrasting responses related to shoot and root growth, nitrogen accumulation, root porosity and adventitious roots formation among the species. Grass pea was reported as being relatively more tolerant to waterlogging stress, compared with pea and lentil (Malik et al., 2015).

Despite the reported grass pea tolerance to both water deficit and waterlogging stresses, there is a considerable gap of knowledge regarding the extent and range of variability of these tolerances, as well as the physiological mechanisms involved and the underlying genetics (Banerjee et al., 2021; Dixit et al., 2016).

The objectives of this work were to set the range of variability in grass pea's physiological response to different water conditions and identify sources of drought and flood tolerance in this legume species. These objectives were attained by quantifying 20 different morpho-physiological parameters in 194 grass pea accessions from a worldwide collection under optimal (well-watered), water deficit and waterlogging conditions. Different phenotypic perspectives, from the individual accession to the subpopulation levels (based on ecotype clustering parameters), were explored.

To the best of our knowledge, this is the first study providing such amount of morpho-physiological data on this species, evaluating its variability of responses to both water deficit and waterlogging stresses. We believe that the insights of this work will help start unravelling the mechanisms behind the reported contrasting water stress tolerance of grass pea, as well as provide useful material for future climate change (multi-water stresses tolerance) breeding programs.

2. Material and methods

2.1. Plant material and growth chamber-controlled conditions

A worldwide collection of 194 *Lathyrus sativus* L. (grass pea) accessions was used in this study. Among these, 87 accessions had a Mediterranean geographical origin, 49 South Asian, 21 East European, 15 Sub-Saharan African, 13 North and Central Asian, 2 Northern American, 2 Southern American and 5 of unknown origin. Within the collection, 92 accessions had light and 102 dark seed color (visually scored); 58 had large and 136 had small seed size. Seed size classification was assigned based on the weight (g) of 100 seeds (below 18 g accessions were considered small, and over 18 g accessions were considered large) (Sampaio et al., 2021). Details on accessions' geographical origin and morphological seed characteristics are shown in Table S1 (Supplementary Material).

Seeds were germinated by placing them on wet filter paper at 4°C in the dark for two days, followed by incubation at 26 ± 2 °C in the dark until germination. Germinated seedlings were transferred to pots (7 × 7 × 8 cm), one per pot, containing 2:1 mixture of universal substrate (turf 95%, green compound 5%, Geolia®) and silica sand (0.4–0.8 mm granulometry, Axton®). Pots were placed in open opaque boxes (450 mm x 270 mm x 250 mm) and grew in a growth chamber under controlled conditions (12 h light/12 h dark photoperiod, 23 ± 01 °C, average top light of 350 μmol/s/m^2, and 50% Relative Humidity).

2.2. Experimental design

The grass pea phenotyping experiment followed a split plot design in randomized complete blocks, with blocks being repeated experiments in rounds, with different water regimes as whole plot treatment and accession as sub plot treatment, with the addition that plants were placed in boxes that were considered to represent an additional incomplete block term (Fig. S1, Supplementary Material). Each box with pots represented an incomplete block containing a subset of 10 accessions (2 plant replicates of each) plus 2 common check accessions (2 plant replicates of each), in such a way that each box contained 24 plants in total. The check accessions, LS87124 and PI568190, were chosen based on contrasting average β-ODAP content in seeds (1.8 and 6.2 mg β-ODAP/g flour, respectively), previously determined from dry seeds harvested from plants grown in the same field environment (Gonçalves, personal communication). The distribution of the boxes in the growth chamber and of the plants within the boxes were randomized.

Plants were watered with tap water according to the treatment – well-watered (WW), water deficit (WD) or waterlogging (WL)- assigned to the respective incomplete block. Regardless of the water treatment imposed, the plants in each box were assessed for several physiologic and morphologic traits at 22 DAG. WW boxes were watered daily, assuring constant soil moisture of about 95% field capacity (FC). WD boxes were similarly watered for 16 days, and watering was withdrawn during the last 5 days until measurement (corresponding to a final soil water content of about 55% FC). WL boxes were well-watered during the first week, and then subjected to waterlogging (partial submergence, with water reaching approximately 1 cm height of the stem) from the 8th day after germination (DAG) until measurement day, hence 14 days of waterlogging stress (Fig. S2, Supplementary Material). The soil water content (SWC) was determined as a weight fraction (Coombs et al., 1987): SWC (%) = 100 * (FW−DW)/DW, where FW was the fresh weight of the soil in each pot and DW was the dry weight of the same soil portion (dried in the oven for at least 4 days, until constant weight). The specific water treatments applied were defined through a preliminary study in which non-lethal levels of stress were investigated, aiming a compromise between plants being stressed, but not too stressed in order to allow the physiological measurements and to detect diversity on their response to the treatments (data not shown).

A total of three experimental rounds were performed (each screening the complete collection of accessions and treatments), each one with 20 incomplete blocks (or boxes), measuring one box each day, what resulted in a total of 6 replicate plants *per* accession, *per* treatment.

2.3. Photosystems and photosynthetic performance assessment

Leaf gas exchange parameters, namely stomatal conductance (gs), transpiration (E) and CO_2 assimilation rate (A), were measured at two photosynthetic active radiations (PAR): 347 μmol/m^2/s and 869 μmol/m^2/s (corresponding respectively to the average light intensity at which the plants are grown and the light intensity close to the maximum A value - Amax - previously determined), using an Infra-Red-Gas-Analyser (IRGA, LCpro+ ADC BioScientific Ltd., Hertfordshire, UK). Instantaneous water use efficiency (WUE = A/E) was calculated for both light intensities. A, E and gs values were adjusted to the leaf area measured inside IRGA's chamber.

Non-destructive measurements of Chlorophyll a fluorescence-related traits, namely the maximum and actual quantum yields of photosystem II – respectively Fv/Fm and Fv/Fo - and performance index expressed on absorption basis – PI_{ABS} - were assessed after a 30 min dark-adaptation period, using an OS-30p+ Chlorophyll Fluorometer (Opti-Sciences).

All the measurements were made between 3 and 6 h after the time set up for light period initiation in the growth chamber, in the top expanded leaf of each plant (i.e. the youngest mature leaf).

2.4. Leaf pigments content

Leaf photosynthetic pigments content - chlorophylls a (C_a), b (C_b), and carotenes + xanthophylls (C_{cx}) - were spectrophotometrically quantified, according to Lichtenthaler (1987), with ethanol (95%) solvent, using leaf segments from the same bifoliate leaf previously used for the IRGA measurements. The sum of C_a and C_b was also calculated (C_{ab}). Pigment concentrations were expressed *per* leaf area unit. A Pharmacia Biotech Ultraspec 4000 UV was used.

In addition, measurements were taken on the same leaves with a SPAD-502 meter (Konica Minolta) and the arithmetic average of the SPAD units calculated for each plant. The SPAD measurement is an index positively correlated with Chlorophyll contents (Coste et al., 2010).

2.5. Leaf water status and biomass partitioning

Following Čatský (1960), leaf relative water content (RWC) was determined for the same leaf used for gas exchange and chlorophyll a fluorescence measurements, i.e. the top expanded leaf of each plant. The plant water status was estimated using the formula RWC = (FW-DW)/(TW-DW), where FW is the fresh weight of a leaf segment with a known area, TW the turgid weight of the leaf segment (after being left for 24 h in water), and DW the dry weight of the leaf segment (dried in the oven for at least 4 days, until constant weight).

Finally, after all the described measurements, plants were uprooted, aerial and root parts separated and carefully washed. Samples were then dried at 80 °C for approximately one week (until constant weight) and dry biomasses (root and aerial) determined. Root to shoot ratio (R/S) and total dry biomass (TDB) were calculated.

2.6. Statistical analysis

Phenotyping data quality control was performed individually for each water treatment (WD, WW and WL) and trait (A_{347}, E_{347}, gs_{347}, A_{869}, E_{869}, gs_{869}, WUE_{347}, WUE_{869}, Fv/Fm, Fv/Fo, PI_{ABS}, RWC, C_a, C_b, C_{cx}, C_{ab}, C_{ab}/C_{cx}, SPAD, R/S and TDB) through graphical inspection of residuals to assess normality and identification of outliers. Linear data transformations were performed whenever needed (Table S2, Supplementary Material).

Linear mixed models were applied to analyse each trait individually, using the model *trait* = *round* + *treatment* + *round.treatment* + *accession* + *treatment.accession* + *incomplete block* + *error*, where *round* is one complete experimental block with all accessions measured in all treatments, *treatment* is the water treatment, *accession* is the genotypic term, *treatment.accession* is the interaction between the accession and the water treatment, *incomplete block* identifies the box in which each plant pot was grown, and *round.treatment* represents the whole plot error.

With the terms *round*, *accession*, *treatment* and *treatment.accession* fitted as fixed terms we obtained the best linear unbiased estimates (BLUEs) for each accession in each treatment, individually per trait. Wald tests for the significance of fixed effects were applied.

With all terms fitted as random, a restricted maximum likelihood (REML) procedure was applied to the model to assess the variance components of the linear mixed model and the broad-sense heritability calculated as Cullis et al. (2006).

To assess whether accessions' variation could be explained by seed color (one of the main grass pea ecotypes clustering parameter), the following linear mixed model was applied: *trait* = *round* + *treatment* + *color* + *treatment.color* (as fixed terms) + *round.treatment* + *incomplete block* (as random terms), where *color* is the seed color class (i.e. light/dark) attributed to the 194 accessions of the collection, and *treatment.color* stands for the interaction between that term and the water treatment. A similar model and data set were used to estimate how much of the accessions' variation was explained by seed *size* and *origin* (the other main grass pea ecotypes clustering parameters), replacing *color* by these terms in the individual analysis. An F-test for the significance of fixed effects and, when applicable, means comparison post hoc LSD tests were performed.

Finally, the BLUEs obtained for all accessions in each trait and treatment were merged in a single data set, Pearson's correlation coefficients were calculated among traits, followed by a principal component analysis (PCA) for an overall multivariate representation.

All data analysis was performed using Genstat® software, 21st edition (VSNI, 2021) and R Studio© (PBC, 2021).

2.7. Identification of contrasting accessions

Delta (Δ) values were calculated for the two water stress treatments (WL and WD) for all the accessions, as the difference between the BLUEs obtained in the respective water stress treatment and the BLUEs for the control treatment (WW), i.e. $\Delta WD = BLUE_{WD}$ - $BLUE_{WW}$ and $\Delta WL = BLUE_{WL}$ - $BLUE_{WW}$. The traits selected for this analysis were: A_{347}, TDB, WUE_{347}, RWC and R/S for ΔWD, and A_{347}, E_{347}, TDB, Fv/Fm and R/S for ΔWL based on literature stating that a more accentuated decrease or stability of these physiologic parameters are reliable indicators of susceptibility or tolerance (respectively) to water stress in plants (Akhtar and Nazir, 2013; Basu et al., 2016; Loreti et al., 2016).

Heatmapper© online tool (Babicki et al., 2016) was used to generate dendrograms and heat maps of trait variation among the accessions, and to visually select representative accessions of susceptible and tolerant responses. For the dendrograms development, the Δ's of A_{347}, TDB and WUE_{347} traits were used as input data for clustering differential WD responses; and the Δ's of A_{347}, E_{347} and TDB traits were used as input data for clustering differential WL responses. The clustering method was set as 'average linkage' and the Euclidean method selected for computing distance.

3. Results

3.1. Contrasting water treatments exposed stress specific ranges of phenotypic variation among the measured traits

We assessed 20 different morpho-physiological parameters, including leaf gas exchange, chlorophyll a fluorescence, pigments content and biomass related traits, in a worldwide representative grass pea collection in response to well-watered (WW), water deficit (WD) and waterlogging (WL) conditions. As a general observation, WD caused mainly withering, prostration of the plants and, not surprisingly, dried out leaves. In terms of stature, WD plants usually had shorter shoots and thinner, but longer, root systems compared to WW plants. In WL, some plants showed yellowish leaves and usually roots were thicker and shorter than in the other water treatments (Fig. S3, Supplementary Material). However, all these visual observations were not uniformly displayed among the different accessions, revealing a clear variability of phenotypic responses under WW, WL and WD within the studied collection.

Gas exchange traits A, E, and gs (at both light intensities) and RWC were the most negatively affected traits under WD; whilst R/S and C_{ab}/C_{cx} were the most negatively affected traits under WL. Regarding WUE (at both light intensities), the range of variability was much higher in

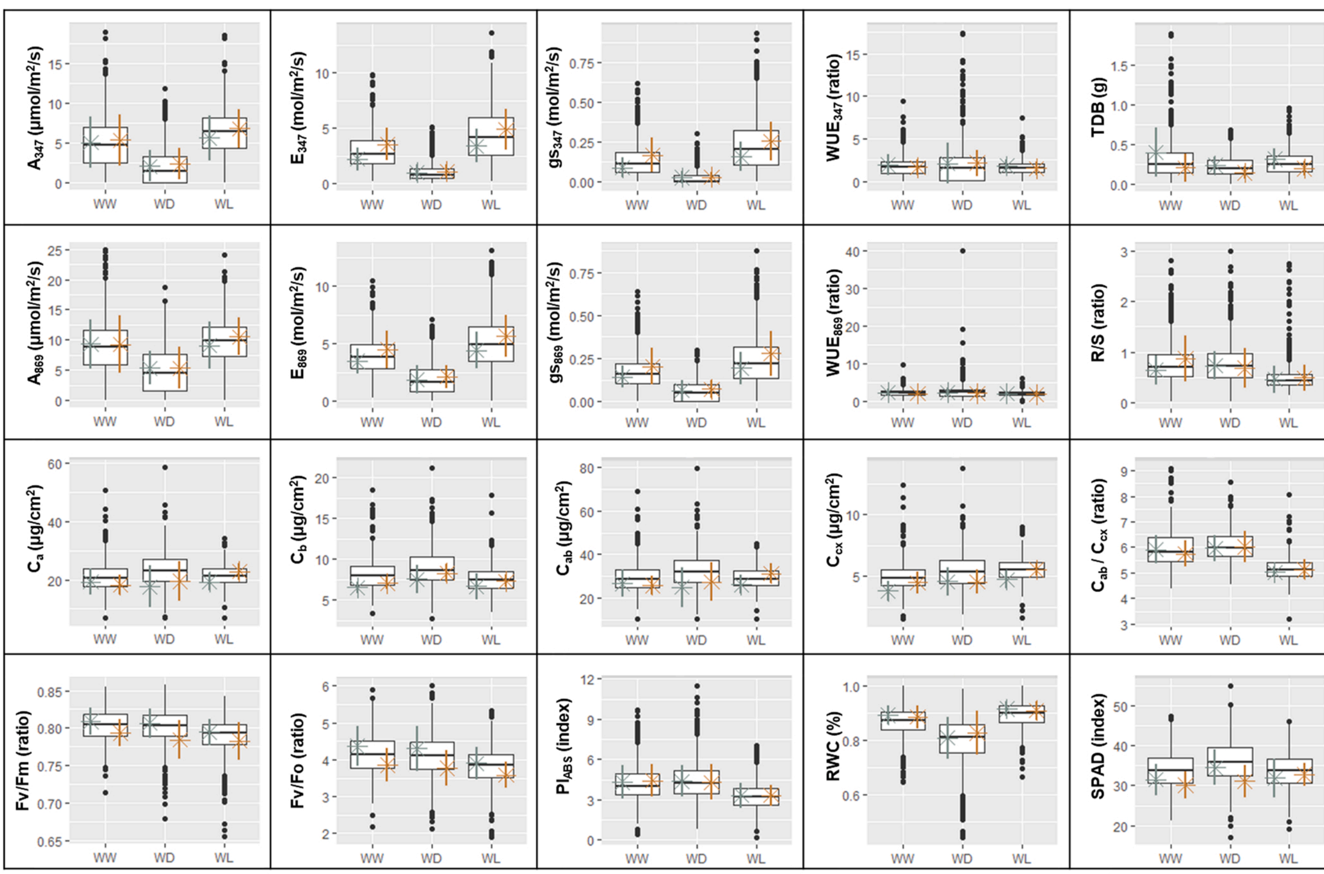

Fig. 1. Boxplot distributions of measured values for A_{347}, E_{347}, gs_{347}, WUE_{347}, A_{869}, E_{869}, gs_{869}, WUE_{869}, Fv/Fm, Fv/Fo, PI_{ABS}, RWC, TDB, R/S, C_a, C_b, C_{ab}, C_{cx}, C_{ab}/C_{cx} and SPAD measured in grass pea plants subject to WW, WD and WL conditions. Average ± standard deviation values of the two check accessions (LS87124 and PI568190) are depicted as stars (grey and orange, respectively) with bars over the boxplots. A347: CO2 assimilation rate at growth light intensity; A869: CO2 assimilation rate at Amax light intensity; E347: transpiration rate at growth light intensity; E869: transpiration rate at Amax light intensity; gs347: stomatal conductance at growth light intensity; gs869: stomatal conductance at Amax light intensity; WUE347: instantaneous water use efficiency at growth light intensity; WUE869: instantaneous water use efficiency at Amax light intensity; TDB: total dry biomass; RWC: leaf relative water content; R/S: root to shoot ratio (dry biomasses); PIABS: performance index on absorbance basis; Fv/Fm: maximum quantum yield of photossystem II; Fv/Fo: actual quantum yield of photossystem II; Ca: chlorophyll a content (spectrophotometrically measured); Cb: chlorophyll b content (spectrophotometrically measured); Cab: chlorophylls a+b content (spectrophotometrically measured); Ccx: xantophyll + carotene content (spectrophotometrically measured); Cab/Ccx: greeness ratio based on spectrophotometrically measured pigment contents; SPAD: greeness index given by SPAD-502 instrument. WW: well-watered; WD: water deficit; WL: waterlogging.

Table 1
Variance components and broad-sense heritability (h^2) for the 20 traits[a] measured in 194 accessions (Acc.) under 3 water treatments (Treat.) tested following a split-plot experimental design.

Trait	Trait mean	Variance components			*Heritability* h^2
		σ^2 *Treat*	σ^2 *Acc*	σ_2 *Treat. Acc*	
A_{347}	1.180 µmol/ m^2/s	4.262	0.265	0.464	0.3065
A_{869}	7.799 µmol/ m^2/s	6.12	0.79	1.26	0.3696
$\sqrt{E_{347}}$	1.537 mol/ m^2/s	0.2967	0.0177	0.0294	0.4594
E_{869}	3.618 mol/ m^2/s	2.441	0.214	0.438	0.4157
$\sqrt{gs_{347}}$	0.130 mol/ m^2/s	0.03272	0.00133	0.00364	0.346
$\sqrt{gs_{869}}$	0.340 mol/ m^2/s	0.01936	0.0013	0.0029	0.3702
$\sqrt{WUE_{347}}$	1.180 (ratio)	0.0004	0.0074	0.0183	0.2118
$\sqrt{WUE_{869}}$	1.414 (ratio)	0.0003	0.0125	0.018	0.3746
TDB	0.269 g	0.00071	0.01016	0.00123	0.8799
RWC	85.1%	0.00244	0.000584	0.00061	0.567
$\sqrt{R/S}$	0.793 (ratio)	0.00678	0.00223	0.00205	0.4297
$\sqrt{PI_{ABS}}$	1.962 (index)	0.02178	0.01271	0.00186	0.649
$(Fv/Fm)^2$	0.640 (ratio)	0.0000661	0.000124	0.0000143	0.6365
Fv/Fo	4.063 (ratio)	0.016	0.0306	0.0037	0.06472
$\sqrt{C_a}$	4.746 µg/ cm^2	0.0047	0.0429	0.0301	0.4671
$\sqrt[3]{C_b}$	2.004 µg/ cm^2	0.0032	0.00449	0.00213	0.5107
C_{ab}	30.957 µg/ cm^2	1.6	7.71	5.18	0.4767
$\sqrt{C_{cx}}$	2.357 µg/ cm^2	0.01075	0.00517	0.01597	0.4933
$\sqrt{C_{ab}/C_{cx}}$	2.347 (ratio)	0.01044	0.00086	0.00135	0.2415
SPAD	35.203 (index)	1.56	5.18	1.9	0.7235

[a] A_{347}: CO_2 assimilation rate at growth light intensity; A_{869}: CO_2 assimilation rate at Amax light intensity; E_{347}: transpiration rate at growth light intensity; E_{869}: transpiration rate at Amax light intensity; gs_{347}: stomatal conductance at growth light intensity; gs_{869}: stomatal conductance at Amax light intensity; WUE_{347}: instantaneous water use efficiency at growth light intensity; WUE_{869}: instantaneous water use efficiency at Amax light intensity; TDB: total dry biomass; RWC: leaf relative water content; R/S: root to shoot ratio (dry biomasses); PI_{ABS}: performance index on absorbance basis; Fv/Fm: maximum quantum yield of photossystem II; Fv/Fo: actual quantum yield of photossystem II; C_a: chlorophyll a content (spectrophotometrically measured); C_b: chlorophyll b content (spectrophotometrically measured); C_{ab}: chlorophylls a+b content (spectrophotometrically measured); C_{cx}: xantophyll + carotene content (spectrophotometrically measured); C_{ab}/C_{cx}: greeness ratio based on spectrophotometrically measured pigment contents; SPAD: greeness index given by SPAD-502 instrument.

WD plants than in WW and WL plants (especially evident for WUE_{347}). Boxplots for the three treatments for all traits, are shown in Fig. 1.

For almost all traits and treatments, both check accessions (LS87124 and PI568190) showed average values within the interquartile range of the respective boxplot, indicating that these accessions' behaviour, in all three water treatments, was not extreme and were adequate as data-normalizers across the screening.

After data transformations (Table S2, Supplementary Material), linear mixed models were applied to analyse each trait individually. In most of them, significant differences between at least two treatments were found. Exceptions were the traits $\sqrt{WUE_{347}}$, $\sqrt{WUE_{869}}$, $(Fv/Fm)^2$, Fv/Fo, TDB, $\sqrt{C_a}$ and C_{ab}. Significant differences among *accessions* and significant *treatment.accession* interactions were detected for all traits, with a significance level of 0.05, not only confirming that there was variability of physiologic performance among accessions, but also indicating that the relative rank of performance of the accessions changed with the watering conditions.

3.2. Gas exchange related traits are highly correlated and together with RWC explain most treatment variability component, while TDB, SPAD and chlorophyll a fluorescence related traits explain most the genetic component

Calculated variance components and broad sense heritability (h^2) for each trait are summarized in Table 1. A higher portion of phenotypic variance was due to differences among *accessions* (σ^2 *Acc*) than to the interaction *treatment.accession* (σ^2 *Treat.Acc*) for SPAD, TDB, $(Fv/Fm)^2$, Fv/Fo, C_{ab}, $\sqrt[3]{C_b}$, $\sqrt{C_a}$, $\sqrt{R/S}$ and $\sqrt{PI_{ABS}}$. The trait with the highest broad sense heritability was TDB, followed by SPAD, $\sqrt{PI_{ABS}}$ and $(Fv/Fm)^2$ (highlighted in Table 1). Therefore, while for these traits we can rely on a big component of the variability being explained by genetics, when looking at gas-exchange related traits, $\sqrt{WUE_{347}}$, $\sqrt{WUE_{869}}$ and RWC we must acknowledge important differences depending on the *treatment*.

A strong correlation (≥0.75) was found among gas exchange traits (A_{347}, $\sqrt{E_{347}}$, $\sqrt{gs_{347}}$, A_{869}, E_{869} and $\sqrt{gs_{869}}$), among chlorophyll fluorescence traits $(Fv/Fm)^2$ and Fv/Fo, and among most spectrophotometric chlorophyll contents ($\sqrt{C_a}$, $\sqrt[3]{C_b}$, C_{ab}, and $\sqrt{C_{cx}}$), when we consider the three water treatments at once (Fig. 2). Interestingly, there were some notorious differences concerning some specific pairwise correlations, when calculated for each treatment separately (Fig. S4, Supplementary Material). For instance, RWC had a stronger positive correlation with gas-exchange traits in WD, while in WW and WL treatments this correlation was negative.

We performed a principal component analysis (PCA) integrating the 20 traits, so that we could more easily visualize the variability and range of responses to the three water treatments on the grass pea collection. The two first principal components explained a total of 54.87% of the observed variance. From the PCA biplot PC-1/PC-2 (Fig. 3) we observe that the three water treatments clustered apart, but with some overlapping among them. The two most differentiated treatments were WL and WD, with WW occupying an intermediate position. The water treatment separation was mainly due to differences in RWC, gas exchange related (A_{347}, $\sqrt{E_{347}}$, $\sqrt{gs_{347}}$, A_{869}, E_{869}, and $\sqrt{gs_{347}}$, WL>WW>WD), Chlorophyll a fluorescence related ($\sqrt{PI_{ABS}}$), $\sqrt{R/S}$ and $\sqrt{C_{ab}/C_{cx}}$ traits (WL<WW<WD), whose eigen vectors had PC-1 as their strongest component (Table S3, Supplementary Material), hence the lack of between-treatments distinction in the biplot PC-2/PC-3. Variation within each treatment was also found for the already mentioned traits, but the main within-treatment variation was due to pigment related ($\sqrt{C_{cx}}$, $\sqrt{C_a}$, $\sqrt[3]{C_b}$, Cab and SPAD), Fv/Fo, $(Fv/Fm)^2$ and TDB traits.

3.3. Seed morphology and geographic origin influence the accessions' phenotype and response to contrasting water treatments

Significant interactions (*p-value* < 0.05) *color.treatment* were detected for most traits, except for $\sqrt{C_{ab}/C_{cx}}$, $\sqrt{R/S}$ and $\sqrt{WUE_{869}}$ (Table S4, Supplementary Material). Also *size.treatment* interactions were significant for all traits except Fv/Fo, $(Fv/Fm)^2$, $\sqrt{C_{ab}/C_{cx}}$ and $\sqrt{R/S}$. A significant phenotypic difference (*p-value* < 0.05) between light and dark seed colors was found for $\sqrt{WUE_{869}}$ and between small and large seed sizes accessions for Fv/Fo and $(Fv/Fm)^2$, despite the lack of significance in the respective *seed trait.treatment* interactions. For instance, light seeds had higher $\sqrt{WUE_{869}}$ and large seeds had higher Fv/Fo and $(Fv/Fm)^2$, across water treatments (Fig. S5, Supplementary Material). Large and light seeds had on average larger TDB (0.41 ± 0.16 g) than small and dark seeds (0.24 ± 0.11 g).

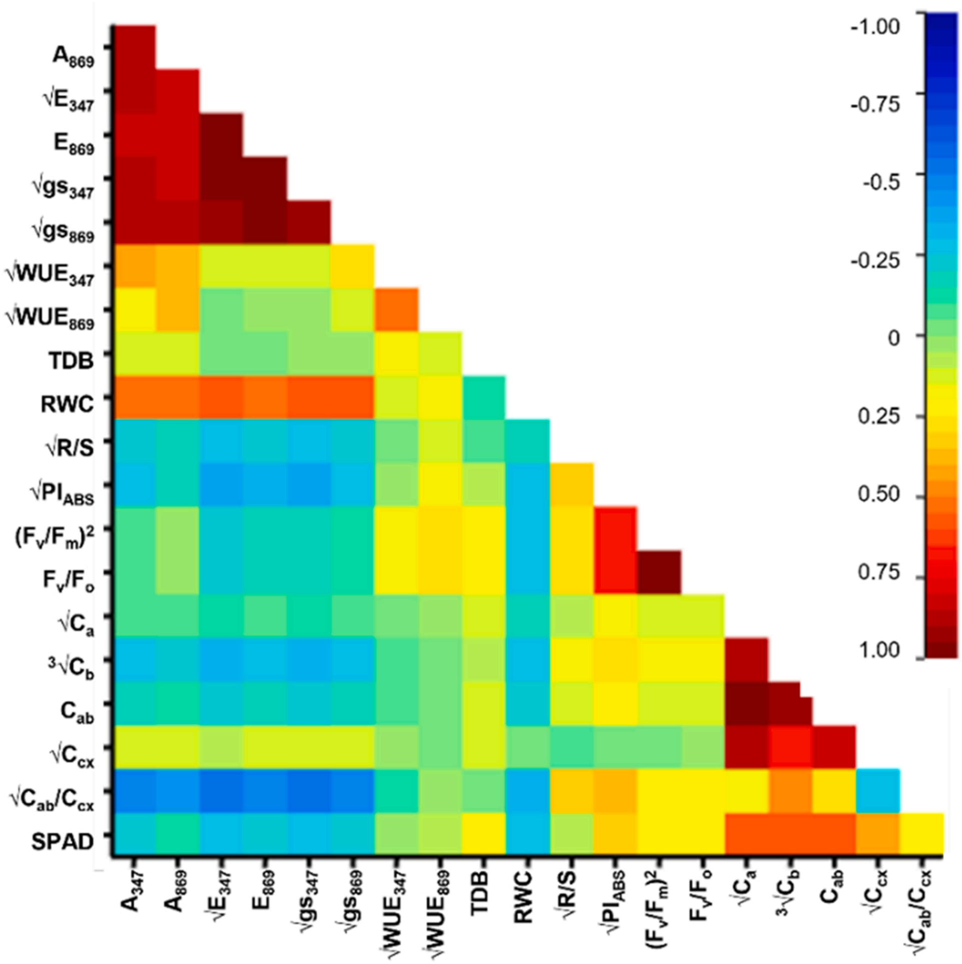

Fig. 2. Correlation matrix heatmap with Pearson coefficients based on BLUEs for 194 grass pea accessions in 3 water treatments (WW, WD and WL), colored by a spectrum from blue (correlation =−1.0) to red (correlation = 1.0). WW: well-watered; WD: water deficit; WL: waterlogging.

We found that dark and small seeds had significantly higher RWC than light and large seeds in WD, but not in WW or WL. Also, small seeds had higher $\sqrt{gs_{869}}$ and $\sqrt{WUE_{869}}$, and lower $\sqrt[3]{C_b}$ and C_{ab} than large seeds in WD, but not in WW or WL. Large seeds had significantly higher $\sqrt{C_a}$ and SPAD in WW and WD, but not in WL. Light seeds had significantly higher SPAD in WD, but not in WW or WL.

Under WD conditions, the highest diversity was observed in small and dark seeded accessions (Fig. S6, Supplementary Material). These accessions were also overall characterized by higher gas-exchange related trait values in WL conditions. Whereas in WW there was a general overlap of the range of RWC values for both categories of seed color and size, in WD and WL the lowest values of RWC were observed in light and large accessions.

We calculated delta (Δ) values for all accessions in both water stress treatments, in such way that the more negative ΔWD or ΔWL is for an accession and a trait, the more affected by the stress that accession was. Overall, dark and small seeded accessions decreased less the gas-exchange parameters and RWC when in WD than light and large seeded accessions (Fig. 4). The accessions that most decreased pigment contents in WL were light and large seeded accessions, however in WD, they increased more R/S and greenness (indicated both by $\sqrt{C_{ab}/C_{cx}}$ and SPAD).

Contrasting with *color.treatment* and *size.treatment* interactions, which were highly significant for most of the traits, *origin.treatment* interactions were only highly significant (*p-value* < 0.01) for $\sqrt{E_{347}}$, E_{869}, $\sqrt{gs_{347}}$, $\sqrt{gs_{869}}$, $\sqrt{PI_{ABS}}$, RWC and SPAD (Table S4, Supplementary Material).

Significant phenotypic differences (*p-value* < 0.05) among the seven seed origins (Sub-Saharan Africa, Mediterranean, East Europe, North and Central Asia, South Asia, North America and South America) were found in almost all the traits, with exception of A_{869}, $\sqrt{C_{ab}/C_{cx}}$, $\sqrt{gs_{347}}$, $\sqrt{gs_{869}}$ and $\sqrt{PI_{ABS}}$. Together these data indicates that pigment contents ($\sqrt{C_a}$, $\sqrt[3]{C_b}$, C_{ab}, and $\sqrt{C_{cx}}$), $(Fv/Fm)^2$, Fv/Fo, $\sqrt{WUE_{869}}$, $\sqrt{R/S}$ and TDB are traits for which accessions with different seed origins perform differently and with little interaction (*p-value* > 0.01) with the water treatment. As an example, and referring to the three seed origins which together account for over 80% of the working collection, South Asian seeds produced plants with significantly less TDB than East European seeds (*p-value*=0.019), and these with significantly less TDB than Mediterranean seeds (*p-value*=0.017) (Fig. 5).

Regarding the analyses by delta values, we observed that most ΔWL for gas-exchange traits were positive, regardless of the seed origin (Fig. S7, Supplementary Material). We also observed, in Fig. S7, that in different physiologic traits some seed origins are less affected by WD and WL stress than others. Sub-saharan Africa and North and Central Asian accessions, for instance, showed less variability of ΔWD and ΔWL. In WD, the most affected accessions in terms of RWC are Mediterranean and East-European, the same origins that correspond to the most affected in WL in terms of pigment contents. Pairwise testing of differences between *origin.treatments* statistically supported most of these data, and revealed further important details: Mediterranean accessions had significantly lower RWC in WD than in WW and also $\sqrt{PI_{ABS}}$ in WL than in WW and WD; and Sub-saharan Africa accessions had significantly higher $\sqrt{C_{cx}}$, $\sqrt{E_{347}}$, and $\sqrt{gs_{347}}$ in WL than in WW (studentized

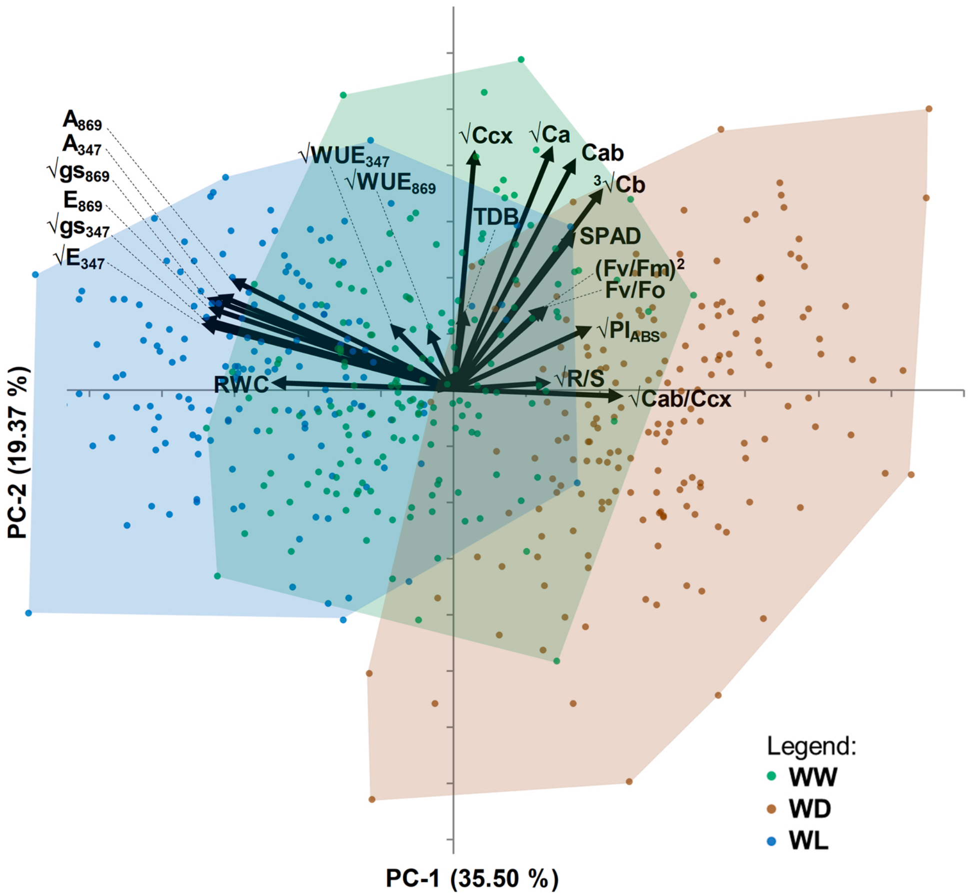

Fig. 3. Principal component analysis biplots representing the distribution of 187 grass pea accessions (small dots) under three water regimens (WW – green dots; WD – brown dots; WL – blue dots), regarding A_{347}, $\sqrt{E_{347}}$, $\sqrt{gs_{347}}$, $\sqrt{WUE_{347}}$, A_{869}, E_{869}, $\sqrt{gs_{347}}$, $\sqrt{WUE_{869}}$, $(Fv/Fm)^2$, Fv/Fo, $\sqrt{PI_{ABS}}$, RWC, TDB, $\sqrt{R/S}$, $\sqrt{C_a}$, $\sqrt[3]{C_b}$, C_{ab}, $\sqrt{C_{cx}}$, $\sqrt{C_{ab}/C_{cx}}$ and SPAD traits, indicated by vectors (eigen vector coordinates multiplied by 8 to better visualization). BLUEs for each accession x treatment were used. Total of 54.87% variation explained by principal components 1 and 2 (PC-1, PC-2). Polygons delimiting the areas of the water treatments were added for better visualization. WW: well-watered; WD: water deficit; WL: waterlogging. A347: CO2 assimilation rate at growth light intensity; A869: CO2 assimilation rate at Amax light intensity; E347: transpiration rate at growth light intensity; E869: transpiration rate at Amax light intensity; gs347: stomatal conductance at growth light intensity; gs869: stomatal conductance at Amax light intensity; WUE347: instantaneous water use efficiency at growth light intensity; WUE869: instantaneous water use efficiency at Amax light intensity; TDB: total dry biomass; RWC: leaf relative water content; R/S: root to shoot ratio (dry biomasses); PIABS: performance index on absorbance basis; Fv/Fm: maximum quantum yield of photossystem II; Fv/Fo: actual quantum yield of photossystem II; Ca: chlorophyll a content (spectrophotometrically measured); Cb: chlorophyll b content (spectrophotometrically measured); Cab: chlorophylls a+b content (spectrophotometrically measured); Ccx: xantophyll + carotene content (spectrophotometrically measured); Cab/Ccx: greeness ratio based on spectrophotometrically measured pigment contents; SPAD: greeness index given by SPAD-502 instrument. BLUEs: Best Linear Unbiased Estimators.

range post hoc LSD test, *p-value*<0.05).

3.4. Contrasting grass pea accessions for tolerance or susceptibility to the water stress treatments were identified; with additional multi resistant and multi susceptible accessions highlighted

Six susceptible and six tolerant accessions for each stress (Table 2) have been selected by analyzing the Heatmaps for the response (ΔWD and ΔWL) and the associated dendrograms (Fig. S8, Supplementary Material) and they are characterized as follows:

WD: accessions that strongly decreased A_{347}, TDB and $\sqrt{WUE_{347}}$ in WD relative to WW were considered as susceptible, contrasting with accessions that mostly maintained A_{347} and $\sqrt{TDB}$, and increased $\sqrt{WUE_{347}}$;
WL: accessions that strongly decreased A_{347} and TDB in WL relative to WW were considered as susceptible, contrasting with accessions that mostly maintained or increased these traits.

Accessions PI283546 and PTLS1001 were identified as being respectively multi stress-tolerant (i.e. tolerant to both WD and WL) and multi stress-susceptible (i.e. susceptible to both WD and WL).

Table 2 also summarizes some characteristics of the 22 selected accessions. The multi-tolerant accession is highlighted in green, and the multi-susceptible accession is highlighted in red. Interestingly, all the accessions identified as WD-susceptible have light and large seeds, while the majority of the WD-tolerant ones have dark and small seeds. Concerning WL contrasting groups, the seed size and origin is balanced among susceptible and tolerant accessions, however we verify that the majority (5/6) of WL-tolerant accessions have dark seeds and the majority (5/6) of WL-susceptible accessions have light seeds.

In Fig. S9 (Supplementary Material) the relative positioning of the selected accessions in the PCA biplot constructed with the ΔWD and ΔWL (Fig. 4) is highlighted.

4. Discussion

Plants response to water stress is a complex phenomenon that includes morphological, physio-biochemical, cellular, and molecular adaptation. The differential responses reflect different tolerance levels, not only among species, but also within a considered tolerant species like grass pea.

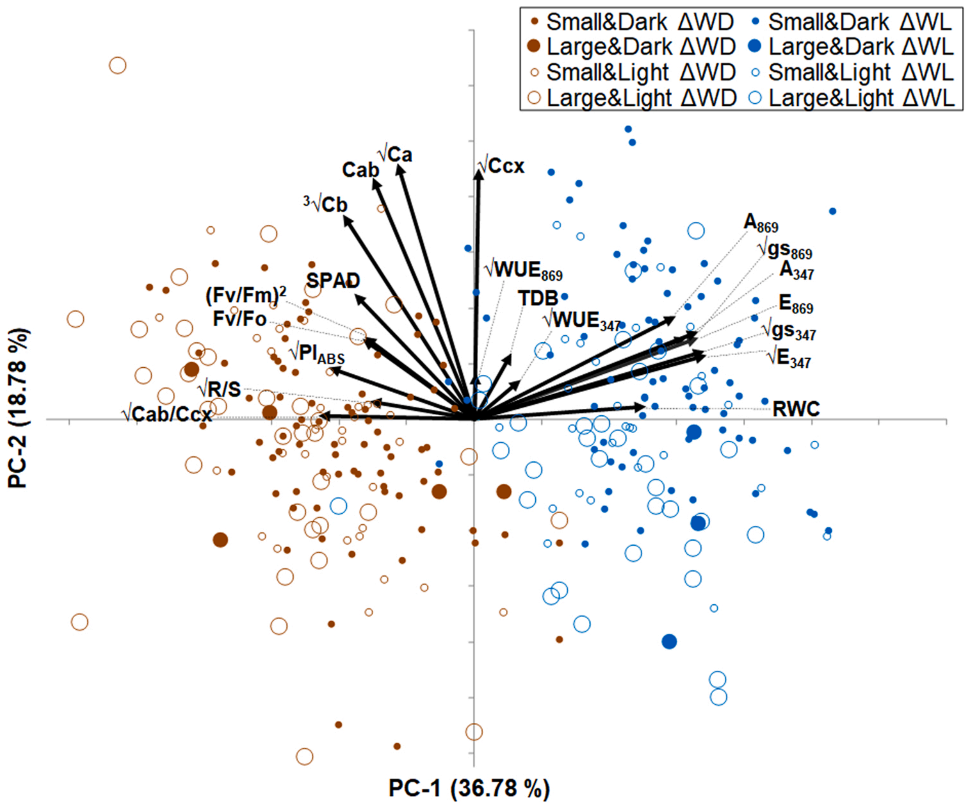

Fig. 4. Principal component analysis biplot representing ΔWD (brown tones) = $BLUE_{WD}$ - $BLUE_{WW}$ and ΔWL (blue tones) = $BLUE_{WL}$ - $BLUE_{WW}$ for 151 grass pea accessions of different seed size and color (large filled circles: large and dark seeded accessions, large empty circles: large and light seeded accessions, small filled circles: small and dark seeded accessions, small empty circles: small and light seeded accessions) regarding A_{347}, $\sqrt{E_{347}}$, $\sqrt{gs_{347}}$, $\sqrt{WUE_{347}}$, A_{869}, E_{869}, $\sqrt{gs_{347}}$, $\sqrt{WUE_{869}}$, $(Fv/Fm)^2$, Fv/Fo, $\sqrt{PI_{ABS}}$, RWC, TDB, $\sqrt{R/S}$, $\sqrt{C_a}$, $\sqrt[3]{C_b}$, C_{ab}, $\sqrt{C_{cx}}$, $\sqrt{C_{ab}/C_{cx}}$ and SPAD traits, indicated by vectors (eigen vector coordinates multiplied by 8 to better visualization). Total of 55.56% variation explained by principal components 1 and 2 (PC-1, PC-2). WW: well-watered; WD: water deficit; WL: waterlogging. BLUEs: Best Linear Unbiased Estimators. A347: CO2 assimilation rate at growth light intensity; A869: CO2 assimilation rate at Amax light intensity; E347: transpiration rate at growth light intensity; E869: transpiration rate at Amax light intensity; gs347: stomatal conductance at growth light intensity; gs869: stomatal conductance at Amax light intensity; WUE347: instantaneous water use efficiency at growth light intensity; WUE869: instantaneous water use efficiency at Amax light intensity; TDB: total dry biomass; RWC: leaf relative water content; R/S: root to shoot ratio (dry biomasses); PIABS: performance index on absorbance basis; Fv/Fm: maximum quantum yield of photossystem II; Fv/Fo: actual quantum yield of photossystem II; Ca: chlorophyll a content (spectrophotometrically measured); Cb: chlorophyll b content (spectrophotometrically measured); Cab: chlorophylls a+b content (spectrophotometrically measured); Ccx: xantophyll + carotene content (spectrophotometrically measured); Cab/Ccx: greeness ratio based on spectrophotometrically measured pigment contents; SPAD: greeness index given by SPAD-502 instrument.

Through the screening of 194 grass pea accessions from a worldwide collection after subjecting it to different watering treatments, a wide range of responses was identified and significant differences among accessions were detected for all the 20 physiologic traits measured. This establishes a good working base for further studies concerning the genetic basis of the observed phenotypic differences in grass pea response to water stresses.

We observed an adaptation on gas-exchange occurring in most of grass pea plants as main response to water deficit conditions, whilst increased shoot growth and carotenoid content was observed in response to waterlogging. Some variability in water treatments' response was explained by inherited seed traits such as size, color and geographical origins.

Additionally, accessions tolerant and susceptible to one or both stresses were identified and constitute interesting material to be explored in further studies and/or integrated in future breeding programs tackling climatic challenges.

At the best of our knowledge, this is the first study providing such amount of morpho-physiological data on this species and evaluating its variability of responses to both water deficit and waterlogging stresses.

4.1. Water treatment clearly reflects on RWC and gas-exchange traits

In the present study, leaf RWC was, as expected, higher in WL plants, followed by WW and then WD plants (Figs. 1 and 3), reaching values as low as 30% in some extreme cases. Lower RWC values were concomitant with lower gas exchange traits gs, A and E, measured both at growth light and Amax light conditions (347 μmol/m^2/s and 869 μmol/m^2/s, respectively), in WD (Figs. 1, 3 and S3). Since stomatal regulation is well established as one of the first physiologic responses of the plants when water scarcity is sensed (Dubois and Inzé, 2020) and A, gs and E rates have been reported to decrease with increasing water scarcity in legumes (Chowdhury et al., 2016; Leitão et al., 2021a; Nadeem et al., 2019), these results were not surprising. However, they had only been reported in grass pea plants subject to drought – water withhold until approx. 65% FC – in podding stage (Kong et al., 2015), hence this study provides new and complementary insights for this species.

In the opposite stress condition (waterlogging), average values of gas-exchange related traits increased from WW to WL conditions (Fig. 1). This may be a clear difference between grass pea and other legume species as cowpea, mungbean, and common bean, where significant decreases in photosynthetic rate, transpiration and stomatal conductance upon similar partial submergence level have been reported (Ahmed et al., 2002; Kumar et al., 2013; Olorunwa et al., 2022; Posso

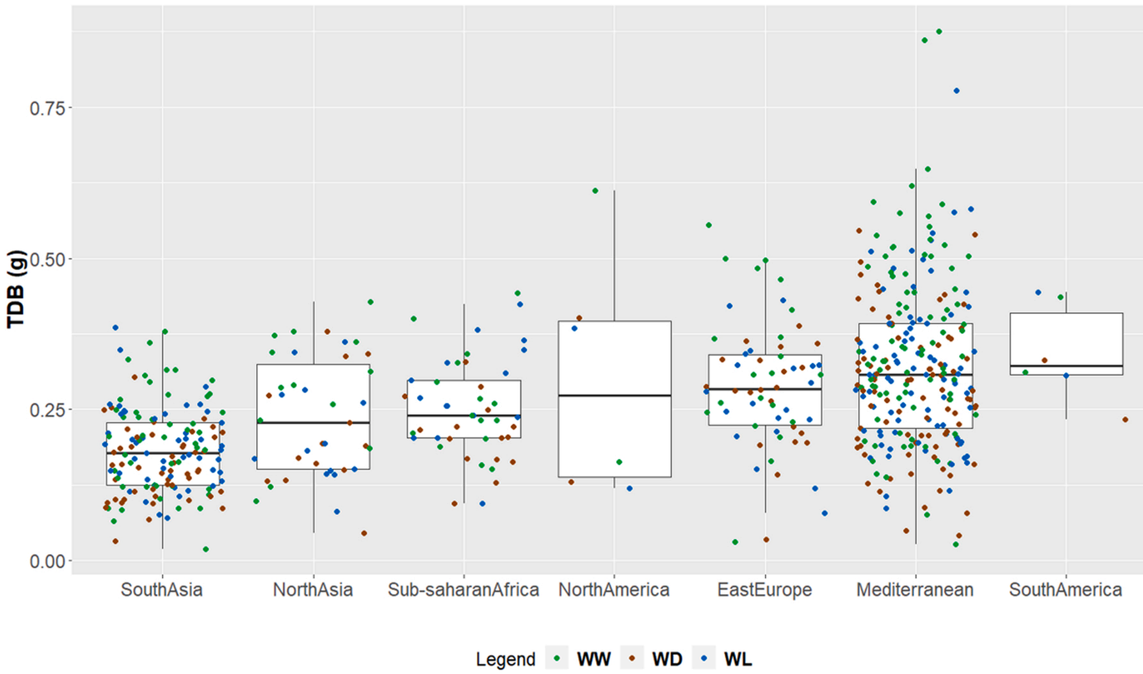

Fig. 5. Boxplot distributions of TDB for accessions of seven seed origins. Green, brown and blue dots represent BLUE values in WW, WD and WL conditions, respectively. BLUEs: Best Linear Unbiased Estimators. WW: well-watered; WD: water deficit; WL: waterlogging.

et al., 2020). In all except common bean, the decrease in gas-exchange parameters was accompanied by decreases in significant chlorophyll fluorescence parameters and consequences on plant height and dry biomass, which was also not the case in our grass pea collection of accessions.

Nevertheless, apart from the global tendency to increase gas-exchange values observed in the grass pea collection under WL conditions, it is important to notice the range of variability among accessions, with lowest A, E and gs in WL overlapping the highest values observed in WW and WD treatments.

4.2. One of the grass pea adaptations to contrasting water conditions is a differential growth rate of shoot and root

In WD, adjustment of plant growth as a tissue dehydration avoidance strategy frequently happens, with reduced shoots and deeper-penetrating roots being frequently observed in legumes and in other crops (Basu et al., 2016; Eziz et al., 2017; Hall, 2012; Kashiwagi et al., 2006; Khan et al., 2010; Sinclair and Muchow, 2001). Though not statistically significant, the highest R/S were indeed observed in grass pea plants subject to WD (Fig. 1). A tendency to increase root density while reducing root diameter in WD, although was not measured as a trait, was visually detected (Fig. S2e, Supplementary Material), which was previously advanced as a possible water scarcity tolerance mechanism in some legume species, namely soybean (Hudak and Patterson, 1996) and chickpea (Kashiwagi et al., 2006). A screen report of root architecture diversity in some grain legumes (soybean, cowpea, pea, common bean, and chickpea) produced by Ye et al. (2018) had already highlighted the intra-species variability and the urge to investigate beneficial root traits towards enhanced drought tolerance and yield in a breeding context. Accessions with higher plasticity of the root system, namely the ability to grow deeper and denser roots under drought stress, showed increased water and nutrient uptake in other legumes (Grzesiak et al., 1997; Jaganathan et al., 2015). Hence, the hereby noticed increase in R/S in some grass pea plants subject to water deficit should not be disregarded and can be further explored.

The increased TDB coupled with reduced R/S in WL (compared with WW and WD treatments) observed in this study may be explained because a common survival strategy in crops to escape low-oxygen environments like in WL conditions, is a rapid elongation of underwater stems. This might enable photosynthetic tissue to outgrow shallow floodwaters (Bailey-Serres et al., 2012), the formation of adventitious roots (Ploschuk et al., 2018) and a root growth closer to the soil surface as to obtain oxygenation more easily (Malik et al., 2015; Voesenek and Bailey-Serres, 2015). Possibly, the partial submergence imposed in this work triggered shoot elongation in the grass pea plants as a preventive adaptation to the eventuality of more drastic flood conditions, like it happens at least in some deepwater rice cultivars (Vergara et al., 1976).

In an interesting comparative study on tolerance to transient waterlogging in four grain legume species, grass pea was highlighted as the most tolerant species in terms of germination and seedling survival, shoot and root growth. One of the justifications was its fast root growth during the recovery phase after submergence (Wiraguna et al., 2021). This indicates that possibly, grass pea root growth rates are quickly adaptable to the soil moisture. Again, it will be worth it to study more root-related traits and plasticity in grass pea that may underlie important mechanisms of water stress tolerance.

4.3. Gas-exchange parameters explain most variability between water treatments, while total dry biomass, chlorophyll fluorescence and pigment related traits are mostly genetically determined

In all gas-exchange parameters (A, E and gs), the highest variance component was water treatment. In fact, 80% of the total treatment variance is explained by the six gas-exchange parameters: A_{347}, E_{347}, gs_{347}, A_{869}, E_{869}, and gs_{869} (Table 1). This reiterates the usefulness of IRGA measurements as effective and reliable indicators of plant relative performance and physiologic state (reviewed by Urban et al., 2017).

For SPAD, TDB, $(Fv/Fm)^2$, Fv/Fo, C_{ab}, $\sqrt[3]{C_b}$, and $\sqrt{C_a}$, the highest portion of phenotypic variance was due to differences among the accessions rather than among treatments. In addition, TDB, SPAD, $\sqrt{PI_{ABS}}$ and $(Fv/Fm)^2$ were the traits with higher broad sense heritability (h^2 ranging between 0.63 and 0.88, Table 1). Under moderate/mild stress conditions (such as the ones in the present study), most chlorophyll fluorescence related traits (such as Fv/Fo, $(Fv/Fm)^2$ and $\sqrt{PI_{ABS}}$) were reported to be quite stable (Silvestre et al., 2014), therefore the lack of

Table 2
Selected accessions with contrasting responses to WD and WL, respective seed characterization (seed color, seed size and geographic origin), and summary of trait[a] differences (Δ). Δ's associated colors range from blue (strong negative difference, i.e. more susceptibility) to white and orange (close to zero- and positive differences, i.e. more tolerance). Highlighted in green is one simultaneously WD- and WL- tolerant accession; highlighted in red is one simultaneously WD- and WL- susceptible accession.

Group	Accessions ID	Seed Color	Seed Size	Seed Geographical Origin	Δ RWC	Δ $\sqrt{WUE_{347}}$	Δ A_{347}	Δ $\sqrt{R/S}$	Δ TDB
WD-tolerant	PI283546	Dark	Large	Mediterranean	-0.091	0.645	-0.211	-0.012	0.095
	PI317443	Dark	Small	South Asian	-0.109	1.446	-0.259	0.022	-0.083
	PI577141	Dark	Small	South Asian	-0.100	0.679	-0.709	-0.070	-0.009
	PI391432	Dark	Small	South Asian	-0.017	0.616	-1.102	-0.136	-0.015
	ACC273	Dark	Small	Unknown	-0.038	0.561	-0.187	0.118	-0.035
	BGE17185	Light	Large	Mediterranean	-0.048	0.565	-1.911	-0.017	0.020
WD-susceptible	PTLS1001	Light	Large	Mediterranean	-0.283	-0.912	-6.106	-0.016	-0.196
	PTLS1009	Light	Large	Mediterranean	-0.181	-0.635	-2.816	0.078	-0.460
	BGE15746	Light	Large	Mediterranean	-0.227	-1.168	-2.861	0.055	-0.048
	BGE23926	Light	Large	Mediterranean	-0.114	-0.958	-5.638	0.191	-0.219
	PI667251_A	Light	Large	East European	-0.071	-0.420	-5.936	0.057	0.002
	STUDENICA	Light	Large	East European	-0.181	-0.732	-5.029	0.079	-0.164

(continued on next page)

Table 2 (*continued*)

Group	Accessions ID	Seed Color	Seed Size	Seed Geographical Origin	Δ $(Fv/Fm)^2$	Δ $\sqrt{E_{347}}$	Δ A_{347}	Δ $\sqrt{R/S}$	Δ TDB
WL-tolerant	PI283546	Dark	Large	Mediterranean	-0.027	0.130	2.656	0.039	0.106
WL-tolerant	PI422526_B	Dark	Large	East European	-0.015	-0.180	2.945	-0.328	-0.015
WL-tolerant	PTLS1007	Light	Large	Mediterranean	-0.043	0.281	2.451	-0.070	-0.011
WL-tolerant	PI358857	Dark	Small	Mediterranean	-0.019	-0.095	2.584	-0.220	0.003
WL-tolerant	PI196001	Dark	Small	Sub-SaharanAfrica	0.002	-0.388	2.073	-0.078	-0.019
WL-tolerant	ACC190	Dark	Small	Unknown	-0.025	0.516	1.556	-0.230	0.084
WL-susceptible	PTLS1001	Light	Large	Mediterranean	-0.033	-1.043	-1.066	-0.563	-0.171
WL-susceptible	BGE1489	Light	Large	Mediterranean	-0.045	-0.253	-2.895	-0.206	-0.112
WL-susceptible	BGE19777	Light	Large	Mediterranean	-0.029	0.491	-3.474	-0.116	-0.137
WL-susceptible	PI283578	Light	Small	Mediterranean	-0.007	0.720	-2.436	-0.163	-0.202
WL-susceptible	PI422534	Light	Small	North Asian	-0.036	0.406	-2.862	-0.302	-0.164
WL-susceptible	PI442795	Dark	Small	South Asian	-0.022	0.122	-3.226	-0.120	-0.084

[a] A_{347}: CO_2 assimilation rate at growth light intensity; E_{347}: transpiration rate at growth light intensity; WUE_{347}: instantaneous water use efficiency at growth light intensity; TDB: total dry biomass; RWC: leaf relative water content; R/S: root to shoot ratio (dry biomasses); Fv/Fm: maximum quantum yield of photossystem II.

substantial differences among treatments was expected. These traits might be good indicators of the inherited predisposition to tolerate water stress situations. Indeed, chlorophyll a fluorescence had been successfully applied to distinguish *Lathyrus* spp. genotypes with different performances under water deficit (Silvestre et al., 2014).

4.4. Pigment content related traits and WUE reveal possible sources of contrasting water stress tolerance in grass pea

The average levels of C_{cx} were significantly increased in WL relative to WW conditions (*p-value*<0.001). As already referred, carotenoids are determinant to avoid redox imbalance and prevent oxidative stress that is induced under water stress (Hasanuzzaman et al., 2020; Pan et al., 2021). In other pigment content- and greenness- related traits (C_a, C_b, C_{ab}, C_{ab}/C_{cx}, and SPAD), a wider variability was observed in WD plants (Fig. 3), being that C_b and SPAD were significantly higher in WD compared to WW. Similar results, namely the increased chlorophyll contents under drought conditions (40% FC imposed to seedling-stage plants) compared to WW have also been reported in common bean (Leitão et al., 2021a). The fact that chlorophyll contents were not significantly reduced in the water stress conditions confirms that grass pea is, overall, considerably robust to at least mild WD and WL stress conditions and pigment biosynthesis is not compromised.

For traits WUE_{347}, WUE_{869}, the interaction *treatment.accession* was significant (Wald tests, *p-value*<0.001) and it was also the variance component that accounted for most of the phenotypic variability (Table 1). This means that for these traits, the ranks of the accessions significantly change according to the water treatment, revealing an adaptability that is particularly relevant in WD, when the plant must make a more efficient use of water (Vadez et al., 2011). Accessions with improved WUE under drought conditions (such as the ones selected in Table 2) are especially useful from a breeding-to-climate-change perspective (Khatun et al., 2021), and in this sense, the present study supports grass pea potential as a climate sustainable crop.

Regarding the multi-tolerant accession that was identified (PI283546), its relative stability in the gas-exchange parameters across the three water conditions, namely the carbon assimilation and transpiration rates, along with the maintenance of photosystem II's vitality (as assessed with chlorophyll a fluorescence traits), guaranteed the continued photosynthate production indirectly observed through TDB and R/S traits. The increase in pigment contents and possibly also an enhanced antioxidant and osmoregulatory capacity (Fratianni et al., 2014; Jiang et al., 2013; Rathi et al., 2019; Zhou et al., 2016) might be important contributors to the aforementioned stability of gas-exchange parameters and photosystem II's vitality.

4.5. Light and large seeds may produce bigger plants, but dark and small seeded accessions seem to be physiologically less affected by contrasting water stresses

Significant phenotypic differences (*p-value* < 0.05) between light and dark seed colors and between small and large seed sizes were found in almost all the traits. While light and large seeds produced plants with higher TDB across all treatments, accessions with dark and small seeds had significantly higher RWC, $\sqrt{gs_{869}}$ and $\sqrt{WUE_{869}}$ in WD. These accessions also showed to not decrease pigment contents in WL as much as light and large seeded accessions (Fig. 4).

The identification of accessions with contrasting responses of tolerance and susceptibility to both water stresses further reaffirmed these seed size and color distinctions: all the accessions identified as WD-susceptible belonged to the light and large seed cluster, while most of the WD-tolerant accessions came from the dark and small seed cluster. In addition, most of WL-tolerant accessions had dark seeds and most of WL-susceptible accessions had light seeds (Table 2).

In a study with the same grass pea collection, on resistance to the foliar biotrophic pathogen *Uromyces pisi*, differential responses according to seed morphology had already been observed: in that case, dark-colored seed accessions were more susceptible to *U. pisi*, than the light-colored seed accessions (Martins et al., 2022). However, Sampaio et al. (2021) studying the wilting soil-borne pathogen *Fusarium oxysporum* concluded that dark and small seed accessions were more tolerant than light and large seed accessions like in the present study. It could be interesting to further explore the patterns of susceptibility and tolerance of different subpopulations of grass pea to simultaneous biotic and abiotic stresses, especially when they share the entrance gate (such are as the roots, for both *F. oxysporum* and water stress), similarly to what has been done in other legume species (Leitão et al., 2021b).

4.6. Different geographical origin may influence levels of grass pea tolerance to contrasting water stresses

Some significant phenotypic differences were observed among seven geographical origins (Sub-Saharan Africa, Mediterranean, East Europe, North and Central Asia, South Asia, North America and South America) within the studied collection. For instance, Mediterranean and East-European grass pea accessions are more affected by both WD (based on ΔWDs for RWC) and WL (based on ΔWLs for pigment contents and PI_{ABS}) stresses, while Sub-saharan Africa and North and Central Asian accessions show less variability of ΔWD and ΔWL across all traits. This may indicate that accessions coming from those African and Asian regions have developed less varied (but efficient) water stress coping mechanisms, what makes sense given the frequent agricultural drought events in sub-saharan regions (Gommes and Petrassi, 1996) and especially the relatively high frequency of 'seesaw events' – the rapid transitioning from intense drought to pluvial spells, or long wet periods followed by sudden dry spells - in both Central Africa, North and Central-Eastern Asia regions (He and Sheffield, 2020).

Sub-saharan Africa accessions in WL have significantly higher $\sqrt{C_{cx}}$ than in WW. Carotenoids are important antioxidants (Gechev et al., 2006), and an enhanced performance of the antioxidant machinery, ROS scavenging and osmotic adjustment mechanisms have been pointed out as a possible basis for the observed tolerance to flooding in grass pea (Malik et al., 2015; Zhou et al., 2016). In this sense, our data indicates a possible antioxidant-related advantage of Sub-saharan Africa accessions for coping with WL, comparing to other geographical origins. Sub-saharan Africa accessions showed also significantly higher $\sqrt{E_{347}}$, and $\sqrt{gs_{347}}$ in WL than in WW, what might be important as an immediate response for equilibrating internal water contents in face of an excessive soil moisture situation. Wiraguna et al. (2020) reported differences in seed germination rates in waterlogged soils depending on geographical origin, being genotypes from Bangladesh (South Asia origin) and Ethiopia (Sub-saharan Africa origin) the most well adapted. In Africa, particularly in Ethiopia, grass pea is grown during the monsoon season in soils with a high clay content; while in southern Asia grass pea is commonly used as relay-sowing crop in rice field – both situations making it highly likely for the seeds to experience transient soil waterlogging from an early developmental stage (Malik et al., 2015; Wiraguna et al., 2020).

Regardless of the water treatment, South Asian accessions produced plants with significantly less TDB than East European accessions, and these with significantly less TDB than Mediterranean accessions (Fig. 5), to refer just the three most common seed origins in the working collection. Since TDB is, as previously discussed, a highly genetically determined trait, these sub-populational distinctions were not surprising. We did not find any other study addressing plant biomass variability across different grass pea geographical origins; however referring to other yield-related traits, Grela et al. (2012) verified that south-central Europe accessions had on average lower pod number per plant and lower number of seeds per pod, but higher thousand seeds weight in comparison with the accessions from east-central Europe (to be noted that both 'south-central Europe' and 'east-central Europe' categories, as defined in the referred study, fit into 'East Europe' seed origin category in our working collection).

5. Conclusions

Given the diversity of water stress tolerance strategies that exist across legumes and other plant species, comprehensive studies accounting for several morphologic and physiologic responses simultaneously are particularly useful as a first approach to address the topic in less well characterized species.

In the current study we observed numerous short-term responses in the majority of grass pea accessions to both mild drought and transient partial submergence – water oscillations evermore frequent in the Mediterranean region. Thus, this study underscores the resilience of grass pea comparatively to other grain legumes and affirms its high potential for the future of Mediterranean agriculture.

Physiologic and phenotypic variability was hereby reported for a considerably diverse grass pea collection subjected to three different water treatments (well-watered, mild water deficit and partial submergence). Hence, this study is a useful starting point for understanding the mechanisms underlying this species' robustness and huge potential under a climatic unpredictability that is steadily increasing, with flood and drought extreme events becoming more and more frequent.

The authors acknowledge that the specificity of the treatments imposed to the plants, the limitation of experimental window to early growth stages and the lack of more directly yield-, root system- and nodulation-related traits necessarily restrict the conclusions and that for instance, multi-stress, recovery-stress or more extreme water stress conditions were not addressed.

Nevertheless, increased root growth and stomatal closure appear to be common features under drought in this species, contributing to improvement of water use efficiency, while increased shoot growth and carotenoid content seems to be important grass pea waterlogging responses.

However, the presented data reveals variability namely in ecotypes characterized by different seed types, suggesting that specific adaptations may be inherited within populations. A Genome Wide Association Study (GWAS) using the collected phenotypic data is ongoing, where we expect to clear out the genetic basis of grass pea water stress tolerance and identify linked genetic markers and candidate genes involved in the water stress responses for future precision breeding of multi-stress tolerance.

Twenty-two accessions with contrasting (tolerant and susceptible) phenotypes were identified in the present study, useful for future breeding and genetic detailed studies considering abiotic stress tolerance. These include one multi stress-tolerant and one multi stress-susceptible accessions, which are also particularly interesting to explore common mechanisms involved in tolerance and susceptibility to both water stresses in this species. An efficient antioxidative machinery and the rapid improvement of WUE may be two of those multi-stress tolerance mechanisms that deserve further investigation. Finally, exploring patterns of susceptibility and tolerance in grass pea facing other abiotic-abiotic and abiotic-biotic stress conjugations, would be interesting and important, given the multi-front nature of current climate change scenario.

CRediT authorship contribution statement

Matilde Sanches: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Ana Margarida Sampaio:** Data curation, Formal analysis, Writing – review & editing. **Susana Araújo:** Conceptualization, Methodology, Writing – review & editing. **Fred van Eeuwijk:** Formal analysis, Methodology, Validation, Writing – review & editing. **Frank Van Breusegem:** Supervision, Writing – review & editing. **M. Carlota Vaz Patto:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Matilde Sanches reports financial support was provided by Foundation for Science and Technology. Matilde Sanches reports financial support was provided by European Union. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agwat.2023.108664.

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