DR ANDREI SMERTENKO (Orcid ID : 0000-0002-5078-4881)

Article type : Original Article

Title: Impact of heat and drought stress on peroxisome proliferation in quinoa

Leonardo Hinojosa[1], Marwa Sanad[2#], David Jarvis[4], Patrick Steel[3], Kevin Murphy[1*], Andrei Smertenko[2*]

[1]Department of Crop and Soil Sciences, Washington State University, PO Box 646340, Pullman, WA 99164, U.S.A.

[2]Institute of Biological Chemistry, Washington State University, PO Box 646340, Pullman, WA 99164, U.S.A.

[3]Department of Chemistry, Durham University, Durham, UK

[4] Plant & Wildlife Sciences, Brigham Young University, Provo, UT 84602, U.S.A.

[#]Current address: Department of Genetics and Cytology, National Research Centre, Giza, Egypt

[*]AS and KM should be considered joint senior authors

Phone: +1-509-592-7209

Fax: + 1 509-335-7643

E-mail: andrei.smertenko@wsu.edu

Key words: *Peroxisomes, heat, drought, stress, hydrogen peroxide, quinoa, adaptation*

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/tpj.14411

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Abstract

Although peroxisomes play a key role in plant metabolism under both normal and stressful growth conditions, the impact of drought and heat stress on the peroxisomes remains unknown. Quinoa represents an informative system for dissecting the impact of abiotic stress on peroxisome proliferation because it is adapted to marginal environments. Here we determined the correlation of peroxisome abundance with physiological responses and yield under heat, drought, and heat plus drought combination stresses in eight genotypes of quinoa. We found that all stresses caused a reduction in stomatal conductance and yield. Furthermore, H_2O_2 content increased under drought and heat plus drought. Principal component analysis demonstrated that peroxisome abundance correlated positively with H_2O_2 content in leaves and correlated negatively with yield. Person correlation coefficient for yield and peroxisome abundance (r=-0.59) was higher than for commonly used photosynthetic efficiency (r=0.23), but comparable to those for classical stress indicators such as soil moisture content (r=0.51) or stomatal conductance (r=0.62). Our work established peroxisome abundance as a sensitive parameter for responses to heat and drought stress in the genetically diverse populations. As heat waves threaten agricultural productivity in arid climates, our findings will facilitate identification of genetic markers for improving yield of crops under extreme weather patterns.

Introduction

Quinoa (*Chenopodium quinoa* Willd.) is an ancient seed crop originating from the Andean region of South America. Quinoa has gained considerable attention worldwide over the past decade due to its positive end-use quality characteristics and its nutritional and health benefits, most notably as a gluten-free, high-protein crop with a well-balanced complement of amino acids and high concentrations of iron, calcium, and phosphorus (Vega-Gálvez *et al.*, 2010; Wu, 2015; Wu *et al.*, 2016, 2017*a*,*b*; Kowalski *et al.*, 2016; Aluwi *et al.*, 2017). Although the natural distribution of quinoa is from southern Colombia to the coast of south-central Chile from 2 °N to 43 °S (Zurita-Silva *et al.*, 2014), quinoa has recently been cultivated in parts of the world with arid and hot growth seasons (Bazile *et al.*, 2016*a*; Murphy *et al.*, 2016). Quinoa adapts to drought conditions (Geerts *et al.*, 2008; Martínez *et al.*, 2009; Razzaghi *et al.*, 2012; Walters *et al.*, 2016; Maliro *et al.*, 2017) by accumulating solutes, reducing leaf stomatal conductance, and modulating root architecture (Bosque Sanchez *et al.*, 2003; Jacobsen *et al.*, 2009; González *et al.*, 2011; Alvarez-Flores *et al.*, 2018). High temperatures

This article is protected by copyright. All rights reserved.

Accepted Article

stimulate quinoa growth (Yang *et al.*, 2016; Becker *et al.*, 2017; Bunce, 2017; Hinojosa *et al.*, 2019) but diminish seed yield, particularly under drought conditions (Fuentes and Bhargava, 2011; Peterson and Murphy, 2015; Walters *et al.*, 2016; Lesjak and Calderini, 2017; Hinojosa *et al.*, 2018). However, the reasons for the high sensitivity of quinoa to the combination of heat and drought remain poorly understood.

The combination of heat and drought stress causes greater yield loss than the individual stresses of drought, heat, freezing, or flooding in all crops including barley, maize, wheat (Suzuki *et al.*, 2014; Zandalinas *et al.*, 2018). Air temperature in the previous century rose by ~0.8 °C, and by the end of this century, the temperature is predicted to increase by ~2-4 °C (IPCC, 2014). Sustaining quinoa yields in climates with frequent heat waves will depend, in large part, on breeding heat-tolerant varieties. These efforts are hindered by the lack of suitable markers that can be used in breeding programs for heat and drought tolerance (Raney *et al.*, 2014; Morales *et al.*, 2017). Here we aim to identify such markers.

Our approach is based on the knowledge that high temperature and drought induce oxidative stress in all thus far analyzed plants by accelerating the generation of reactive oxygen species (ROS) including singlet oxygen, superoxide radical, hydrogen peroxide, and hydroxyl racial (Mittler, 2002; Sharma *et al.*, 2012). Low concentrations of ROS contribute to the induction of adaptive responses to abiotic stresses such as ozone, UV, high light intensity, dehydration, wounding, and temperature extremes (Mittler *et al.*, 2011; Baxter *et al.*, 2014; Suzuki *et al.*, 2014; Choudhury *et al.*, 2017). However, accumulation of ROS also inflicts damage to cellular components through peroxidation of membrane lipids and oxidation of proteins, DNA and RNA (Mittler, 2002; Sharma *et al.*, 2012; Choudhury *et al.*, 2017). Cells deploy ROS-scavenging enzymes and anti-oxidants to ameliorate this oxidative damage.

One of the key components of the ROS scavenging system are peroxisomes, the small ubiquitous globular organelles of approximately 1 μm in diameter bounded with a single membrane (De Duve and Baudhuin, 1966). Peroxisomes contain an efficient ROS scavenging system that includes the antioxidants ascorbate and glutathione, and several antioxidant enzymes including dehydro- and monodehydro-ascorbate reductase, glutathione peroxidase, glutathione reductase, ascorbate peroxidase, peroxiredoxins, superoxide dismutase, and catalase (Nyathi and Baker, 2006; Cruz de

This article is protected by copyright. All rights reserved.

Carvalho, 2008; Corpas *et al.*, 2017*a*). Catalase was shown to play a role in scavenging hydrogen peroxide (H_2O_2) under stress conditions including drought in all thus far examined plant species including maize, sunflower and rice (Apel and Hirt, 2004; Cruz de Carvalho, 2008).

Abiotic stresses including drought, soil salinity, high light intensity, and heavy metals are known to increase peroxisome abundance in *Arabidopsis* (Desai and Hu, 2008; Sinclair *et al.*, 2009; Rodríguez-Serrano *et al.*, 2016; Fahy *et al.*, 2017) and drought increases peroxisome abundance in wheat (Sanad et al. 2019). Peroxisomes were proposed as a cellular proxy of drought and stresses that induce ROS production (Smertenko, 2017). Peroxisomes can form *de novo* from the endoplasmic reticulum or proliferate through fission (Hu *et al.*, 2012; Kao *et al.*, 2018). Peroxisome proliferation begins with peroxisome elongation mediated by the peroxisomal membrane-binding protein peroxin11 (*PEX11*) (Orth *et al.*, 2007; Sinclair *et al.*, 2009; Rodríguez-Serrano *et al.*, 2016). The transcription of *PEX11 genes* showed correlation with peroxisome abundance in response to salt stress in *Arabidopsis* (Mitsuya *et al.*, 2010; Fahy *et al.*, 2017). Drought stress also promotes transcription of *PEX11* genes *Arabidopsis* and wheat (Ebeed et al. 2018; Li and Hu 2015). Completion of the fission is facilitated by *FISSION1* proteins *FIS1A* and *FIS1B* (Lingard *et al.*, 2008; Hu *et al.*, 2012), and the dynamin-related proteins 3A and 3B (*DRP3A*, *DRP3B*) (Lingard *et al.*, 2008). The peroxisomal and mitochondrial division factor 1 (*PMD1*) also plays a role in peroxisomal fission (Aung and Hu, 2011); however, whether PMD1 works within the PEX11 pathway remains unclear.

The overall goals of this study are: (i) to determine the reasons for quinoa yield losses in response to heat, drought, and heat plus drought combination in eight quinoa genotypes from diverse geographical locations; and (ii) to test the suitability of using peroxisome abundance as a marker of response to heat, drought and heat and drought combination. We measured plant responses under field and greenhouse conditions using several established traits such as seed yield, ROS accumulation, stomatal conductance, leaf greenness index, photosynthetic activity, and quantification of peroxisome abundance. Our data shows that quinoa has limited capacity to avoid accumulation of reactive oxygen species under all stresses. Hence, protection of quinoa yield losses in arid hot climates requires breeding varieties with more efficient ROS homeostasis. We demonstrate that peroxisome peroliferation under our experimental conditsions correlates with transcription level of genes for peroxisome fission proteins *PEX11C* and *FIS1A*.

This article is protected by copyright. All rights reserved.

Accepted Article

Materials and Methods

Field experiments

Eight quinoa genotypes were selected out of 120 in our preliminary experiments in which plants were germinated in the greenhouse and then exposed to 40°C for 3 hours each day for 12 days starting at the flowering stage. Leaf greenness index (LGI) was measured on three random leaves from the middle of each plant by a Minolta SPAD-502 M (Konica Minolta Sensing, Tokyo, Japan) on the day that the plants were moved to the high temperature conditions (day 0) and 12 days after the heat treatment. The reduction of LGI was calculated as the difference between LGI on day 12 of the heat treatment and Day 0. The yield was calculated at maturity and measured per individual plant. Based on the grain yield and the LGI, four accessions were selected as putative heat-tolerant genotypes (QQ74, Pison, Baer, and BGQ 352) and four selected as putative heat-susceptible genotypes (Japanese Strain, 3 UISE, 17GR, and La Molina; **Table 1**).

The above eight genotypes were grown at Spillman Agronomy Farm of Washington State University (WSU) in Pullman, WA, USA (46°41'54"N 117°08'48"W, altitude 760 m), from June to October 2016 in sandy loam soil with pH of 5.7 and 3.96 % organic matter. The plants were seeded in the greenhouse, and one-month-old seedlings were transplanted in the field. The field was divided into distinct irrigated and non-irrigated treatments; 250 mm of supplementary water was delivered using drip irrigation in the irrigated treatment. Irrigation was supplied at least two days per week from the time of transplanting until the fruit development stage. Four replicates per genotype were grown in each irrigation treatment. The hottest temperature recorded was 35.1°C coinciding with the flowering stage, and the total rainfall was 45.72 mm (**Supplementary Fig. S1**). Soil moisture content and soil temperature were obtained using a 5TM soil sensor and recorded with a ProCheck data logger (Decagon Devices, Inc. Pullman, WA, USA).

Stomatal conductance, LGI, and chlorophyll *a* fluorescence were used as general indicators of stress. Chlorophyll *a* fluorescence was measured using a pulse amplitude modulation portable fluorometer MINI-PAM Walz GmbH (Effeltrich, Germany); leaves were dark-adapted for 30 min prior to the measurements with a leaf clip (DCL-8, Walz GmbH). Stomatal conductance was measured in the same leaves with a SC-1 Decagon porometer (Pullman, WA, USA) in fully expanded leaves from the

This article is protected by copyright. All rights reserved.

Accepted Article

middle portion of the plant. LGI was measured with a Minolta SPAD-502 M. To assess the impact on yield components, plant height, and grain weight parameters were recorded. The seeds were harvested by hand and cleaned using a blower type 4110.21.00 (200 mm) with inlet cup of 125 mm model 4110.20.09 (Seed Processing Holland B.V. Enkhuizen, The Netherlands).

Greenhouse and growth chamber experiments

The experiment was conducted in greenhouses at WSU, using the same eight quinoa genotypes that were used in the field experiment. Quinoa plants were grown in 2.6 L pots, filled with Sunshine Professional Growing Mix that includes 70-80% Canadian Sphagnum peat moss and dolomitic limestone remnants (Agawam, MA). Approximately five seeds per pot were sown. Seedlings were thinned to one plant per pot 10 days after seeding (DAS). Fertilization was performed 20 DAS with 2 g of Osmocote® classic (13-13-13; Everris, NA, Inc.) per pot. Plants were watered daily, and soil moisture content and soil temperature were monitored and stored daily using a 5TM probe and a ProCheck Sensor Read-Out (**Supplementary Fig. S2**). The growth chamber conditions were as follows: 14 h daytime, 700 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD), 25/19 °C daytime maximum/nighttime minimum temperatures, three hours of a ramping period between day and night, 40-70% relative humidity.

The stress treatments were applied 45 DAS when plants were at the flowering stage. Heat and heat × drought treatments were performed in the phenomics growth chamber facility of WSU, whereas plants in the drought and control treatments remained in the greenhouse. The growth conditions in the phenomics chamber were identical to those in the greenhouse during acclimation for three days before imposing the stress. The temperature was then increased to 35/30 °C daytime maximum/nighttime minimum, and relative humidity was maintained at 20-45%. To induce drought stress, watering was withheld until the soil water content decreased to ~0.08 m^3/m^{-3}. Plants were exposed to the drought, heat, and heat × drought stress treatments for five days. The temperature was then reduced to the original temperature (25/19 °C), and the plants were re-watered. After three days of recovery from the heat stress, the plants were moved from the phenomics growth chamber to the original greenhouse until harvesting. Air temperature and relative humidity were recorded every 30 min using a Lascar EL-USB-2-LCD USB Humidity Data Logger w/ LCD Display (MicroDAQ.com, Contoocook, NH) both in the greenhouse and the growth chamber (**Supplementary Fig. S2**). Chlorophyll fluorescence was imaged in dark-adapted plants with FluorCam 2701 LU camera

This article is protected by copyright. All rights reserved.

(Photon Systems Instruments, Drásov, Czech Republic). The maximum quantum efficiency of PSII (Fv/Fm) and non-photosynthetic quenching (NPQ) were determined from the images using Fluorcam software (version 7.0, 2007–2012, Photon Systems Instruments) according to manufacturer recommendations. Chlorophyll *a* fluorescence was measured with a pulse amplitude modulation portable fluorometer MINI-PAM Walz GmbH. LGI was measured as above on day 0 (plants were moved to the high temperature conditions) and day 5 of the heat treatment. The reduction of LGI was calculated as the difference between LGI on Day 5 of the heat treatment and Day 0. Stomata conductance and yield were measured as described above.

Microscopy and image analysis

Peroxisome staining was performed in young leaves approximately 1 cm in diameter using 1 μM N-BODIPY solution (Nitro-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene), which was prepared freshly from 1 mM stock solution in DMSO (Fahy et al., 2017). The images were acquired after 15 min of staining with Leica SP8 confocal microscope in the resonant scanning mode (12000 Hz) with six averages, 512 × 512 pixels image resolution.

Measuring peroxisome abundance

Peroxisome abundance was measured using N-BODIPY as described by Fahy *et al.* (2017). In the field experiments, the leaves were collected 57 days after transplanting to the field. The average temperature during this period was 31.4 °C. In the growth chamber experiments, the leaves were collected on the fifth day of the heat stress. Leaves were ground in liquid nitrogen, and total protein was extracted using a buffer containing 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 8 M urea. Cell debris was removed by centrifugation for 10 min at 14,000 rpm at room temperature. Then 20 μl of the protein extract of each sample was mixed with 80 μl of freshly prepared 2 μM solution of N-BODIPY and 100 μl of water in 96-well plates and incubated for 10 min. The fluorescence intensity was measured at 490 nm excitation wavelength and 530 nm emission wavelength using spectrofluorimeter Synergy Neo B (Biotek Instrument, Inc). Three technical and four biological replicates were performed per genotype in each treatment. The background was measured as (i) 20 μl of N-BODIPY supplemented with 180 μl of water per 96-well plate, and (ii) 20 μl of the protein extract in 180 μl of water per extract. Both background values were subtracted from the N-BODIPY fluorescence signal value. The fluorescence intensity was normalized by the protein concentration as

This article is protected by copyright. All rights reserved.

Accepted Article

measured with the Bradford Reagent (Biorad Laboratories) using a calibration curve constructed with solutions of known concentration of Bovine Serum Albumin. Fluorescence intensity was calculated in arbitrary units per 1 mg of protein.

Hydrogen peroxide content

Hydrogen peroxide content was measured according to Junglee, Urban, Sallanon & Lopez-Lauri (2014), with modification. The same leaf material was used for measuring peroxisome abundance and H_2O_2 content. Ground leaf material (150 mg) was extracted in 1 ml of buffer containing 0.1% (w/v) Trichloroacetic acid (TCA), 1 M KI and 10 mM KH_2PO_4 buffer, 5.8 pH at 4 °C for 10 min. A control was prepared with the same buffer but without KI. The extracts were protected from direct light. The homogenate was centrifuged at 12,000 × g for 15 min at 4 ˚C. Then, 200 µL of supernatant from each tube was placed in UV-microplate wells, incubated at room temperature (20 °C – 22 °C) for 20 min, and absorbance was measured at 320 nm. Three technical repeats were conducted per each sample. H_2O_2 concentration was determined using a calibration curve.

Gene transcription analysis

Peroxisome genes in quinoa were identified by performing a BLAST search of Arabidopsis sequences against the quinoa pseudomolecule genome assembly (Jarvis *et al.*, 2017) using default parameters (**Supplementary Fig. S3**). Syntenic regions of coding sequences surrounding homoeologous genes pairs in quinoa were identified and visualized using the CoGE GEvo tool (Lyons and Freeling, 2008).

RNA was extracted from leaves using the RNeasy Plant Mini Kit (Qiagen Inc, CA, USA). cDNA was synthesized using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.). Transcripts of quinoa peroxisome biogenesis genes *Peroxin11A, B,* and *C*; *FISSION1 (FIS1A)*; dynamin-related proteins (*DRPs*), and the peroxisomal and mitochondrial division factor 2 (*PMD2*) were annotated from the quinoa genome sequence (Jarvis *et al.*, 2017). The qPCR primers were designed to target both homoeologs for each peroxisome genes and primer-blast software was used to design them. (**Supplementary Table S1)**. Glyceraldehyde 3-phosphate dehydrogenase (*CqGAPDH*) was used as the reference as it was reportedly provides a suitable reference for analysis of gene expression under abiotic stress (Ruiz-Carrasco *et al.*, 2011) (**Supplementary Table S1)**. Three biological and three technical replicates were performed per

This article is protected by copyright. All rights reserved.

Accepted Article

sample. The relative quantification (RQ) values were calculated using ΔC_T mean and normalized by with *CqGAPDH* values.

Phylogenetic analysis

Phytozome v12 (https://phytozome.jgi.doe.gov) database was used to download amino acid sequences for *CqPEX11*, *CqDRP*, *CqFIS1,* and *CqPMD2* (**Supplementary Table S2**). The alignment of sequences was performed using the ClustalX software package (Thompson *et al.*, 1997). The phylodendrogram was constructed using the Bootstrap/Jackknife method of PAUP 4.0 software (Sinauer Associates). Bootstrap values were calculated from 1000 replicates; only groups with bootstrap scores of 60 or above were retained in the phylodendrogram. The alignment of the sequences is included as **Supplemental Dataset 1**. *Homo sapiens HsPEX11A* (NP_003838.1), *HsPEX11B* (NP_001171724.1), *HsPEX11C* (NP_542393.1), and *Drosophila melanogaster DmPEX11* (NP_611071.1) were used as an outgroup for constructing PEX11 phylodendrogram. *Arabidopsis thaliana AtDRP1E* and *AtDRP1C* were used as an outgroup for constructing DRP phylodendrogram.

Statistical analysis

For the field experiment, a split block design was used in order to evaluate the effects of irrigation and genotype. Irrigation was the main plot and genotype the sub-plot. Four blocks were used (2.77 m^2 per plot), and 30 plants were grown per plot, with 30 cm distance between plants.

For the growth chamber experiment, four treatments (control, drought, heat, and heat × drought combined stress) and four biological replicates (plant/pot) were used for each genotype. The data were subjected to two-way ANOVA for the growth chamber and field experiment. Standard error was shown as an estimate of variability. One-way ANOVA for each genotype was performed for peroxisome abundance, H_2O_2 content, and yield from the growth chamber experiment.

Post-hoc means separation for significant ANOVA factor effects were performed using the Tukey HSD test for genotypes, and LSD test for irrigation factor at the probability level of 0.05. Pearson correlation analysis was carried out among variables evaluated using all the genotypes excepted La

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

Molina and BGQ 352 for the field experiment. In the growth chamber experiment, Principal Component Analysis (PCA) and Pearson correlation were calculated for heat or drought stress using data from all genotypes. Combination of heat and drought abolished the yield in several genotypes and for this reason data from this condition could not be included in the analysis. In addition, PCA and Pearson correlation were performed for the genotypes Pison and QQ74 separately. This experiment included the gene transcription data.

Field experiment data were analyzed with the R statistical programing language (R Core Team, 2015) using the 'agricolae' package (de Mendiburu, 2017). Growth chamber data were analyzed using JMP (version 8.0, SAS Institute, Inc., Cary, NC). Principal component analysis (PCA) and Pearson correlation were performed using the R packages 'FactoMinerR' (Lê *et al.*, 2008), 'factoextra' (Kassambara and Mundt, 2016), and 'reshape2' (Wickham, 2007).

Results

Field experiment

Different physiological and agronomical parameters were measured in eight genotypes of quinoa that were grown in irrigated and non-irrigated plots during the hot season (**Table 1**; **Fig. 1; Supplementary Fig. S1; Supplementary Table S3**). Soil moisture in the non-irrigated plots was lower throughout the season (**Fig. 1A**). Chlorophyll fluorescence (F_v/F_m) was not significantly different among genotypes ($P = 0.22$) or among treatments ($P = 0.12$) (**Fig. 1B**). On the other hand, stomatal conductance was significantly lower in plants from the non-irrigated treatment (**Fig. 1C**). A dramatic decline in the stomatal conductance in both treatments coincided with the highest temperatures of July and August (**Supplementary Fig. S1**). Overall, drought did not affect leaf temperature, chlorophyll fluorescence, or LGI, whereas plant height and yield were lower under drought conditions relative to the irrigated controls (**Supplementary Table S3**). We found significant differences among genotype for LGI, plant height, and yield, but not for the other parameters (**Supplementary Table S3**).

This article is protected by copyright. All rights reserved.

The genotype QQ74 attained the highest mean grain yield of 961.10 kg/ha under the irrigated treatment, whereas BGQ 352 and La Molina did not produce any seeds in either the irrigated or the non-irrigated treatments (**Fig. 1D, Supplementary Table S3**). As the reduction of yield under stress conditions was not accompanied by changes in chlorophyll fluorescence, we tested the impact of stress on ROS homeostasis by measuring H_2O_2 content in leaves. Although mean H_2O_2 increased under stress conditions, we found significant variability among the genotypes: Japanese Strain, 17GR, BGQ 352, and 3 UISE accumulated more H_2O_2 under non-irrigated conditions, Pison contained more H_2O_2 under irrigated conditions, and no significant changes were detected in Baer and QQ74 (**Fig. 2A**).

As reactive oxygen species promote peroxisome proliferation, we examined the correlation between H_2O_2 content and peroxisome abundance in leaves. We have previously shown that peroxisomes can be labeled in cells and quantified in total extracts using the small fluorescent probe N-BODIPY (Landrum *et al.*, 2010; Fahy *et al.*, 2017; Frick and Strader, 2018). However, this technique was not yet used in quinoa. First, we tested the ability of N-BODIPY to stain peroxisomes in cells. As in other systems, N-BODIPY stained peroxisome-like structures in quinoa leaves (**Fig. 2B**), which exhibit rapid motions (**Movie S1**) as was shown for peroxisomes in *Arabidopsis* (Rodríguez-Serrano *et al.*, 2014). Measuring emission of N-BODIPY in a total leaf extract at 490 nm excitation produced the emission spectrum with a maximum of 530 nm (**Fig. 2C**). No fluorescent signal was detected if protein extract was used alone. Peroxisome abundance was higher in the non-irrigated treatment. The genotypes Japanese Strain, 17GR, and 3 UISE had significantly higher peroxisome abundance under the non-irrigated treatment (**Fig. 2D**). These genotypes also exhibited higher content of H_2O_2 in leaves.

Pearson correlation between individual parameters revealed that peroxisome abundance correlated positively with H_2O_2 content and leaf temperature, and negatively with yield, LGI, plant height, and stomatal conductance (**Supplementary Fig. S4**). Yield losses under stress in genotypes with elevated (17GR, 3 UISE, Japanese Strain, BGQ 352) or normal (Baer) H_2O_2 content suggests that oxidative stress was not the sole factor responsible for yield losses. BGQ 352 and La Molina are not adapted to the long-day photoperiod conditions found in Pullman. Consequently, both genotypes failed to produce seeds in both the irrigated and non-irrigated treatments due to late flowering. For the remaining genotypes, the flowering time in July coincided with a period of hot weather (**Supplementary Fig. S1A**). Thus, heat could be one of the unaccounted factors for the yield loss in

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

genotypes with efficient ROS homeostasis under drought. To examine the interaction between drought and heat stress, we performed experiments under the controlled greenhouse or growth chamber conditions.

Greenhouse and growth chamber experiment

Impact of stresses on photosynthesis and yield

We started by analyzing the impact of stresses on chlorophyll fluorescence parameters. The maximum quantum efficiency of PSII (*F*v/*F*m) and non-photosynthetic quenching (NPQ) were affected by the combination of heat and drought, but not heat alone (**Fig. 3A, C).** The least affected genotype was BGQ 352. In the course of the stresses, the *F*v/*F*m and NPQ values declined gradually under the combination of heat and drought (**Fig. 3B, 3D**), whereas *F*v/*F*m was not affected by the heat stress. Both parameters recovered after re-hydration.

Stomatal conductance, leaf temperature, and LGI were measured in all treatments with the exception of the heat × drought combination as this treatment made leaves excessively frail (**Fig. 3E, Supplementary Table S4**). Overall, both heat and drought caused reduction of stomatal conductance, though the impact of drought was more pronounced. Stomatal conductance was not affected by heat in Pison, QQ74, and 17GR (**Fig. 3E**). LGI was not significantly affected by the stress treatments, but differences were observed among the genotypes. On the other hand, leaf temperature was affected by each of the stress treatments, but no differences were detected among the genotypes (**Supplementary Table S4**).

Each stress treatment caused significant yield reduction in all genotypes, albeit to a different degree (**Fig. 3F**). The combination of drought and heat caused complete yield loss in all genotypes with exception of Pison, QQ74, and 17GR. Notably, BGQ 352 did not produce seeds under drought and heat stress despite steady *F*v/*F*m and higher NPQ values. Furthermore, similar changes of the chlorophyll fluorescence parameters were accompanied by dramatically different yield in QQ74, La Molina, Japanese Strain, and 3 UISE. This indicates that photosynthetic parameters alone are insufficient for discriminating stress-tolerant genotypes in quinoa.

This article is protected by copyright. All rights reserved.

Hydrogen peroxide and peroxisome proliferation

All stress treatments increased H_2O_2 content in leaves, but the most profound increase was detected in response to the heat × drought treatment (**Fig. 4A**). Analysis of the individual genotypes showed the highest content of H_2O_2 under the heat × drought treatment in all genotypes with the exception of BGQ 352, La Molina, and 17GR (**Fig. 4B**). In agreement with the higher level of hydrogen peroxide, all stresses promoted peroxisome proliferation on the population level and in the individual genotypes (**Fig. 4C, D**). However, peroxisome abundance under the combination of heat and drought was lower than heat and drought alone in all genotypes with exception of Japanese Strain.

As peroxisome abundance increased in response to all stresses, we determined the impact of stresses on the transcription level of peroxisome proliferation factors. Analysis of the quinoa genome revealed homologues of *Arabidopsis* genes known to function in peroxisome proliferation (**Fig. 5A**). Specifically, we identified pairs of homoeologous genes (one gene from each quinoa sub-genome; **Supplementary Fig. S3**) that share sequence homology with *PEX11A* (designated *CqPEX11A-1* and *CqPEX11A-2*), *PEX11B* (*CqPEX11B-1* and *CqPEX11B-2*), *PEX11C* (*CqPEX11C-1* and *CqPEX11C-2*) (Lingard and Trelease, 2006; Lingard *et al.*, 2008), *FIS1A* (*CqFIS1A-1* and *CqFIS1A-2*), *DRP3A* (*CqDRP3A-1* and *CqDRP3A-2*), and *PMD2* (*CqPMD2-1* and *CqPMD2-2*) (Aung and Hu, 2011). We identified three genes with homology to *DRP3B* (Lingard *et al.*, 2008): two genes (*CqDRP3B-2* and *CqDRP3B-3*) are homoeologous genes from each sub-genome, whereas the third gene (*CqDRP3B-1*) appears to be a duplication of *CqDRP3B-2*. We also identified three genes with homology to *DRP5B* (Zhang and Hu, 2010); however, further investigation indicated that one of the genes (*CqDRP5B-1*) is homoeologous to the other two (AUR62027388 and AUR62027388), which were incorrectly annotated and should be combined as a single gene (*CqDRP5B-2*; **Supplementary Fig. S3G**) . We did not identify a homologue of *PMD1*.

We measured transcription of peroxisome biogenesis genes in genotypes QQ74 and Pison, which showed strong peroxisome proliferation and produced seeds under all stresses in the field and greenhouse situations. Transcription of *CqPEX11A, CqPEX11B, CqDRP3A*, and *CqPMD2* remained constant. *CqPEX11B* did not show a consistent response to stresses (**Fig. 5B**). *CqPEX11C, CqFIS1A*, and *CqDRP3B* were up-regulated in response to the combination of heat and drought (**Fig. 5B, C, D**), and *CqPEX11C* and *CqFIS1A* were also upregulated in response to drought in both genotypes (**Fig. 5B, C, D**). Heat stress promoted transcription of *CqPEX11C* and *CqFIS1A* in QQ74. In Pison, transcription

This article is protected by copyright. All rights reserved.

Accepted Article

of both genes was already high under normal growth conditions (**Fig. 5B, C, D**). These data indicate that (i) the classical PEX11-fission pathway contributes to proliferation of peroxisomes under drought and the combination of heat and drought (Reumann and Bartel, 2016); and (ii) an alternative pathway (e.g. by *de novo* formation) could plays a role in peroxisome proliferation under heat stress.

Analysis of correlation between the traits

To examine the relationships between parameters evaluated under drought and heat stress in the growth chamber and greenhouse experiments we performed Pearson correlation and PCA. The correlation between yield and other parameters under combination of drought and heat could not be analysed because five genotypes failed to produce seeds. Combined data from both stresses showed negative correlation of peroxisome abundance with stomatal conductance and yield (r = -0.68 and -0.59, respectively) and positive correlation with hydrogen peroxide content in leaves (r = 0.39) (**Fig. 6A**). There was a strong negative correlation between hydrogen peroxide content and soil moisture, stomatal conductance, or yield (r = -0.81, -0.73, and -0.50, respectively). Finally, yield values correlated positively with stomatal conductance and soil moisture (r = 0.62 and 0.51, respectively; **Fig. 6A**). PCA of heat and drought stress individually showed that peroxisome abundance correlated positively with hydrogen peroxide content and negatively with yield (**Fig. 6B-C**).

Considering that genotypes Pison and QQ74 produced yield under all stress conditions, we could analyse correlation of all traits with the transcription level of peroxisome fission genes. There experiments showed significant correlation of hydrogen peroxide content in leaves with the transcription of peroxisome biogenesis genes and with peroxisome abundance. In particular, *CqPEX11B* transcription correlated positively with hydrogen peroxide content under heat stress (r = 0.88) (**Fig. S5A**). Under drought stress transcription of *CqDRP3B* and *CqFIS1A* correlated positively with hydrogen peroxide (r = 0.85 and 0.83 respectively) (**Fig. S5B**). *CqDRP3B* and *CqPEX11C* correlated positively with hydrogen peroxide content under the combination of heat and drought, (r = 0.68 and 0.79, respectively; **Fig. S5C**). Peroxisomes abundance correlated positively with hydroxide peroxide content under all type of stresses with a r value between 0.46 to 0.99 (**Fig. S5A-C**). Peroxisome abundance correlated positively with the transcription of peroxisome fission genes and negatively with yield under all stresses.

This article is protected by copyright. All rights reserved.

Discussion

Impact of stresses on H_2O_2 content and peroxisome abundance

ROS play a dual role in stress response: low ROS levels initiate adaptation mechanisms (del Río *et al.*, 2006; Corpas, 2015; del Río and López-Huertas, 2016; Raja *et al.*, 2017; Corpas *et al.*, 2017*a*; Corpas and Barroso, 2018) whereas high ROS levels cause oxidative damage to cellular components, which compromise plant viability (Foyer and Noctor, 2009; Sharma *et al.*, 2012; Das and Roychoudhury, 2014; Mittler, 2017). In our experiments, all examined stress treatments caused a significant increase in H_2O_2 content in quinoa leaves. Furthermore, H_2O_2 content in leaves correlated negatively with yield. We also found a negative correlation between ROS content in leaves and yield under drought in wheat varieties (Sanad *et al*., 2019). Together, these observations support the idea that ROS accumulation in leaves can be used as an indicator of drought-susceptibility.

Although ROS accumulation and activity of ROS scavengers were used for evaluating stress-tolerance (Foyer & Noctor 2009; Sharma *et al.* 2012; Das & Roychoudhury 2014; Mittler 2017), measuring H_2O_2 in populations of hundreds individuals could be laborious. It has been hypothesized that peroxisome abundance can be used as a proxy for ROS content (Smertenko, 2017). Here, we tested this hypothesis by correlating peroxisome abundance with H_2O_2 accumulation and other traits. In quinoa, peroxisome abundance in leaves increased in response to drought, heat, and the heat × drought treatment. Furthermore, peroxisome abundance correlated positively with H_2O_2 content in leaves and negatively with yield. Peroxisome proliferation was shown to be induced by soil salinity (Mitsuya *et al.*, 2010; Fahy *et al.*, 2017), light (López-Huertas, Charlton, Johnson, Graham & Baker 2000; Ferreira, Bird & Davies 1989; Desai & Hu 2008), ozone (Oksanen E. *et al.*, 2004), and cadmium treatments (Rodríguez-Serrano *et al.*, 2016; Corpas *et al.*, 2017*b*). Our findings support the notion that peroxisome abundance can be used as a cellular indicator of drought and heat stress response.

Peroxisome proliferation and peroxisome biogenesis genes

New peroxisomes can form *de novo* from the ER or through fission of existing peroxisomes (Hu *et al.*, 2012; Cross *et al.*, 2016). While we still lack reliable markers of the *de novo* mechanism, the markers of fission are known. In *Arabidopsis thaliana*, peroxisome fission depends on *PEX11*, which is encoded by a family of five genes, *PEX11A-E*, that can be divided into three subfamilies, *PEX11A,*

This article is protected by copyright. All rights reserved.

PEX11B, and *PEX11C-E* (Lingard and Trelease, 2006; Orth *et al.*, 2007; Lingard *et al.*, 2008). *PEX11* works together with *FIS1A*, *FIS1B*, *DRP3A*, *DRP3B* (Lingard *et al.*, 2008), *DRP5B* (Zhang and Hu, 2010) and *PMD1* (Aung and Hu, 2011). The quinoa genome contains homologues of known peroxisome fission factors: three *PEX11* genes (*PEX11A-C*), three *DRPs* (*DRP3A, DRP3B,* and *DRP5B*), and two *FIS1.* The lack of *PMD1,* but presence of *PMD2* homologue, which in *Arabidopsis* localizes to mitochondria and functions mitochondria fission (Aung and Hu, 2011), suggests that PMD1-dependent peroxisome fission pathway is not conserved in other species.

Analysis of the PEX11 family members in *A. thaliana* shows functional specialization. PEX11A plays an important role in the formation of peroxisome extensions, known as peroxules, under heavy metal stress in *A. thaliana* (Rodríguez-Serrano *et al.*, 2016). Transcription of *PEX11A* and *PEX11C* increases under salt-stress in wild-type *A. thaliana* (Col-0) but not in the salt-susceptible mutants (Fahy *et al.*, 2017). Transcription of *PEX11* genes was also up-regulated by drought *Arabidopsis* and wheat (Ebeed et al. 2018; Li and Hu 2015). In our experiments, *CqPEX11C* and *CqFIS1A* transcripts were up-regulated in both genotypes examined (Pison and QQ74) under drought and the combination of heat and drought. Principal component analysis demonstrated a positive correlation between *CqPEX11C* transcription and peroxisome abundance and a negative correlation with yield under combination of heat and drought stress. *CqPEX11A* and *CqPEX11B* transcripts remained unaffected by the combination of heat and drought, though *PEX11B* was up-regulated by drought in QQ74. Our data point out that: (i) quinoa *PEX11* gene family members have distinct organ-specific transcription and play different roles in response to stresses; and (ii) transcription of *PEX11C* and *FIS1A* correlates with higher peroxisome abundance under the drought or combination of heat and drought stress.

The combination of heat and drought caused the highest transcription levels of *FIS1A*, *DRP3B*, and *PEX11C*; however, peroxisome abundance appeared lower than under drought or heat alone. Assuming that transcription level of these genes represents the activity of the peroxisomal fission machinery, this indicates that the higher rate of peroxisome proliferation under the combination of heat and drought is compensated by the higher rate of peroxisome degradation. Damaged peroxisomes are eliminated by a specialized type of autophagy named pexophagy (Farmer *et al.*, 2013; Shibata *et al.*, 2013). Consequently, cells of *Arabidopsis* autophagy mutants *atg5-1* contain more peroxisomes (Yoshimoto *et al.*, 2014; Fahy *et al.*, 2017).

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Reduced peroxisome abundance under the combination of drought and heat stress could be the consequence of higher autophagic flux. Increasing autophagic flux is known to cause better fitness in response to stresses including oxidative stress (Minina *et al.*, 2018). Whether autophagy plays a role in drought adaptation remains unknown; however, survival of drought and heat together may require much higher autophagic flux than each stress alone. Alternatively, the activity of the fission machinery may not be the peroxisome proliferation rate-limiting factor. This factor could be unstable under the heat and drought stress or be produced at lower levels. Inability to maintain peroxisome abundance and H_2O_2 content could be the reasons for the dramatic yield losses under these circumstances.

Correlation of physiological parameters and peroxisome abundance with seed yield

Although quinoa is adapted to marginal environments, its yield can be diminished by high temperature (Bhargava *et al.*, 2006; Pulvento *et al.*, 2010; Hirich *et al.*, 2014; Peterson and Murphy, 2015; Bazile *et al.*, 2016*b*; Eisa *et al.*, 2017; Lesjak and Calderini, 2017; Hinojosa *et al.*, 2019) or drought (Walters *et al.*, 2016; Al-Naggar *et al.*, 2017). However, warm temperatures (28/20°C) were shown to increase plant height, photosynthesis rate, stomatal size, and seed yield in quinoa (Yang *et al.*, 2016; Becker *et al.*, 2017). The yield losses from heat and drought could be reduced through breeding tolerant varieties. Existing quinoa germplasm contains useful stress-tolerance traits. For example, genotypes Red Head and Salcedo produced higher seed yield than Cherry Vanilla at 35/29 °C (day/night) relative to the control conditions 20/14°C (Bunce, 2017). We also found significant yield losses in all genotypes in the field experiments despite many genotypes maintained ROS homeostasis, as evident from the measurements of the ROS content and peroxisome abundance (**Figure 2 A,D**). These findings highlight (i) susceptibility of all analyzed germplasm to these stresses; (ii) complexity of the stress-tolerance trait; and (iii) importance of using diverse parameters for assessing stress responses.

Out of eight genotypes, QQ74 and Pison performed consistently better relatively other genotypes under stress in the field and the greenhouse conditions. In agreement with our findings, QQ74 produced the highest yield amongst all tested varieties during warm summer in Pullman in 2011 (Peterson and Murphy, 2015). These genotypes could be used as the starting material for breeding varieties with better performance under combination of heat and drought.

This article is protected by copyright. All rights reserved.

PCA demonstrated the positive correlation of stomatal conductance with yield. We found that stomatal conductance was profoundly reduced in all genotypes under drought stress, whereas heat stress had a less significant impact. In Pison, QQ74, and 17GR, stomatal conductance was not affected by heat stress. Opened stomata at high temperature (40/24 °C day/night) in QQ74 and 17GR cool the leaves through transpiration (Hinojosa *et al.*, 2019). High temperature caused stomatal opening in quinoa cultivars 'Titicaca' and 'Achachino', and in other species such as *Lens culinaris*, *Hordeum vulgare*, *Nicotiana tabacum*, and *Arabidopsis thaliana* (Rizhsky *et al.*, 2002, 2004; Yang *et al.*, 2016; Cantalapiedra *et al.*, 2017; Sehgal *et al.*, 2017; Becker *et al.*, 2017).

Stomatal closure under drought and heat stress can reduce photosynthesis rate (Reddy *et al.*, 2004; Wahid *et al.*, 2007; Hasanuzzaman *et al.*, 2013). Higher stomatal conductance is related with the highest photosynthesis rate in quinoa (González *et al.*, 2011). Chlorophyll fluorescence parameters such as *F*v/*F*m and NPQ are commonly used for evaluating abiotic stress (Sharma *et al.*, 2015; Zhou *et al.*, 2015, 2018). Overall, we found a positive correlation between chlorophyll fluorescence parameters (*F*v/*F*m and *NPQ*) and yield. However, heat stress did not inhibit the *F*v/*F*m in all genotypes evaluated, as reported in *Arabidopsis* (Rizhsky *et al.*, 2004). On the contrary, NPQ seems to be a genotype-dependent parameter.

LGI indicates leaf chlorophyll content (Richardson *et al.*, 2002). The ability to maintain higher chlorophyll content (the stay-green trait) associated with heat tolerance in wheat (Cossani and Reynolds, 2012), and LGI was proposed as a high throughput measure to screen wheat germplasm under heat conditions (Ristic, Bukovnik & Prasad 2007). However, LGI correlated negatively with drought tolerance in potatoes (Rolando *et al.*, 2015; Ramírez *et al.*, 2015). In our experiments in both the field and in the greenhouse LGI was not influenced by heat or drought and poorly correlated with the yield. Perhaps, the relatively short exposure to the stresses (five days) was insufficient to induce significant changes.

Peroxisome abundance correlated positively with ROS content, but both parameters correlated negatively with yield. Moreover, peroxisome abundance and ROS content increased under all types of stress. As the accumulation of ROS and peroxisomes are symptomatic of oxidative stress, we conclude that all genotypes in our study have poor ability to maintain ROS homeostasis under stress. However, the genotypes QQ74 and Pison performed better than the other six genotypes under the

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

combination of heat and drought stress in both field and greenhouse conditions. Consistent with this conclusion, drought and the combination of heat and drought caused significant yield losses in the greenhouse experiments. Accumulation of ROS could be the consequence of stomatal closure, which prevents CO_2 uptake. This suggests that breeding varieties with more efficient ROS scavenging could be an effective strategy to prevent yield losses in quinoa production in arid climates.

Acknowledgements:

We thank to Deirdre Fahy and Dr. Derek Pouchnik for their advice on the gene transcription analysis. Moreover, we acknowledge the Plant Phenomics and Plant Growth Facilities stuff from WSU for their help in the greenhouse and growth chamber experiments. We thank Luz Gomez, Mario Tapia, and National Plant Germplasm System for share the quinoa seeds used in our experiments. We also acknowledge the generous financial support of USDA NIFA OREI #2016-51300-25808, USDA Award #2016-68004-24770, USDA NIFA Award #2017-67013-26200, The Orville A. Vogel Wheat Research Fund, and SENESCYT-Ecuador. The authors declare no conflict of interests.

Conflict of interests: The authors declare no conflict of interests.

Data Statement: This work does not contain large datasets. All materials are provided in the main text or supplemental information.

Short supporting legends:

Fig. S1. Average temperature and precipitation in Pullman during 2016.

Figure S2. Air temperature, soil temperature, and soil moisture in the greenhouse and growth chamber experiments.

This article is protected by copyright. All rights reserved.

Accepted Article

Figure S3. Conserved syntenic relationship between homoeologous regions surrounding peroxisome genes in quinoa.

Figure S4. Pearson correlation matrix of relationships between the measured parameters.

Figure S5. Pearson correlations and principal component analyses of all parameters in genotypes Pison and QQ74.

Table S1. Sequence of primers used in this study.

Table S2. Amino acid sequences of quinoa peroxisome fission genes.

Table S3.

Table S4.

Supplemental Dataset 1.

Movie S1. Mobility of N-BODIPY stained peroxisomes in quinoa leaves.

This article is protected by copyright. All rights reserved.

Accepted Article

References

Al-Naggar AM, M Abd El-Salam R, Badran A, El-Moghazi M. 2017. Genotype and drought effects on morphological, physiological and yield traits of quinoa (*Chenopodium quinoa Willd*.). Asian Journal of Advances in Agricultural Research **3**, 1–15.

Aluwi NA, Murphy KM, Ganjyal GM. 2017. Physicochemical characterization of different varieties of quinoa. Cereal Chemistry **94**, 847–856.

Alvarez-Flores R, Nguyen-Thi-Truc A, Peredo-Parada S, Joffre R, Winkel T. 2018. Rooting plasticity in wild and cultivated Andean *Chenopodium* species under soil water deficit. Plant and Soil **425**, 479–492.

Apel K, Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology **55**, 373–399.

Ashraf M. 2009. Biotechnological approach of improving plant salt tolerance using antioxidants as markers. Biotechnology Advances **27**, 84–93.

Aung K, Hu J. 2011. The Arabidopsis tail-anchored protein PEROXISOMAL AND MITOCHONDRIAL DIVISION FACTOR1 is involved in the morphogenesis and proliferation of peroxisomes and mitochondria. The Plant Cell **23**, 4446–4461.

Baxter A, Mittler R, Suzuki N. 2014. ROS as key players in plant stress signalling. Journal of Experimental Botany **65**, 1229–1240.

Bazile D, Jacobsen S-E, Verniau A. 2016*a*. The global expansion of quinoa: Trends and limits. Frontiers in Plant Science **7**, 622.

Bazile D, Pulvento C, Verniau A, *et al.* 2016*b*. Worldwide evaluations of quinoa: Preliminary results from post International Year of Quinoa FAO projects in nine countries. Frontiers in Plant Science **7**, 850.

Becker VI, Goessling JW, Duarte B, Caçador I, Liu F, Rosenqvist E, Jacobsen S-E. 2017. Combined effects of soil salinity and high temperature on photosynthesis and growth of quinoa plants (*Chenopodium quinoa*). Functional Plant Biology **44**, 665–678.

Bhargava A, Shukla S, Ohri D. 2006. *Chenopodium quinoa*—An Indian perspective. Industrial Crops and Products **23**, 73–87.

Bosque Sanchez H, Lemeur R, Damme PV, Jacobsen S-E. 2003. Ecophysiological analysis of drought and salinity stress of quinoa (*Chenopodium quinoa* Willd.). Food Reviews International **19**, 111–119.

Bunce JA. 2017. Variation in yield responses to elevated CO2 and a brief high temperature treatment in quinoa. Plants **6**, 26.

This article is protected by copyright. All rights reserved.

Cantalapiedra CP, García-Pereira MJ, Gracia MP, Igartua E, Casas AM, Contreras-Moreira B. 2017. Large differences in gene expression responses to drought and heat stress between elite barley cultivar Scarlett and a Spanish landrace. Frontiers in Plant Science **8**, 647.

Choudhury FK, Rivero RM, Blumwald E, Mittler R. 2017. Reactive oxygen species, abiotic stress and stress combination. The Plant Journal **90**, 856–867.

Corpas FJ. 2015. What is the role of hydrogen peroxide in plant peroxisomes? Plant Biology **17**, 1099–1103.

Corpas FJ, Barroso JB. 2018. Peroxisomal plant metabolism – an update on nitric oxide, Ca2+ and the NADPH recycling network. J Cell Sci **131**, jcs202978.

Corpas FJ, Barroso JB, Palma JM, Rodriguez-Ruiz M. 2017*a*. Plant peroxisomes: A nitro-oxidative cocktail. Redox Biology **11**, 535–542.

Corpas FJ, Pedrajas JR, Palma JM, Valderrama R, Rodríguez-Ruiz M, Chaki M, del Río LA, Barroso JB. 2017*b*. Immunological evidence for the presence of peroxiredoxin in pea leaf peroxisomes and response to oxidative stress conditions. Acta Physiologiae Plantarum **39**, 57.

Cossani CM, Reynolds MP. 2012. Physiological traits for improving heat tolerance in wheat. Plant Physiology **160**, 1710–1718.

Cross LL, Ebeed HT, Baker A. 2016. Peroxisome biogenesis, protein targeting mechanisms and PEX gene functions in plants. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1863**, 850–862.

Cruz de Carvalho MH. 2008. Drought stress and reactive oxygen species. Plant Signaling & Behavior **3**, 156–165.

Das K, Roychoudhury A. 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Frontiers in Environmental Science **2**, 53.

De Duve C, Baudhuin P. 1966. Peroxisomes (microbodies and related particles). Physiological Reviews **46**, 323–357.

Desai M, Hu J. 2008. Light induces peroxisome proliferation in Arabidopsis seedlings through the photoreceptor phytochrome A, the transcription factor HY5 HOMOLOG, and the peroxisomal protein PEROXIN11b. Plant Physiology **146**, 1117–1127.

Ebeed HT, Stevenson SR, Cuming AC, Baker A 2018. Conserved and differential transcriptional responses of peroxisome associated pathways to drought, dehydration and ABA. J Exp Bot **69,** 4971-4985.

Eisa SS, Eid MA, Abd E-S, *et al.* 2017. *Chenopodium quinoa* Willd. A new cash crop halophyte for saline regions of Egypt. Australian Journal of Crop Science **11**, 343–351.

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

Fahy D, Sanad MNME, Duscha K, *et al.* 2017. Impact of salt stress, cell death, and autophagy on peroxisomes: quantitative and morphological analyses using small fluorescent probe N-BODIPY. Scientific Reports **7**, 39069.

Farmer LM, Rinaldi MA, Young PG, Danan CH, Burkhart SE, Bartel B. 2013. Disrupting autophagy restores peroxisome function to an *Arabidopsis* lon2 mutant and reveals a role for the LON2 protease in peroxisomal matrix protein degradation. The Plant Cell **25**, 4085–4100.

Ferreira RMB, Bird B, Davies DD. 1989. The effect of light on the structure and organization of lemna peroxisomes. Journal of Experimental Botany **40**, 1029–1035.

Foyer CH, Noctor G. 2009. Redox regulation in photosynthetic organisms: Signaling, acclimation, and practical implications. Antioxidants & Redox Signaling **11**, 861–905.

Frick EM, Strader LC. 2018. Kinase MPK17 and the peroxisome division factor PMD1 Influence salt-induced peroxisome proliferation. Plant Physiology **176**, 340–351.

Fuentes F, Bhargava A. 2011. Morphological analysis of quinoa germplasm grown under lowland desert conditions. Journal of Agronomy and Crop Science **197**, 124–134.

Geerts S, Raes D, Garcia M, *et al.* 2008. Introducing deficit irrigation to stabilize yields of quinoa (Chenopodium quinoa Willd.). European Journal of Agronomy **28**, 427–436.

González JA, Bruno M, Valoy M, Prado FE. 2011. Genotypic variation of gas exchange parameters and leaf stable carbon and nitrogen isotopes in ten quinoa cultivars grown under drought. Journal of Agronomy and Crop Science **197**, 81–93.

Hasanuzzaman M, Nahar K, Alam MM, Roychowdhury R, Fujita M. 2013. Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. International Journal of Molecular Sciences **14**, 9643–9684.

Hinojosa L, González J.A, Barrios-Masias F.H, **Fuentes F**, **Murphy K**. 2018. Quinoa abiotic stress responses: A review. Plants **7**, 106.

Hinojosa L, Matanguihan J, Murphy K. 2019. Effect of high temperature on pollen morphology, plant growth and seed yield in quinoa (*Chenopodium quinoa* Willd.). Journal of Agronomy and Crop Science **201**, 33-45.

Hirich A, Choukr-Allah R, Jacobsen S-E. 2014. Quinoa in Morocco – Effect of sowing dates on development and yield. Journal of Agronomy and Crop Science **200**, 371–377.

Hu J, Baker A, Bartel B, Linka N, Mullen RT, Reumann S, Zolman BK. 2012. Plant peroxisomes: biogenesis and function. The Plant cell **24**, 2279–2303.

IPCC. 2014. *Climate Change 2014 – Impacts, adaptation, and vulnerability: Regional aspects*. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

This article is protected by copyright. All rights reserved.

Accepted Article

Jacobsen S-E, Liu F, Jensen CR. 2009. Does root-sourced ABA play a role for regulation of stomata under drought in quinoa (*Chenopodium quinoa* Willd.). Scientia Horticulturae **122**, 281–287.

Jarvis DE, Ho YS, Lightfoot DJ, *et al.* 2017. The genome of Chenopodium quinoa. Nature **542**, 307–312.

Junglee S, Urban L, Sallanon H, Lopez-Lauri F. 2014. Optimized assay for hydrogen peroxide determination in plant tissue using potassium iodide. American Journal of Analytical Chemistry **5**, 730.

Kao Y-T, Gonzalez KL, Bartel B. 2018. Peroxisome function, biogenesis, and dynamics in plants. Plant Physiology **176**, 162–177.

Kassambara A, Mundt F. 2016. *factoextra: Extract and visualize the results of multivariate data analyses. R package*.

Kowalski RJ, Medina-Meza IG, Thapa BB, Murphy KM, Ganjyal GM. 2016. Extrusion processing characteristics of quinoa (*Chenopodium quinoa* Willd.) var. Cherry Vanilla. Journal of Cereal Science **70**, 91–98.

Landrum M, Smertenko A, Edwards R, Hussey PJ, Steel PG. 2010. BODIPY probes to study peroxisome dynamics in vivo. The Plant Journal **62**, 529–538.

Lê S, Josse J, Husson F. 2008. FactoMineR: An R package for multivariate analysis. Journal of Statistical Software **25**, 1–18.

Lesjak J, Calderini DF. 2017. Increased night temperature negatively affects grain yield, biomass and grain number in Chilean quinoa. Frontiers in Plant Science **8**, 352.

Li J, Hu J 2015, Using Co-Expression Analysis and Stress-Based Screens to Uncover Arabidopsis Peroxisomal Proteins Involved in Drought Response. PLoS One **10,** e0137762.

Lingard MJ, Gidda SK, Bingham S, Rothstein SJ, Mullen RT, Trelease RN. 2008. Arabidopsis PEROXIN11c-e, FISSION1b, and DYNAMIN-RELATED PROTEIN3A cooperate in cell cycle–associated replication of peroxisomes. The Plant Cell **20**, 1567–1585.

Lingard JM, Trelease NR. 2006. Five Arabidopsis peroxin 11 homologs individually promote peroxisome elongation, duplication or aggregation. Journal of Cell Science **119**, 1961–1972.

Lyons E, Freeling M. 2008. How to usefully compare homologous plant genes and chromosomes as DNA sequences. The Plant Journal **53**, 661–673.

Maliro MFA, Guwela VF, Nyaika J, Murphy KM. 2017. Preliminary studies of the performance of quinoa (*Chenopodium quinoa* Willd.) genotypes under irrigated and rainfed conditions of central Malawi. Frontiers in Plant Science **8**, 227.

This article is protected by copyright. All rights reserved.

Accepted Article

Martínez EA, Veas E, Jorquera C, San Martín R, Jara P. 2009. Re-Introduction of quinoa into Arid Chile: Cultivation of two lowland races under extremely low irrigation. Journal of Agronomy and Crop Science **195**, 1–10.

de Mendiburu F. 2017. *agricolae: Statistical Procedures for Agriculture Research*. R package version 1.2-8.

Minina EA, Moschou PN, Vetukuri RR, *et al.* 2018. Transcriptional stimulation of rate-limiting components of the autophagic pathway improves plant fitness. Journal of Experimental Botany **69**, 1415–1432.

Mitsuya S, El-Shami M, Sparkes IA, Charlton WL, Lousa CDM, Johnson B, Baker A. 2010. Salt stress causes peroxisome proliferation, but inducing peroxisome proliferation does not improve NaCl tolerance in Arabidopsis thaliana. PLOS ONE **5**, e9408.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science **7**, 405–410.

Mittler R. 2017. ROS are good. Trends in Plant Science **22**, 11–19.

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F. 2011. ROS signaling: the new wave? Trends in Plant Science **16**, 300–309.

Morales A, Zurita-Silva A, Maldonado J, Silva H. 2017. Transcriptional responses of Chilean quinoa (*Chenopodium quinoa* Willd.) under water deficit conditions uncovers ABA-independent expression patterns. Frontiers in Plant Science **8**, 216.

Murphy KM, Bazile D, Kellogg J, Rahmanian M. 2016. Development of a worldwide consortium on evolutionary participatory breeding in quinoa. Frontiers in Plant Science **7**, 608.

Nyathi Y, Baker A. 2006. Plant peroxisomes as a source of signalling molecules. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1763**, 1478–1495.

Oksanen E., Häikiö E., Sober J., Karnosky D. F. 2004. Ozone-induced H2O2 accumulation in field-grown aspen and birch is linked to foliar ultrastructure and peroxisomal activity. New Phytologist **161**, 791–799.

Orth T, Reumann S, Zhang X, Fan J, Wenzel D, Quan S, Hu J. 2007. The PEROXIN11 protein family controls peroxisome proliferation in *Arabidopsis*. The Plant cell **19**, 333–350.

Peterson A, Murphy KM. 2015. Quinoa cultivation for temperate North America: Considerations and areas for investigation. In: Murphy K,, In: Matanguihan J, eds. Quinoa: Improvement and Sustainable Production. Hoboken, NJ, USA: John Wiley & Sons, Inc, 173–192.

Pulvento C, Riccardi M, Lavini A, D'Andria R, Iafelice G, Marconi E. 2010. Field trial evaluation of two *Chenopodium quinoa* genotypes grown under rain-fed conditions in a typical Mediterranean environment in south Italy. Journal of Agronomy and Crop Science **196**, 407–411.

This article is protected by copyright. All rights reserved.

R Core Team. 2015. *R: A language and environment for statistical computing*. Vienna, Australia: R Foundation for Statistical Computing.

Raja V, Majeed U, Kang H, Andrabi KI, John R. 2017. Abiotic stress: Interplay between ROS, hormones, and MAPKs. Environmental and Experimental Botany **137**, 142–157.

Ramírez DA, Rolando JL, Yactayo W, Monneveux P, Mares V, Quiroz R. 2015. Improving potato drought tolerance through the induction of long-term water stress memory. Plant Science **238**, 26–32.

Raney JA, Reynolds DJ, Elzinga DB, Page J, Udall JA, Jellen EN, Bonfacio A, Fairbanks DJ, Maughan PJ. 2014. Transcriptome analysis of drought induced stress in *Chenopodium quinoa*. American Journal of Plant Sciences **5**, 338–357.

Razzaghi F, Plauborg F, Jacobsen S-E, Jensen CR, Andersen MN. 2012. Effect of nitrogen and water availability of three soil types on yield, radiation use efficiency and evapotranspiration in field-grown quinoa. Agricultural Water Management **109**, 20–29.

Reddy AR, Chaitanya KV, Vivekanandan M. 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. Journal of Plant Physiology **161**, 1189–1202.

Reumann S, Bartel B. 2016. Plant peroxisomes: recent discoveries in functional complexity, organelle homeostasis, and morphological dynamics. Current Opinion in Plant Biology **34**, 17–26.

Richardson AD, Duigan SP, Berlyn GP. 2002. An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytologist **153**, 185–194.

del Río LA, López-Huertas E. 2016. ROS generation in peroxisomes and its role in cell signaling. Plant and Cell Physiology **57**, 1364–1376.

del Río LA, Sandalio LM, Corpas FJ, Palma JM, Barroso JB. 2006. Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling. Plant Physiology **141**, 330–335.

Ristic Z, Bukovnik U, Prasad PVV. 2007. Correlation between heat stability of thylakoid membranes and loss of chlorophyll in winter wheat under heat stress. Crop Science **47**, 2067–2073.

Rizhsky L, Liang H, Mittler R. 2002. The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiology **130**, 1143–1151.

Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R. 2004. When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. Plant Physiology **134**, 1683–1696.

Rodríguez-Serrano M, Pazmiño DM, Sparkes I, Rochetti A, Hawes C, Romero-Puertas MC, Sandalio LM. 2014. 2,4-Dichlorophenoxyacetic acid promotes S-nitrosylation and oxidation of actin affecting cytoskeleton and peroxisomal dynamics. Journal of Experimental Botany **65**, 4783–4793.

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

Rodríguez-Serrano M, Romero-Puertas MC, Sanz-Fernández M, Hu J, Sandalio LM. 2016. Peroxisomes extend peroxules in a fast response to stress via a reactive oxygen species-mediated induction of the peroxin PEX11a. Plant Physiology **171**, 1665–1674.

Rolando JL, Ramírez DA, Yactayo W, Monneveux P, Quiroz R. 2015. Leaf greenness as a drought tolerance related trait in potato (*Solanum tuberosum* L.). Environmental and Experimental Botany **110**, 27–35.

Ruiz-Carrasco K, Antognoni F, Coulibaly AK, Lizardi S, Covarrubias A, Martínez EA, Molina-Montenegro MA, Biondi S, Zurita-Silva A. 2011. Variation in salinity tolerance of four lowland genotypes of quinoa (*Chenopodium quinoa* Willd.) as assessed by growth, physiological traits, and sodium transporter gene expression. Plant Physiology and Biochemistry **49**, 1333–1341.

Sanad M, Smertenko A, Garland-Campbell K. 2019. Differential dynamic changes of reduced trait model for analyzing the plastic response to drought phases: a case study in spring wheat f. Frontiers in Plant Science. Frontiers in Plant Sciences, Frontiers Plant Science. **10**, 504.

Sehgal A, Sita K, Kumar J, Kumar S, Singh S, Siddique KHM, Nayyar H. 2017. Effects of drought, heat and their interaction on the growth, yield and photosynthetic function of lentil (*Lens culinaris* Medikus) genotypes varying in heat and drought sensitivity. Frontiers in Plant Science **8**, 1776.

Sharma DK, Andersen SB, Ottosen C, Rosenqvist E. 2015. Wheat cultivars selected for high Fv/Fm under heat stress maintain high photosynthesis, total chlorophyll, stomatal conductance, transpiration and dry matter. Physiologia Plantarum **153**, 284–298.

Sharma P, Jha AB, Dubey RS, Pessarakli M. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany **2012**, 1–27.

Shibata M, Oikawa K, Yoshimoto K, Kondo M, Mano S, Yamada K, Hayashi M, Sakamoto W, Ohsumi Y, Nishimura M. 2013. Highly oxidized peroxisomes are selectively degraded via autophagy in *Arabidopsis*. The Plant Cell **25**, 4967–4983.

Sinclair AM, Trobacher CP, Mathur N, Greenwood JS, Mathur J. 2009. Peroxule extension over ER-defined paths constitutes a rapid subcellular response to hydroxyl stress. The Plant Journal **59**, 231–242.

Smertenko A. 2017. Can peroxisomes inform cellular response to drought? Trends in Plant Science **22**, 1005–1007.

Suzuki N, Rivero RM, Shulaev V, Blumwald E, Mittler R. 2014. Abiotic and biotic stress combinations. New Phytologist **203**, 32–43.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X Windows Interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research **25**, 4876–4882.

This article is protected by copyright. All rights reserved.

Accepted Article

Vega-Gálvez A, Miranda M, Vergara J, Uribe E, Puente L, Martínez EA. 2010. Nutrition facts and functional potential of quinoa (*Chenopodium quinoa* Willd.), an ancient Andean grain: a review. Journal of the Science of Food and Agriculture **90**, 2541–2547.

Wahid A, Gelani S, Ashraf M, Foolad MR. 2007. Heat tolerance in plants: An overview. Environmental and Experimental Botany **61**, 199–223.

Walters H, Carpenter-Boggs L, Desta K, Yan L, Matanguihan J, Murphy K. 2016. Effect of irrigation, intercrop, and cultivar on agronomic and nutritional characteristics of quinoa. Agroecology and Sustainable Food Systems **40**, 783–803.

Wickham H. 2007. Reshaping data with the reshape package. Journal of Statistical Software **21**, 1–20.

Wu G. 2015. Nutritional properties of quinoa. In: Murphy K,, In: Matanguihan J, eds. Quinoa: Improvement and sustainable production. Hoboken, NJ, USA: John Wiley & Sons, Inc, 193–210.

Wu G, Morris CF, Murphy KM. 2017*a*. Quinoa starch characteristics and their correlations with the texture profile analysis (TPA) of cooked quinoa. Journal of Food Science **82**, 2387–2395.

Wu G, Peterson AJ, Morris CF, Murphy KM. 2016. Quinoa seed quality response to sodium chloride and sodium sulfate salinity. Frontiers in Plant Science **7**, 790.

Wu G, Ross CF, Morris CF, Murphy KM. 2017*b*. Lexicon development, consumer acceptance, and drivers of liking of quinoa varieties. Journal of Food Science **82**, 993–1005.

Xue T, Li X, Zhu W, Wu C, Yang G, Zheng C. 2009. Cotton metallothionein GhMT3a, a reactive oxygen species scavenger, increased tolerance against abiotic stress in transgenic tobacco and yeast. Journal of Experimental Botany **60**, 339–349.

Yang A, Akhtar SS, Amjad M, Iqbal S, Jacobsen S-E. 2016. Growth and physiological responses of quinoa to drought and temperature stress. Journal of Agronomy and Crop Science **202**, 445–453.

Yoshimoto K, Shibata M, Kondo M, Oikawa K, Sato M, Toyooka K, Shirasu K, Nishimura M, Ohsumi Y. 2014. Organ-specific quality control of plant peroxisomes is mediated by autophagy. J Cell Sci **127**, 1161–1168.

Zandalinas SI, Mittler R, Balfagón D, Arbona V, Gómez-Cadenas A. 2018. Plant adaptations to the combination of drought and high temperatures. Physiologia Plantarum **162**, 2–12.

Zhang X, Hu J. 2010. The Arabidopsis chloroplast division protein DYNAMIN-RELATED PROTEIN5B also mediates peroxisome division. The Plant cell **22**, 431–442.

Zhou R, Hyldgaard B, Yu X, Rosenqvist E, Ugarte RM, Yu S, Wu Z, Ottosen C-O, Zhao T. 2018. Phenotyping of faba beans (*Vicia faba* L.) under cold and heat stresses using chlorophyll fluorescence. Euphytica **214**, 68.

This article is protected by copyright. All rights reserved.

Accepted Article

Zhou R, Yu X, Kjaer KH, Rosenqvist E, Ottosen C-O, Wu Z. 2015. Screening and validation of tomato genotypes under heat stress using Fv/Fm to reveal the physiological mechanism of heat tolerance. Environmental and Experimental Botany **118**, 1–11.

Zurita-Silva A, Fuentes F, Zamora P, Jacobsen S-E, Schwember AR. 2014. Breeding quinoa (*Chenopodium quinoa* Willd.): potential and perspectives. Molecular Breeding **34**, 13–30.

Tables

Table 1. Eight quinoa genotypes evaluated in the field and the growth chamber experiments under abiotic stresses.

Name	PI number USDA	Seed source	Origin
QQ74	PI 614886	USDA	Maule – Chile
Pison	Ames 13746	USDA	NA
Japanese Strain	NSL 92331	USDA	NA
Baer	PI 634918	USDA	Cajon – Chile
3 UISE	Ames 13756	USDA	NA
17GR	Ames 13735	USDA	NA
BGQ 352	-	Germplasm Bank “La Molina”	Perú
La Molina	-	Mario Tapia (Perú)	Perú

This article is protected by copyright. All rights reserved.

Accepted Article

Figures legends

Figure 1. **Impact of stress on the agronomical and physiological parameters of eight quinoa genotypes in field experiments.**

A, Soil moisture content.

B, Chlorophyll fluorescence on day 27-July-2016 (no significant difference was found).

C, Stomatal conductance measured at four time points.

D, Impact of drought stress on yield.

Data are presented as means (±SE, n = 4). Points with "*" are statistically different at $P < 0.05$; points with "**" are statistically different at $P < 0.01$). Irrigated: heat stress only. Non-irrigated: combined stresses (heat × drought).

Figure 2. **Hydrogen peroxide content and peroxisome proliferation in quinoa leaves from field experiment under irrigated and non-irrigated conditions.**

A, Average hydrogen peroxide content in all genotypes (left) or in individual genotypes (right) ("La Molina" genotype was not recorded).

B, Peroxisomes in a leaf pavement cell stained with N-BODIPY. Scale bar 5 μm.

C, Emission spectra at 480 nm excitation of total quinoa leaf extract incubated with 5 μM N-BODIPY (blue) or with water (red).

D, Average leaf peroxisome abundance of all genotypes (left) or in individual genotypes (right). Data are presented as means (±SE, n = 4). Columns with "*" and "**" are statistically different at the $P < 0.05$ and $P < 0.01$ levels, respectively.

Figure 3. Impact of stresses on photosynthesis, stomatal conductance, and yield.

A and **C**, Maximum quantum efficiency of PSII (Fv/Fm) and non-photosynthetic quenching (NPQ) on the fifth day of stress;

B and **D**, Average of Fv/Fm and NPQ in all quinoa genotypes through five days of

This article is protected by copyright. All rights reserved.

Accepted Article

the stress and two days of recovery.

E, Stomatal conductance in the fifth day of stress. Measuring stomatal conductance in plants under combination of heat and drought stress was not feasible because leaves became frail.

F, Yield per plant.

Data are presented as means (±SE, n = 4), the points with "*" has a statistical difference (**P** < 0.05) and the points with "**" has a statistical difference (**P** < 0.01). Different letters had significant difference (**P** < 0.05) using Tukey HSD test between the treatments.

Figure 4. Impact of stresses on H_2O_2 content and peroxisome abundance.

A, **B**, H_2O_2 content in the population (A) and individual genotypes (B).

C, **D**, Peroxisome abundance in the population (C) and individual genotypes (D).

Error bars represent standard error (n = 4), different letters indicate significant difference between the values (**P** < 0.05).

Figure 5. Impact of stresses on the transcription of peroxisome biogenesis genes.

A, Phylodendrogram of peroxisome fission factors PEX11A and DRPs from *Chenopodium quinoa* (Cq), *Arabidopsis thaliana* (At), *Homo sapiens* (Hs), and *Drosophila melanogaster* (Dm) **B**, Heat map of *FIS1A, DRP3B, PEX11B,* and *PEX11C* transcription in Pison and QQ74 under control, drought, heat and their combination.

Figure 6. Pearson correlations and principal component analyses.

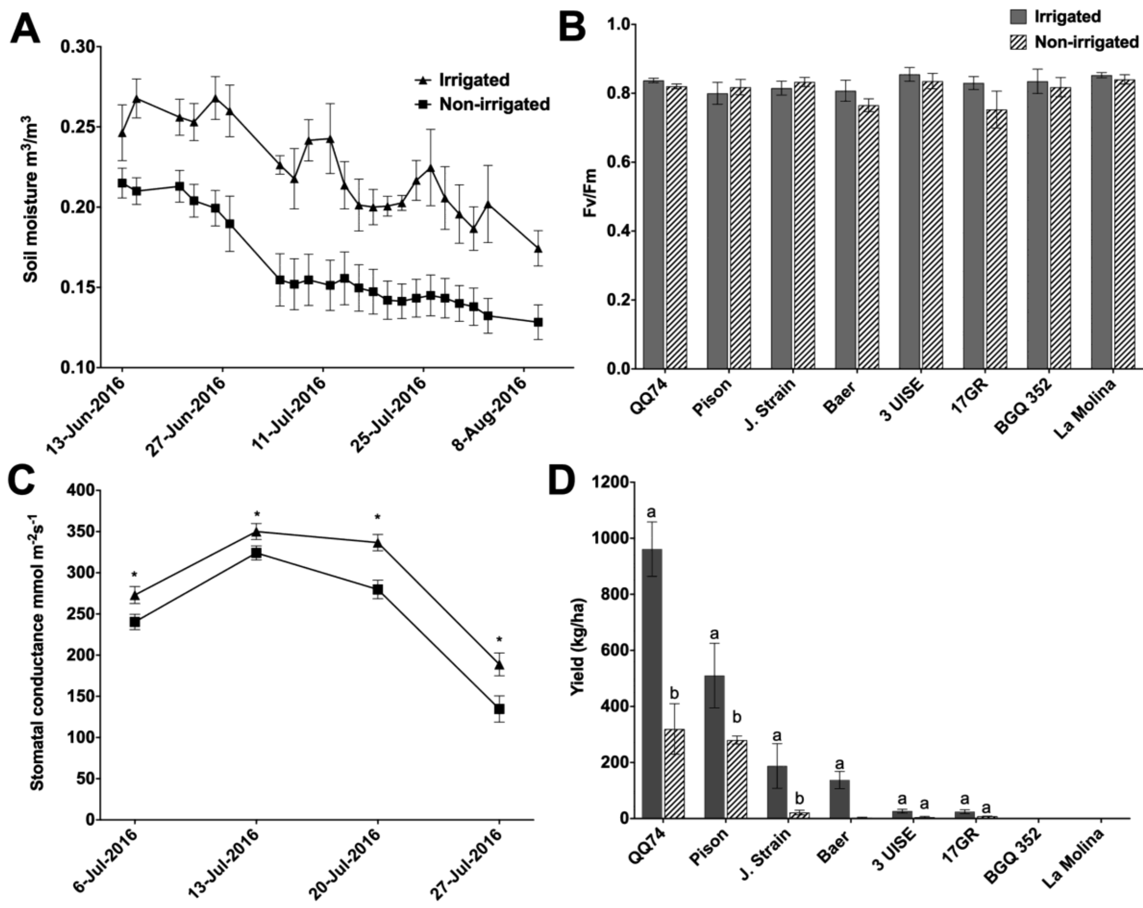
A, Pearson correlation matrix for the parameters evaluated in all the stress conditions.

B-D, Principal component analysis (PCA) biplot using all parameters evaluated under drought (**B**), heat (**C**), and the combination of heat and drought (**D**).

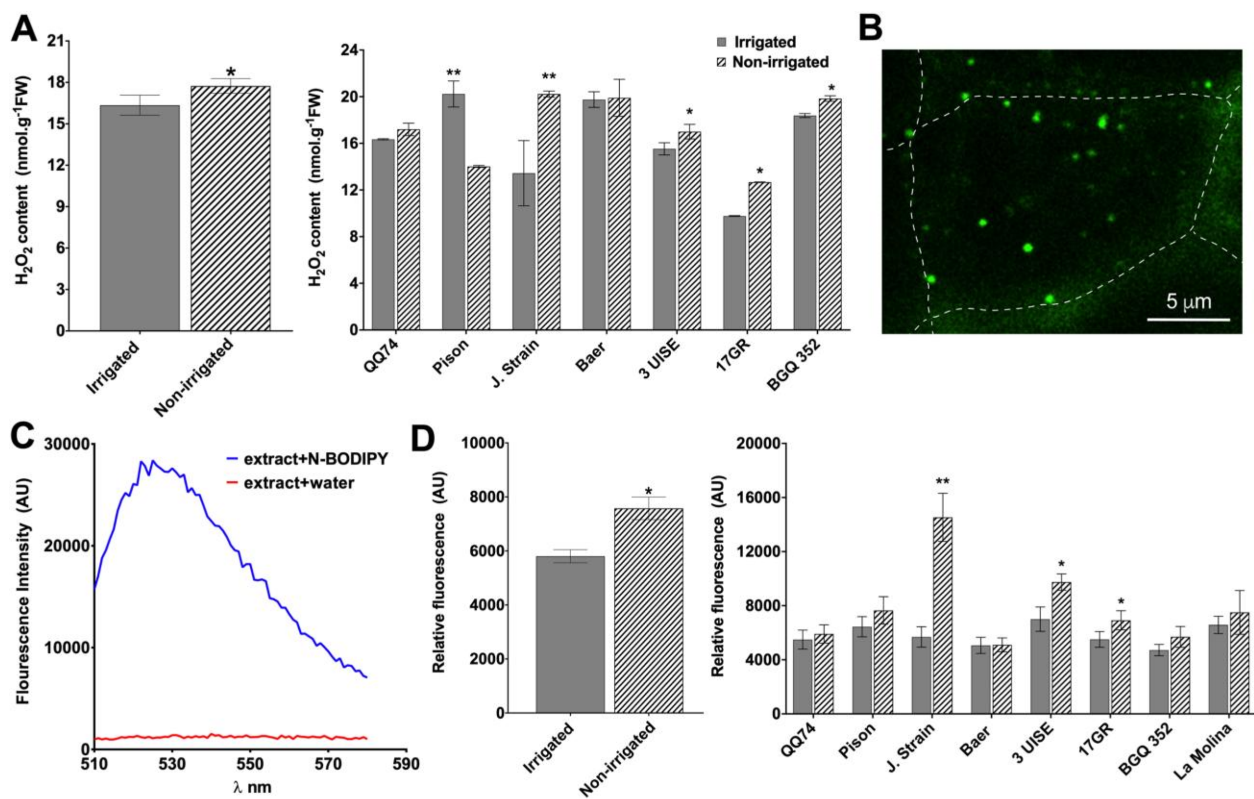
Plant height (P. Height), soil moisture (S. moist.), peroxisome abundance (PEROX), soil temperature (S. Temp.), leaf temperature (Leaf temp.), and stomatal conductance (St. Conductance).

This article is protected by copyright. All rights reserved.

Accepted Article



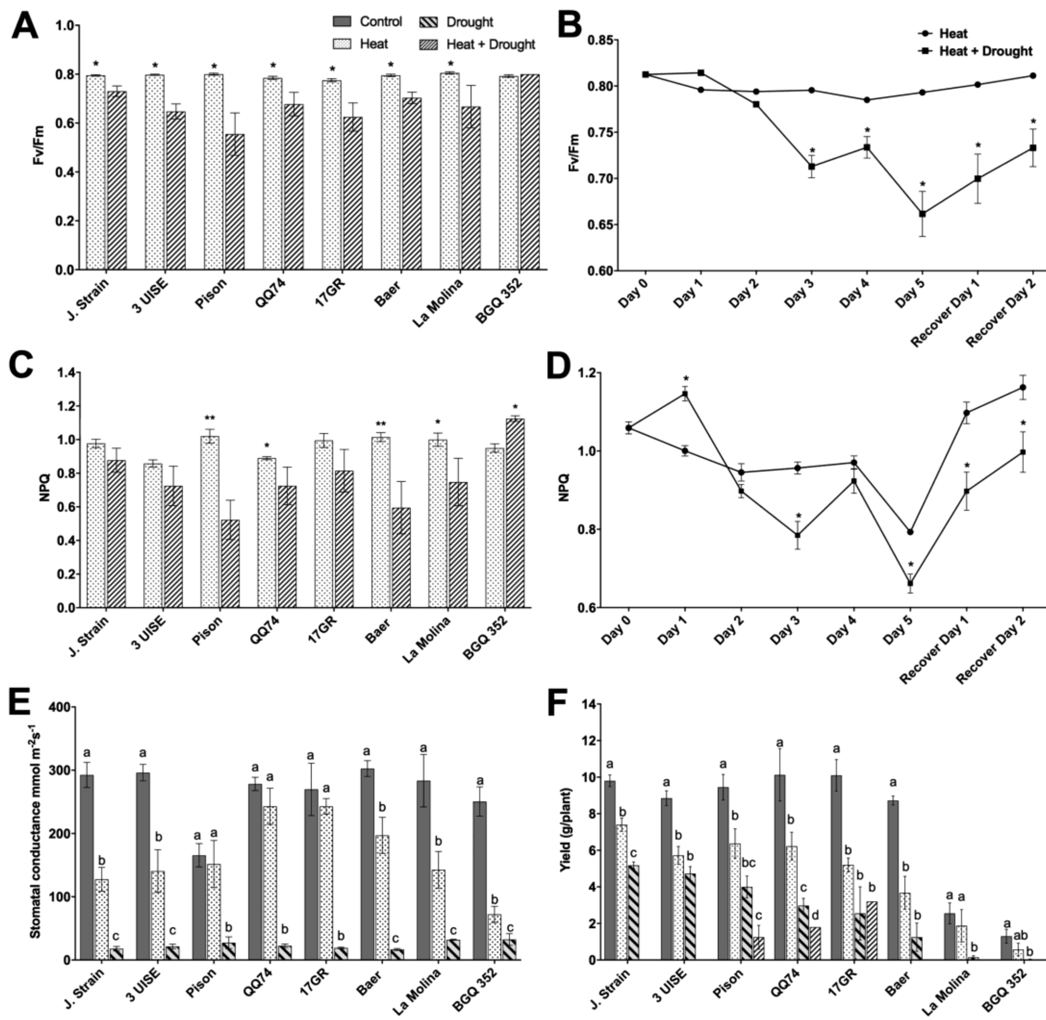
This article is protected by copyright. All rights reserved.



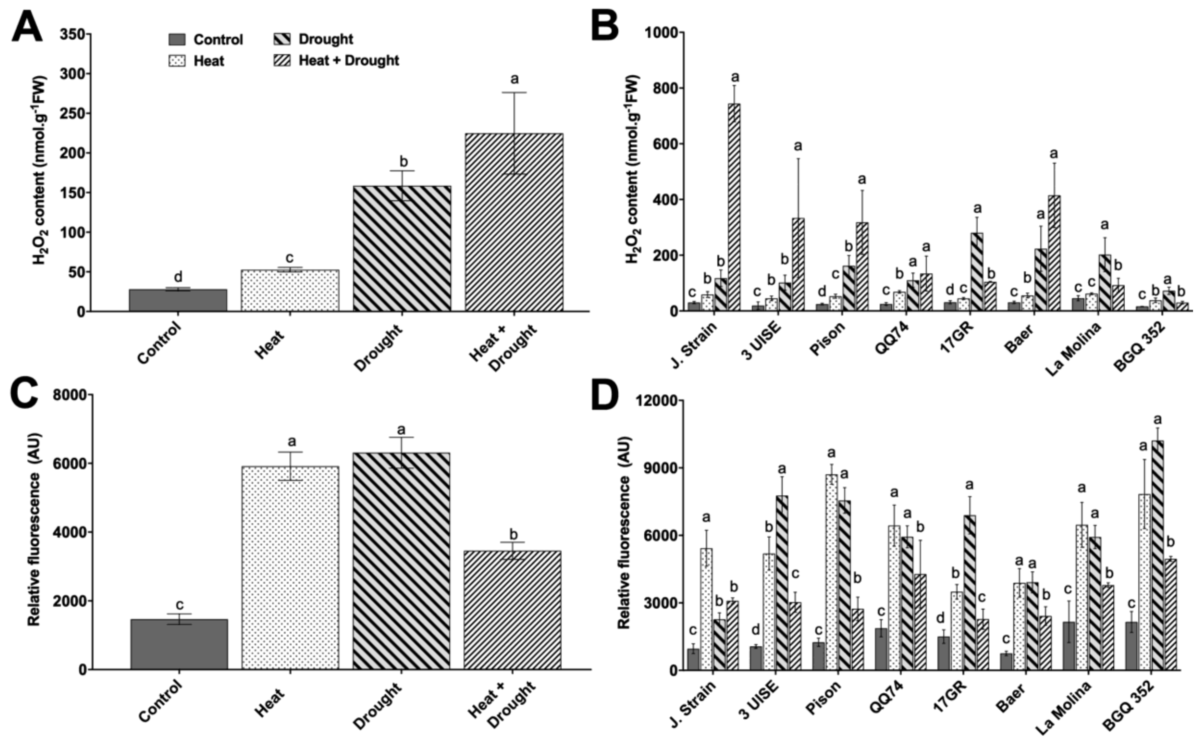
Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

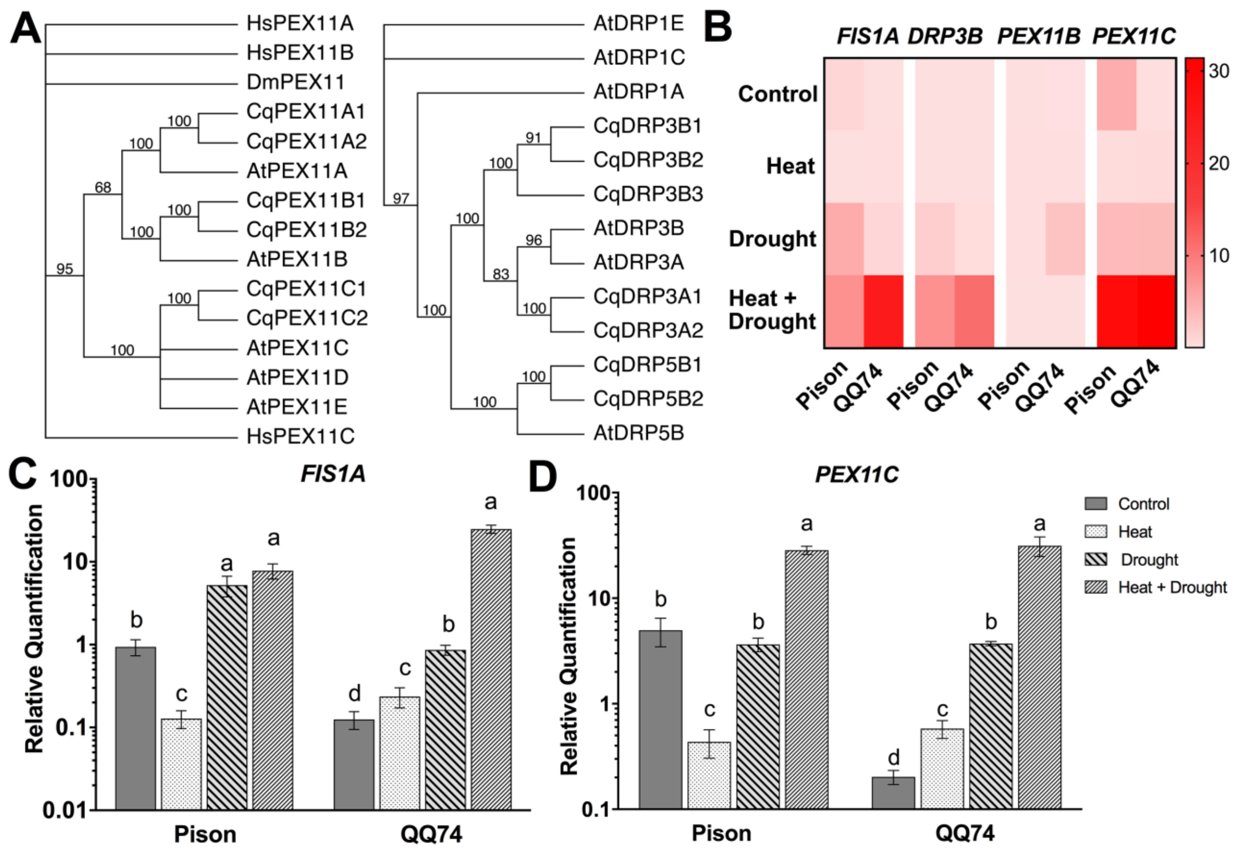


This article is protected by copyright. All rights reserved.



Accepted Article

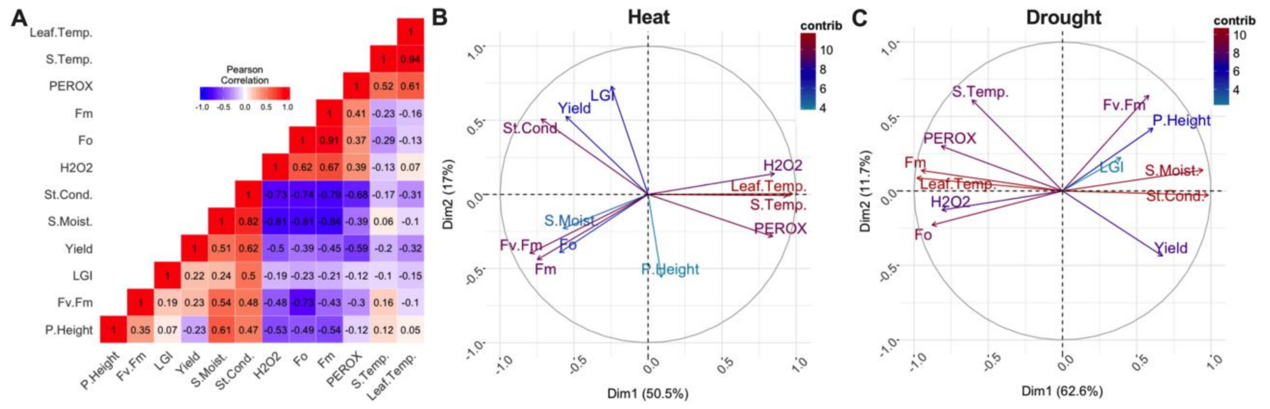
This article is protected by copyright. All rights reserved.



Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article



This article is protected by copyright. All rights reserved.