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Research paper

Drought affects the heat-hardening capacity of alpine plants as indicated by changes in xanthophyll cycle pigments, singlet oxygen scavenging, α-tocopherol and plant hormones

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ABSTRACT

Alpine environments in Europe are increasingly affected by more erratic precipitation patterns, and more frequent drought and heat waves. Heat-hardening capacity is a key feature for survival of these abiotic stress factors, but it is poorly understood how heat and drought affect plant performance when combined. The main objectives of this study were (1) to determine maximum heat hardening capacity in 14 selected plant species and (2) to study how alpine plants respond to combined heat and drought stress compared to heat alone. (3) For risk assessment maximum leaf temperatures were measured in the field and (4) important methodological aspects of testing heat tolerance were evaluated. Heat hardening capacity was assessed by T_c, the heat threshold of photosystem II (PS II), and by heat tolerance tests based on visual inspection of leaf tissue damage or potential quantum efficiency of PS II (F_v/F_m). A purpose-built Heat Tolerance Testing System (HTTS) was used, which allows for controlled heat exposure of whole plants under nearly natural conditions. Additionally, in two species from contrasting habitats, *Senecio incanus* and *Primula minima*, the dynamics of heat hardening was studied during and after 8 days exposure to heat (H), or to a combination of heat and severe drought (H+D) within a light-transmissive heat hardening chamber at the alpine field site. In both species, H treatment significantly increased heat tolerance (LT_{50}), determined by the HTTS, to 58.0 °C and 54.9 °C, respectively, and was accompanied by elevated production of abscisic acid (ABA) and salicylic acid (SA), whereas jasmonic acid (JA) levels decreased. Under H+D the LT_{50} was only 56.5 °C and 51.6 °C, respectively, and levels of ABA were higher in *S. incanus* and SA lower in both species in comparison to H. Changes in xanthophyll cycle pigments, α-tocopherol and carotenoids:chlorophyll ratio were more pronounced in *P. minima* than in *S. incanus*. In *P. minima* both H and H+D significantly increased singlet oxygen ($^1O_2$) scavenging capacity, determined by electron paramagnetic resonance spectroscopy (EPR). In the field, the maximum half-hourly mean (HHM) leaf temperature of *P. minima* (32.2 °C) was significantly lower than of *S. incanus* (46.5 °C, a potentially harmful temperature). We conclude that the investigated species are well adapted to the prevailing temperature conditions in the field. They also possess an outstanding heat hardening capacity, but this can be curtailed when heat is combined with drought. As drought further increases leaf temperatures, the risk of suffering lethal heat damage of some species may increase in the future, particularly at south exposed, ruderal alpine sites with uncertain water supply.

1. Introduction

In the Northern Hemisphere the years between 1983 and 2012 very likely represent the warmest 30 year period in the last 800 years (IPCC, 2013). Heat waves and concomitant climate characteristics such as drought and heat are predicted to become more frequent and severe (Beniston, 2004; Ballester et al., 2010).

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Between 1890 and 1998, the temperature increase in the European Alps (+1.1 K) was twice the mean global surface increase (+0.55 K) (Böhm et al., 2001), and the years 2014 and 2015, when the study was conducted, were among the warmest in Austria since records began in 1767 (ZAMG, 2014, 2015).

Although mean air temperature decreases with altitude according to the adiabatic lapse rate (troposphere: ca. −0.6 K/100 m), alpine plants may still suffer from heat stress (Neuner and Buchner, 2012). Prostrate growth forms, such as rosettes and cushions, effectively decouple plant body temperature from cool ambient air temperatures (Larcher, 1980; Körner and Cochrane, 1983). Such morphological adaptations provide significant thermal benefits on colder days (Cernusca, 1976; Körner and Larcher, 1988; Larcher and Wagner, 2010). However, on clear summer days with high solar irradiation and no wind, leaf temperatures can exceed critical thresholds (e.g. in *Rhododendron ferrugineum;* Buchner et al., 2015), even leading to lethal damage, as observed in *Silene acaulis* (Gauslaa, 1984), *Saxifraga paniculata* (Neuner et al., 1999) and *Minuartia recurva* (Buchner and Neuner, 2003). Above the tree line herbaceous alpine plants and shrubs protect against erosion, and there is value in enhancing our knowledge of maximum leaf temperatures that plants experience (and tolerate), because leaf temperatures cannot be directly derived from air temperatures.

Plants are subjected to different kinds of stresses that can reduce opportunities for growth and reproduction (Larcher, 2003). Through the exposure of organisms to one or more stresses, typical reactions and phases can be distinguished (Kranner et al., 2010). **Heat stress** is associated with enhanced respiration, suppressed photosynthesis, e.g. through reduced activation of rubisco activase, increased photo-protective mechanisms and metabolic changes that increase macro-molecular stability, i.e. through sugar and proline production and heat-shock protein synthesis (Larcher, 2003; Rizhsky et al., 2004; Salvucci and Crafts-Brandner, 2004; Wahid et al., 2007; Hüve et al., 2011; Lipiec et al., 2013). Heat stress can be mitigated by transpiration cooling, but only when plants are not suffering drought. Heat stress also involves activation of heat-hardening processes that, in the short-term, increase thermal tolerance of the photosynthetic apparatus and of leaf tissue (Alexandrov, 1977; Gauslaa, 1984; Neuner and Buchner, 2012). Heat-hardening occurs at high rates in alpine species, for example +4.7 K d^{-1} were observed in *Silene acaulis* (Neuner et al., 2000), +4.8 K d^{-1} in *Loiseleuria procumbens* (Braun et al., 2002) and even +9.5 K d^{-1} in *Minuartia recurva* (Buchner and Neuner, 2003). Driving forces of heat-hardening in nature are not fully understood but exposure to high leaf temperatures (>30 °C, Neuner et al., 2000) and, in some species, water deficiency (Alexandrov, 1977) are effective.

Long-term heat stress results in uncoupled electron transport in the mitochondria and chloroplasts leading to elevated ROS production, exhaustion or inactivation of antioxidant defences and viability loss (Locato et al., 2008; Sgobba et al., 2015). Despite the potentially destructive nature of ROS, the heat-shock response includes an oxidative burst of ROS from plasma membrane-located NADPH oxidases (Larkindale et al., 2005), which is involved in the acquisition of heat tolerance (Suzuki and Mittler, 2006). For example, hydrogen peroxide (H_2O_2) upregulates transcription of heat-shock genes during heat stress (Volkov et al., 2006; Königshofer et al., 2008). Redox signalling functions alongside increased production of phytohormones, such as salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) which are major players in stress signalling in general (Larkindale and Knight, 2002; Kotak et al., 2007; Clarke et al., 2009; De Pinto et al., 2015).

Drought leads to osmotic stress, turgor loss, inhibition of photosynthesis, and like heat stress is widely considered to be accompanied by ABA and SA synthesis, as well as oxidative stress when severe (Munné-Bosch and Peñuelas, 2003; Cutler et al., 2010; Noctor et al., 2014). The production of the ROS singlet oxygen ($^1O_2$) by photosystem II (PS II) can increase under high light when photosynthetic electron transport is limited (i.e. from low CO_2), and contributes to photoinhibition leading to losses in photosynthetic efficiency (Raven, 2011; Tyystjärvi, 2013; Pospíšil and Prasad, 2014; Roach and Krieger-Liszkay, 2014). One mechanism used to prevent $^1O_2$ production under stress is non-photochemical quenching (NPQ), which is activated in response to the trans-thylakoid proton gradient (ΔpH). Induction of NPQ in plants partially occurs through the xanthophyll cycle, in which violaxanthin is de-epoxidized to antheraxanthin and then zeaxanthin, enabling excess light energy to be harmlessly dissipated as heat (for review see Demmig-Adams et al., 2014). However, some $^1O_2$ production by the highly charged PS II in the oxygen-rich environment is inevitable and lipophilic antioxidants, such as α-tocopherol and carotenoids, help protecting the thylakoid membrane from damage (Krieger-Liszkay and Trebst, 2006).

Co-occurrence of abiotic stress factors is frequently observed in nature. Compared to single stress responses, stress combinations have been studied more rarely. Simultaneous abiotic stress factors may or may not lead to cumulative damage, but several examples are known where moderate levels of one stress can improve tolerance to another, e.g. heat and drought (Havaux, 1992; Rizhsky et al., 2004). Simultaneous heat and drought stress was investigated in *Arabidopsis thaliana*, *Nicotiana tabacum* and some crop plants, showing that the combined response clearly differs from either heat or drought stress alone (e.g. Rizhsky et al., 2002, 2004; Vile et al., 2012; Lipiec et al., 2013).

The objectives of this study were to investigate how two contrasting alpine plant species, *Primula minima* and *Senecio incanus*, which have different soil-moisture and thermal niche preferences, respond to combined heat and drought compared to heat alone. Maximum heat hardening capacity was studied and compared to maximum leaf temperatures in the field. Finally, results from different methods for determination of heat tolerance were evaluated. We hypothesized that (1) exposure to heat under well-watered conditions (H) will induce heat-hardening (leaf tissue and PS II thermal tolerance) to the species-specific maximum heat tolerance related to the thermal niche of a species. (2) Simultaneous heat and severe drought stress (H+D) was expected to further affect the heat-hardening capacity in both species. The response of *S. incanus*, which has a lower soil-moisture and warmer thermal niche preference than *P. minima* was expected to differ from *P. minima*. Moreover, it was tested if the applied heat-hardening scenarios (H and H+D) lead to species-specific responses in terms of (3) controlling $^1O_2$ production, by (4) changes in photosynthetic pigments and xanthophyll cycle activity and (5) of the production of known stress-related plant hormones.

2. Material and methods

Heat-hardening capacity was investigated in 14 alpine plant species, of which two herbaceous species (*Primula minima* and *Senecio incanus*) were chosen for a detailed study of the response to heat stress (H) and a combination of heat and severe drought stress (H+D). Heat treatments were chosen to simulate severe heat waves lasting for 8 days. Heat-hardening was measured by changes of leaf tissue heat tolerance and of thermal tolerance of PS II. For assessment of the risk of naturally occurring heat damage, leaf temperatures were recorded *in situ* during the hottest summer months to characterize the species-specific thermal environment. Xanthophyll cycle pigments, α-tocopherol contents, chlorophylls and carotenoids, and plant hormones were measured alongside changes in singlet oxygen ($^1O_2$) scavenging capacity of plant extracts obtained from maximum heat-hardened and non-heat-

hardened control plants. A detailed graphical overview of the measurement procedures is shown in Suppl. 1.

2.1. Plant material and study site

The study was mainly conducted on the two 'alpine ruderalia' (Grabherr et al., 1988) *Primula minima* L. and *Senecio incanus* ssp. *carniolicus* Willd. *P. minima* is a vigorous perennial plant that can be found over non-calcareous rock in the Eastern Alps, the Carpathians and the Balkan from ca. 1500–3000 m a.s.l. It forms glabrous, small and densely packed rosettes with a maximum height of ca. 4 cm, a strong and branched rhizome and prefers snow beds and still debris, and often grows as a pioneer on bare soil at disturbed locations. *S. incanus*, endemic to the Eastern European Alps and the Carpathians, also inhabits a variety of habitats such as grasslands, stable screes, moraines and fell-fields above the treeline up to 3000 m a.s.l. (Reisigl and Pitschmann, 1958). Its densely haired leaf rosettes (height ca. 5–15 cm) can often be found as a pioneer in alpine erosion zones. *P. minima* tends to prefer north-facing slopes, whereas *S. incanus* is particularly frequent on ridges and south-facing slopes with strong solar irradiation. *P. minima* prefers permanently moist soils. The requirements of *S. incanus* in terms of soil moisture are much more flexible (see Oberdorfer, 1962; Landolt, 1992) as *S. incanus* can also be abundant at hot and dry sites, where *P. minima* is missing.

Soil bricks containing individual plants were carefully excavated just beneath the summit of Mt. Patscherkofel (ca. 2150 m a.s.l.; *P. minima*: N 47°12′35"/E 11°27′45"; *S. incanus*: N 47°12′24"/E 11°27′26") and potted (50 for *P. minima* and 80 for *S. incanus*, pot dimensions: 8 × 8 × 7 cm; soil volume per pot: 0.3 l). In the nearby Alpine Garden of the University of Innsbruck located on Mt. Patscherkofel (ca. 1960 m a.s.l) the plants were monitored and regularly watered for at least 10 days before the experiments were started. Additionally, 12 other alpine and nival plant species were taken from Mt. Patscherkofel or near the Timmelsjoch (Ötztal Alps, 2550 m a.s.l) and treated in a similar way before testing their heat tolerance (for further details on the investigated species see Suppl. 2).

2.2. Microclimate and infrared thermography

During summer, soil and leaf temperatures of *P. minima* (n = 9) and *S. incanus* (n = 7) were measured at the natural growing site using thermocouple sensors (diameter: 0.3 mm; TT-Ti-40, Omega Engineering Inc., Stamford, USA) that were mounted to the abaxial side of the leaves by small magnetic leaf clamps, allowing for unhindered exposure to solar irradiation and leaf transpiration (Buchner et al., 2013). For measuring air and soil temperature thermocouple sensors (GG-Ti-28, Omega Engineering Inc., Stamford, US) and soil temperature sensors (Probe 107, Campbell Scientific, Loughborough, UK) were used. Photosynthetically active Photon Flux Density (PPFD) was measured by quantum sensors (SKP 215, Skye Instruments Ltd., Llandrindod Wells, UK). Temperature and PPFD data were recorded by data loggers (CR10X, CR1000, Campbell Scientific, Loughborough, UK) at 1 min - intervals.

In addition, on a clear summer day (4 July 2015) on Mt. Patscherkofel the spatial patterns of leaf temperatures in *P. minima* and *S. incanus* were visualized *in situ* by infrared thermography using a ThermaCAM™ S60 and the ThermaCAM™ researcher software package (both: FLIR Systems AB, Danderyd, Sweden). On the same day, potted plants were also exposed to natural solar irradiation under H or H+D conditions and randomly selected leaves (n = 15 for each species) were used for leaf temperature measurements and for calculating the leaf temperature difference between H and H+D plants.

2.3. Determination of heat tolerance

2.3.1. Non-invasive assessment of tissue heat tolerance using whole plants

After 8 d of heat-hardening heat tolerance was determined by a purpose-built Heat Tolerance Testing System (HTTS) as described by Buchner et al. (2013). Controlled heat exposure (30 min) was conducted on whole individuals under natural solar irradiation. Then the plants were exposed to the natural environmental conditions for 2 days, and leaf damage was visually assessed in 15 randomly selected individuals (*P. minima*) or 15 randomly selected leaves (*S. incanus*) per temperature regime. As the system needs relatively long preparation before the heat exposure can start, this method was only applied at the end of each heat-hardening run. The intention was to determine the species-specific maximum heat tolerance with high ecological relevance under near-natural conditions, but also to show potential differences between the methods applied.

2.3.2. Invasive measurement of tissue heat tolerance using detached leaves

Before and after 8 d of heat-hardening, detached leaves (n = 10 per temperature level) were fixed to heat stable transparencies by adhesive tape (3M™ Transpore™, 3 M, Perchtoldsdorf, Austria), sprayed with water and then immediately exposed to stepwise arranged target temperatures (20 °C for control, 38 °C, 40 °C, 42 °C, 44 °C, 46 °C, 48 °C, 50 °C, 52 °C, 54 °C, 56 °C, 58 °C and 60 °C) for 30 min (Kreeb, 1990) in full darkness, using the field portable heat tolerance testing equipment as described by Buchner and Neuner (2001). Then, the transparencies with the leaves were placed on wet filter paper and transferred into small plastic bags that were exposed to room temperature, and moderate indirect solar irradiation for 2 days, allowing tissue damage to manifest and become apparent. The percentage of leaf damage (tissue necrosis method) of each leaf was assessed either by graphical analysis software (Optimas 6.5, Optimas Corp., Seattle, USA) or by naked eye (the visual estimation method; Buchner et al., 2013). In addition, viability was assessed by measuring maximum quantum efficiency of PS II (F_v/F_m) using chlorophyll fluorescence (Mini PAM, Walz, Effeltrich, Germany). Values for each leaf recorded prior to heat treatment were used as references. Using logistic function, lethal parameters, such as LT_5 and LT_{50}, that describe which temperatures lead to leaf damage of 5% and 50%, respectively, were calculated with Fig. P 2.7 software (Biosoft, Durham, USA) according to Buchner et al. (2013).

2.3.3. Invasive assessment of the heat threshold of PS II using detached leaves

The critical heat thresholds of PS II were determined according to the method described by Schreiber and Berry (1977) and Bilger et al. (1984). Briefly, 15 dark adapted leaves per species and treatment were heated from 25 °C up to 65 °C (1 K min^{-1}) using controllable water baths (CC1, Huber, Offenburg, Germany) and several chlorophyll fluorometers (PAM 101, MiniPAM, PAM 2000, all from Walz, Effeltrich, Germany) for measuring basic fluorescence (F_0). The leaves were mounted to the fibre optic conductors, which were submersed into the water baths, and leaf temperatures and the output signals (mV) of the fluorometers were recorded in 1 s intervals and stored by a datalogger (CR10, Campbell Scientific, Loughborough, UK). From the resulting F_0/T-curves the critical high temperature thresholds of PS II (T_c) were determined, which is the temperature that causes a sharp increase of F_0.

2.4. The heat-hardening process

Heat-hardening of the potted plants was done within a heat-hardening chamber that was manufactured from a small cold frame (Biostar 1500, Juwel H. Wüster GmbH, Imst, Austria) made of double-walled light-transmissive 8 mm Plexiglas panels. The dimensions of the base area were 150 × 80 cm and the mean height of the single pitch roof was 45 cm. For the experiments on *P. minima* and *S. incanus* the chamber was divided into two equally sized rooms, one for the well-watered plants (H) and one for the drought stressed plants (H+D), by a vertically standing partition wall (Plexiglas). Each of the two rooms was heated by 5 ceramic heaters (12 V/150 W; AT858, Conrad Electronics, Hirschau, Germany). Each room was cooled with fresh air by 5 axial fans (120 × 120 × 38 mm; RD12038B12H, Conrad Electronics, Hirschau, Germany), which were embedded into the external walls. Heat hardening of the 12 additional alpine and nival plant species was conducted only under H conditions. Usually, 4 different species were heat treated simultaneously.

Inside the heat-hardening chamber PPFD was continuously measured by a quantum sensor (SKP 215, Skye Instruments Ltd., Llandrindod Wells, UK). Thirteen thermocouple sensors for each experimental condition (H and H+D) were used for controlling mean leaf temperature, thereby ensuring that the plants of both (H and H+D) had near identical leaf temperatures during heating. For measuring leaf temperatures, a control unit was manufactured containing a data-logger and multiplexers (CR1000, 2× AM 25 T, Campbell Scientific, Loughborough, UK) to which a 16 channel relays unit was connected. The ceramic heaters and fans were automatically operated and heating was only provided during the day. Mean leaf temperature was increased from 32 °C on day 0 to ca. 38 °C by day 8, which was sufficient to induce maximum heat-hardening, but did not cause any leaf damage.

Heat tolerance of leaf tissue (chapter 2.3.1, 2.3.2) was determined before and immediately after the heat-hardening process. Furthermore, every day (pre-dawn) heat thresholds of PS II (chapter 2.3.3) were recorded daily. Additionally, the F_v/F_m, a very sensitive heat stress indicator (Willits and Peet, 2001) of dark adapted (minimum: 30 min) leaves, was measured every morning before sunrise and during the midday hours (Mini PAM, Walz, Effeltrich, Germany) to monitor any changes in photosynthetic performance associated to heat-hardening.

2.5. Determination and adjustment of leaf water potentials

Every morning (pre-dawn) and midday total actual leaf water potential Ψ_{act} of small leaf discs (12 discs per species; diameter 5 mm) from both stress treatments (H and H+D) was determined. For this, the PSΨPRO™ water potential system to which eight C52 sample chambers were connected was used. The chambers were regularly calibrated by osmolality standards of 100, 290 and 1000 mmol kg^{-1}. All devices and standard solutions were obtained from Wescor Inc., Logan, USA. Additionally, fresh weight (FW) and dry weight (DW) of the leaf discs were determined to calculate percentage leaf water content (WC) (Eq. (1)).

$$WC[\%FW] = 100\% \cdot \frac{FW - DW}{FW} \quad (1)$$

In preliminary experiments potted plants were not watered while exposed to room temperature (ca. 22 °C) until a certain Ψ_{act} was attained. After re-watering, recuperation of the leaves was monitored over 6 days by determining F_v/F_m and by visual assessment of drought injury. $\Psi_{actcrit}$, the critical total leaf water potential from which full recovery is still possible, was determined. During heat-hardening the H plants were watered every morning and if necessary, also at midday or in the afternoon. The H+D plants were only watered if the Ψ_{act} was in the range of $\Psi_{actcrit}$ and/or in case of critical turgor loss of the leaves.

2.6. Biochemical analyses

2.6.1. Sample preparation

After 8 days of treatment leaves from control (untreated) and from heat hardened (H, H+D) individuals were taken at midday, immediately frozen in liquid nitrogen before storage at –80 °C, and then freeze dried (Lyovac GT 2, Leybold-Heraeus, Köln, Germany) for 3 days. For *S. incanus* and *P. minima* approximately 30 and 100 leaves per replicate (n = 4), respectively, were randomly selected from approximately 10 and 30 plants, respectively. Freeze dried leaves were ground to a fine powder in liquid nitrogen-cooled Teflon capsules (TissueLyser II, Qiagen, Düsseldorf, Germany) containing two 2 mm glass beads at 30 Hz for 6 min. The powder was stored in Eppendorf tubes over silica gel at −80 °C until further processing.

2.6.2. HPLC analysis of photosynthetic pigments and tocopherols

Photosynthetic pigments (absorbance at 440 nm) and tocopherols (fluorescence excitation: 295 nm, emission: 325 nm) were measured simultaneously according to the solvent gradient and method in Remias et al. (2005). 50 mg of lyophilized leaf powder were extracted in 1 ml of ice-cold acetone by shaking (TissueLyser II, Qiagen, Düsseldorf, Germany) at 30 Hz for 2 min with two 2 mm glass beads before centrifugation at 26,000*g* for 45 min. 10 µl of the supernatant was injected into an Agilent 1100 HPLC system equipped with a LiChrospher 100 RP-18 (125 × 4 mm, 5 µm) column (Agilent Technologies, Santa Clara, California, USA). Pigments and tocopherols were identified and quantified using external standards. Authentic standards of chlorophyll a, b (Sigma-Aldrich, St. Louis, USA), lutein, zeaxanthin (Carl Roth, Karlsruhe, Germany), antheraxanthin, neoxanthin, violaxanthin (DHI LAB Products, Hørsholm, Denmark), β-carotene (Merck, Darmstadt, Germany) and of α-, γ- and δ-tocopherol (Sigma-Aldrich, St. Louis, MO, USA) were used.

2.6.3. LC–MS/MS analysis of hormones

20 mg of lyophilized leaf powder were extracted in 1.5 ml of ice-cold acetone/water/acetic acid (80:20:1, v:v:v) after addition of 25 µl stable isotopically labelled internal standard (IS) solution (1 µM ABA-d6, 5 µM SA-d4) by shaking (TissueLyser II, Qiagen, Düsseldorf, Germany) at 30 Hz for 5 min using one 5 mm glass bead per Eppendorf tube, followed by centrifugation at 10,000*g*, 4 °C for 12 min. Supernatants were evaporated to dryness using a SpeedVac SPD111 vacuum concentrator (Thermo Fisher Scientific Inc., Waltham, MA, USA), followed by resuspension in 150 µl of ACN/water (50:50, v:v), supported by 5 min ultrasonication in an ice-cooled water bath. The extracts were filtered through 0.2 µm PTFE filters before injection into the UHPLC–MS/MS system.

Selected plant hormones, i.e. ABA, SA and JA were identified and quantified by LC–MS, using an ekspert ultraLC 100 UHPLC system (Eksigent, Dublin, CA, USA) coupled to a QTRAP 4500 mass spectrometer (AB SCIEX, Framingham, MA, USA). For compounds separation a reversed-phase column (NUCLEODUR C18 Pyramid, EC 50/2, 50 × 2 mm, 1.8 µm, Macherey-Nagel, Düren, Germany) with a 4 × 2 mm guard column connected ahead was used. Run duration was set to 8 min. The mobile phases contained 0.1% formic acid (v/v) (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). Samples were injected starting with 5% solvent B followed by a gradient to 70% solvent B (5 min), rinsing at 100% solvent B (5:01 to 6 min) and equilibration to 5% solvent B (6:30 to 8 min). The injection volume was set to 1 µl, the flow rate to 0.5 ml min^{-1} and the column temperature to 30 °C. Compounds

were detected by the mass spectrometer operated in negative ion mode using multiple reaction monitoring (MRM) (see Suppl. 3). Ion spray voltage was set to −4.5 kV, gas 1 to 40 psi and gas 2 to 50 psi at a temperature of 500 °C. Both quadrupole mass analyzers were operated at unit resolution. Peaks were automatically detected based on the retention time and MRM transition. Peak areas were normalized relative to the internal standards to account for variations during sample preparation and analysis and concentrations were calculated according to the calibration curves created with the authentic samples using the software Analyst and MultiQuant (AB SCIEX, Framingham, MA, USA).

2.6.4. Singlet oxygen-scavenging capacity

Singlet oxygen production was measured with electron paramagnetic resonance (EPR) using the spin-trap 2,2,6,6-tetramethyl-4-piperidone (TEMP) hydrochloride at 85 mM. The TEMP spin adduct of $^1O_2$ (TEMPO) was measured with a Miniscope MS400 X-band EPR (Magnatech, Freiberg Instruments, Germany). Ground lyophilized leaves were extracted in 80% (v/v) acetone and diluted to 10 μg chlorophyll ml^{-1} according to Porra et al. (1989). Singlet oxygen was produced using the photosensitizer Rose Bengal (45 μM in 50 mM potassium phosphate buffer, pH 7.5) in the presence of leaf extracts (final acetone concentration was 67% v/v), which were illuminated for up to 10 min with a 3 W green LED (525 nm, chosen to specifically excite Rose Bengal, but not chlorophyll) in a 50 μl capillary tube. The modulation frequency was 8.63 GHz, and modulation amplitude was 0.1 mT with a central field of the TEMP adduct at 335.95 mT. For the kinetic analysis of signal increase during illumination, corresponding to the amount of trapped $^1O_2$, an average of 2 scans was made over a 0.4165 mT range, each with a sweep time of 28 s.

2.7. Statistics

Data were analysed by SPSS-Statistics 21 (IBM, New York, NY, USA). After testing for normality by the Shapiro-Wilks test, the significance of differences between mean values at $P < 0.05$, unless stated otherwise, was tested either by the Independent Samples *t*-test or by One-Way-Anova, followed by Duncan's or Games-Howell's post-hoc test. One-Way Anova was performed individually for each species and for each parameter. Details regarding the respective sample sizes are given in the text and in Suppl. 1.

3. Results

3.1. Microclimate and field leaf temperatures

3.1.1. Field leaf temperatures during summer 2015

The summer of 2015 was the second warmest summer in Austria since the beginning of instrumental recording in 1767 (ZAMG, 2015). It was dominated by several consecutive heat waves and less than average precipitation, especially in August. The maximum leaf temperature recorded in *P. minima* was 44.9 °C (21 July 11:43) and 57.2 °C in *S. incanus*, which was lethal (7 Aug 13:20).

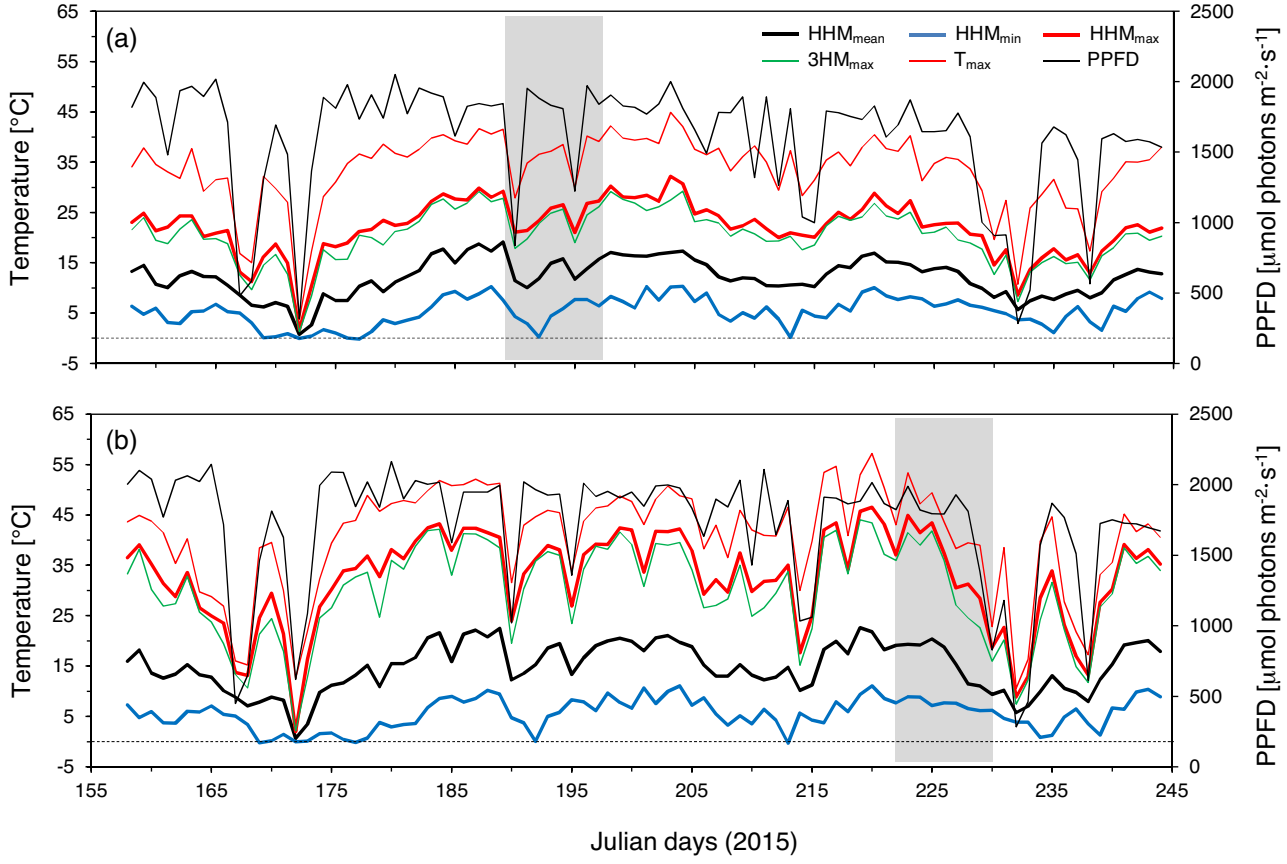

Fig. 1. Leaf temperatures of *Primula minima* and *Senecio incanus* in the field during summer 2015. Leaf temperatures were recorded at the summit area of Mt. Patscherkofel (2246 m a.s.l) from 6 June–31 Aug (Julian days 157–243). **(a)** *P. minima* (n = 9), **(b)** *S. incanus* (n = 7). **Bold lines:** Half hourly mean values (HHM); black: daily mean (HHM$_{mean}$), blue: daily minimum (HHM$_{min}$) and red: daily maximum (HHM$_{max}$) half hourly mean values. **Thin lines:** green: daily maximum 3 hourly mean values (3HM); red: maximum leaf temperature occurring at least once per day (measurement interval: 1 min), black: daily maximum (HHM) photosynthetically active photon flux density (PPFD). Vertical grey bars indicate the time span when the heat-hardening runs were performed.

The maximum half hourly mean values (HHM) were significantly lower for *P. minima* (32.2 °C) than for *S. incanus* (46.5 °C). Due to continuous solar irradiation in this period (maximum PPFD (HHM) 2050 and 2164 μmol photons m^{-2} s^{-1} for *P. minima* and *S. incanus*, respectively) the maximum three hourly mean values (3HM) of leaf temperature for each species were similar to HHM (*P. minima*: 29.2 °C, *S. incanus*: 44.0 °C) (Fig. 1).

In *P. minima* between 6 June and 31 August high maximum HHM of leaf temperatures (HHM_{max}) ≥ 30 °C (this is the threshold where heat hardening starts) occurred only on 4.6% of the days, compared to 69% of the days for *S. incanus*. In the latter species HHM_{max} ≥ 46.5 °C and $3HM_{max}$ ≥ 43.5 °C occurred with a relative frequency of 1.1% (Suppl. 4). In summary, leaf temperatures clearly document the distinctly different thermal niches of the two species, with *S. incanus* experiencing much greater frequency of high leaf temperatures.

In *P. minima* the maximum recorded leaf temperature was far from inducing heat damage to the leaves, whereas in *S. incanus* the difference between HHM_{max} and LT_{50}, as determined by the HTTS, was only 10 K (H+D) and 11.5 K (H). Based on LT_5 values for initial leaf damage, this difference was 8.9 K (H+D) and 10.3 K (H). However, it should be noted that these continually measured leaves were not necessarily the hottest of the plants. Indeed, in *S. incanus* lethal leaf damage was observed several times, especially on dry sites over bare soil on calm days when solar irradiation was high. In particular prostrate and older leaves in the rosettes had higher temperatures (Fig. 2a and b), unless they were protected by shading from younger and more upright standing leaves.

3.1.2. Effect of drought on leaf temperature

In *S. incanus* drought stressed leaves (Ψ_{act} = −1.8 MPa) heated up to >50 °C within 1.5 h upon exposure to direct sunlight, and were on average +6 K warmer compared to a well-watered plant (Ψ_{act} = −1.3 MPa). In *P. minima* this temperature difference was initially lower, despite the greater difference between the water potential of the drought stressed (Ψ_{act} = −2.9 MPa) and well-watered plants (Ψ_{act} = −1.2 MPa) (Fig. 2c–f).

3.2. Effects of heat and drought stress treatments on leaf temperature, water potential and photosystem II, and leaf tissue heat tolerance

3.2.1. Leaf temperature during heat-hardening

The stress treatments lasted 10–12 h per day. During daytime leaf temperatures (HHM) of the plants were kept nearly identical with a daily average difference (H minus H+D) of −0.5 K (*P. minima*) and −0.3 K (*S. incanus*). In both species heat-hardening started at a mean leaf temperature of 32 °C (day 0) and continuously increased each day up to ca. 37–39 °C (day 8). Maximum leaf temperature (HHM) was 39.6 °C in *P. minima* and 42.0 °C in *S. incanus*. During the night mean leaf temperature never fell below 7 °C in *P. minima* and 11 °C in *S. incanus* (Fig. 3a and b).

3.2.2. Total leaf water potentials, leaf water content and F_v/F_m during heat-hardening

The preliminary drying experiment revealed that leaves of both species were able to recover from immense turgor loss and very low leaf water potentials ($\Psi_{actcrit}$ ca. −3.3 MPa and −2.6 MPa in *P.*

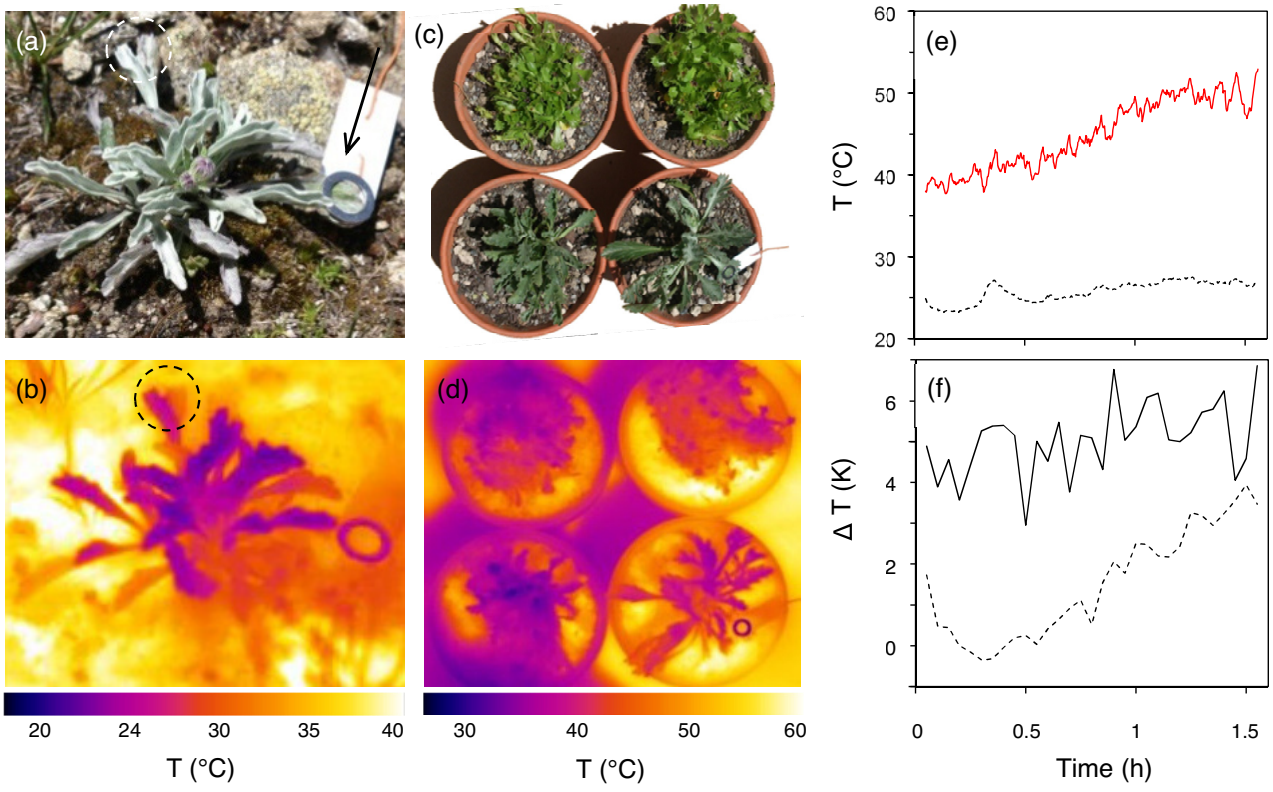

Fig. 2. Visualization of leaf temperatures of *Primula minima* and *Senecio incanus* with infrared thermography and the effect of drought. **(a)** Photograph of *S. incanus* on the summit of Mt. Patscherkofel (2246 m a.s.l.) in full midday sun, 4 July 2015. A thermocouple, used to measure the reference leaf temperature, was attached to the underside of a leaf by a magnetic leaf clamp (arrow). **(b)** Infrared thermal image showing a broad range (>15 K) of leaf temperatures. The dotted circle shows an example of an old prostrate leaf, which was warmer than the more upright young leaves. **(c)** Photograph and **(d)** infrared thermal image during 1.5 h sun exposure of potted *P. minima* (top) and *S. incanus* (bottom), either non drought-stressed (left) or drought-stressed (right). **(e)** Air (dotted line) and leaf temperature (red solid line) of drought-stressed *S. incanus* during measurements from 13:55 (GMT + 2 h) when plants were moved from the shade to the sun (=0 h). **(f)** Differences in leaf temperatures between drought-stressed and non-drought stressed plants of *P. minima* (dotted line) or *S. incanus* (solid line), as measured in (d).

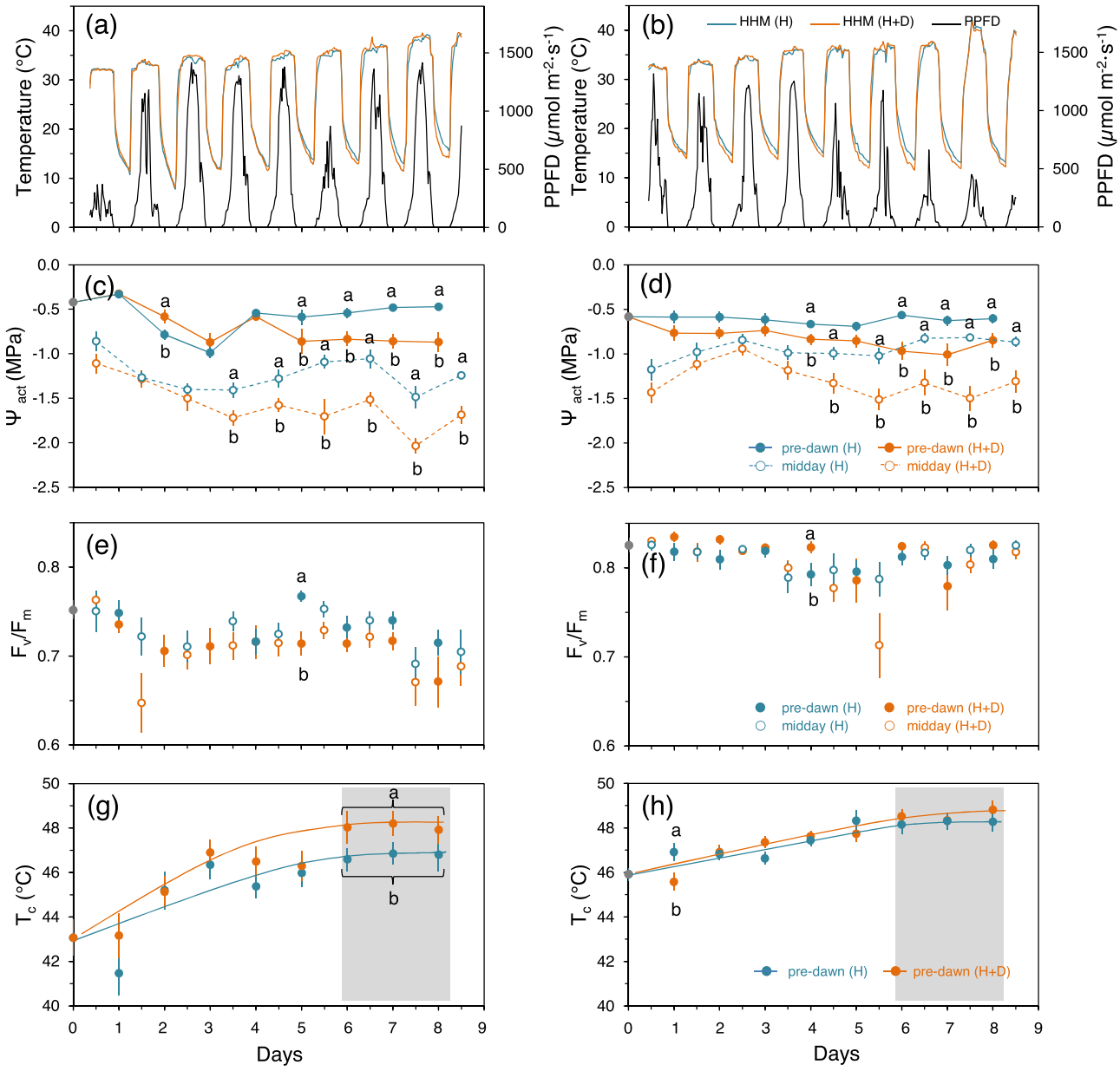

Fig. 3. Controlled heat-hardening of *Primula minima* and *Senecio incanus*. Potted plants were exposed to increased heat (H, blue) or to increased heat and drought (H+D, orange) within a controllable heat-hardening chamber on Mt. Patscherkofel (1960 m a.s.l.) for 8 days during summer 2015. Left column: *P. minima*, right column: *S. incanus*. **(a-b)** Half hourly mean values (HHM) of leaf temperature (n = 13) and PPFD (black lines). **(c-d)** Actual leaf water potentials (Ψ_{act}) pre-dawn (solid symbols) and during the midday hours (open symbols, n = 12 ± SE), **(e-f)** Maximum quantum efficiency (F_v/F_m) of photosystem II of the same samples used in (c-d), n = 12 ± SE. **(g-h)** critical heat thresholds of PS II (T_c), as determined pre-dawn in darkness, n = 15 ± SE. Grey boxes indicate the stable phase when maximum T_c was achieved. Significant differences ($P<0.05$; *t*-test) between mean values are indicated by different letters (g-h: results from day 6–8 were analysed collectively).

minima and *S. incanus*, respectively). However, under combined H +D, comparable turgor loss occurred earlier so that plants had to be watered whenever the condition of the leaves indicated the imminent risk of irreversible drought damage. In both species, this was already the case when Ψ_{act} fell below ca. −1.5 MPa. From day 3–8 (*P. minima*) and day 4–8 (*S. incanus*) the midday Ψ_{act} of the H +D plants was significantly ($P < 0.05$) lower compared to the H plants, with a mean difference $\Delta\Psi_{act}$ of −0.45 MPa (*P. minima*) and −0.44 MPa (*S. incanus*). Similarly, Ψ_{act} of the H+D plants when determined pre-dawn was significantly lower from day 5–8 (*P. minima*) and day 6–8 (*S. incanus*) than Ψ_{act} of the H plants showing mean $\Delta\Psi_{act}$ of −0.34 MPa in both species (Fig. 3c and d).

The amount of added water relative to the soil volume required to keep the plants alive during 8 d of heat-hardening was in *P. minima* 213% (H) and 15% (H+D) and in *S. incanus* 133% (H) and 4% (H+D). Accordingly, the relative leaf WC (% FW basis) of the H+D plants was significantly lower than that of the H plants; e.g. during the last 4 days (day 5–8) mean WC during midday was 80.1% and 77.7% in H and H+D in *P. minima* ($P = 0.011$), respectively, and similar values were calculated for *S. incanus* of 80.1% and 74.5% ($P < 0.001$), respectively.

In *P. minima* on day 0 mean F_v/F_m was 0.752, indicating a relatively intact PS II. During the stress treatments F_v/F_m showed a minor decrease and was 0.689 (H+D) and 0.705 (H) at the end of

heat-hardening. Although the F_v/F_m was marginally lower in H+D plants (Fig. 3e), the F_v/F_m between the H+D and H plants was insignificantly different (exception: day 5). In *S. incanus* mean F_v/F_m was 0.825 at the beginning of the heat treatment and showed a maximum reduction to 0.713 (H+D) and 0.787 (H) on midday of day 5, followed by a complete recovery by midday on day 8, with F_v/F_m 0.817 (H+D) and 0.825 (H) (Fig. 3f).

3.2.3. Heat tolerance of PS II during heat-hardening

In both species maximum heat-hardening of PS II was achieved by the end of the heat treatments, as indicated by T_c, which remained stable during the last 3 days of heat treatments (Fig. 3g and h).

In *P. minima* T_c (mean ± SD) was 43.1 °C ± 3.1 at the beginning of the heat treatment, increased steadily up to day 6, and then plateaued. When the stable values for the last 3 days were pooled, average T_c values of 46.7 °C ± 2.1 (H) and 48.0 °C ± 2.4 (H+D) were recorded. In this last period, mean maximum T_c of the H+D plants was significantly ($P = 0.01$) higher by 1.3 K compared to the H plants, indicating that in combination, H+D enhanced the heat hardening capacity of PS II in *P. minima* compared to H alone (Fig. 3g).

In *S. incanus* T_c was 45.9 °C ± 1.1 on day 0, and as in *P. minima*, increased steadily and then plateaued. When the stable values for the last 3 days were pooled, average T_c values of 48.2 °C ± 1.5 (H) and 48.6 °C ± 1.3 (H+D) were recorded for the final 3 days of heat treatment. Mean maximum T_c of the H+D plants did not differ significantly ($P = 0.298$) from that of the H plants (Fig. 3h).

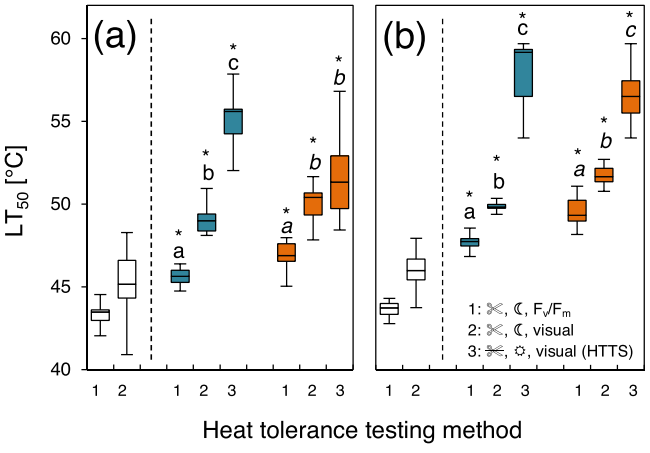

Fig. 4. Maximum heat tolerance of *Primula minima* and *Senecio incanus* and its dependence upon the test applied. Potted plants of **(a)** *P. minima* and **(b)** *S. incanus* were exposed to increasing heat (H, blue) or to increasing heat and drought (H+D, orange) within a controllable heat-hardening chamber on Mt. Patscherkofel (1960 m a.s.l.) for 8 days during summer 2015. Maximum heat tolerance (day 8, LT_{50}) was determined by different testing methods: (1) Detached (✂) leaves (n = 10) were exposed to stepwise arranged temperatures in the dark (☾) and viability was assessed 2 d later by the percentage reduction of potential quantum efficiency of PS II (F_v/F_m). (2) Detached (✂) leaves (n = 10) were treated according to (1) but viability was assessed by visual assessment of leaf damage. (3) Whole plant individuals were exposed under natural solar irradiation (☼) to stepwise arranged temperatures using the HTTS and viability of the non-detached (✂) leaves (n = 15) was assessed 2 d later by visually rating of leaf damage. Open boxes indicate the heat tolerance of the two species before heat-hardening (day 0). One-way ANOVA followed by Duncans's or Games-Howell's post-hoc test was performed separately for H and H+D plants of each species, and significant differences ($P < 0.05$) between the LT_{50} values derived by the different methods are indicated by different letters (regular: H, italics: H+D). Significant differences ($P < 0.05$, *t*-test) between H and H+D plants as derived by the same method are indicated by asterisks.

3.2.4. Maximum heat tolerance of leaf tissue: comparisons of methods applied

In both species along with achieving maximum heat tolerance of PS II (Fig. 3g and h), significant heat-hardening of leaf tissue occurred (Fig. 4). Heat tolerance (LT_{50} ± SD) was highest when determined by the non-invasive method using the HTTS where whole plants were heat-treated under solar irradiation, and the H plants showed significantly higher LT_{50} values than the H+D plants: *P. minima*: 54.9 °C ± 2.1 (H) and 51.6 °C ± 2.5 (H+D) ($P = 0.001$); *S. incanus*: 58.0 °C ± 1.8 (H) and 56.5 °C ± 1.8 (H+D) ($P = 0.03$).

In comparison to HTTS, heat tolerance was lower when determined by the invasive method using detached leaves treated in the dark and contrary, the H plants showed lower LT_{50} values than the H+D plants: *P. minima*: 49.0 °C ± 0.9 (H) and 50.1 °C ± 1.2 (H+D) ($P = 0.036$); *S. incanus*: 49.8 °C ± 0.4 (H) and 51.7 °C ± 0.6 (H+D) ($P < 0.001$).

The determined LT_{50} values were even lower when leaf viability from the same leaves was assessed by calculating the percentage reduction of F_v/F_m and here again the H plants showed lower LT_{50} values than the H+D plants: *P. minima*: 45.6 °C ± 0.6 (H) and 46.9 °C ± 0.9 (H+D) ($P = 0.001$); *S. incanus*: 47.8 °C ± 0.7 (H) and 49.5 °C ± 0.9 (H+D) ($P < 0.001$). The trend of lower thermal stability of PS II in comparison to tissue tolerance was also seen in the 12 other alpine plant species tested after heat-hardening treatments (Table 1).

3.3. Effects of heat and drought stress treatments on photosynthetic pigments, singlet oxygen scavenging and plant hormones

3.3.1. Changes in photosynthetic pigments and leaf α-tocopherol

In both *P. minima* and *S. incanus* heat-hardening slightly reduced the chlorophyll a:b ratio, but had no significant effects on the total chlorophyll contents measured on a DW basis (Table 2). Heat-hardening in the presence of drought (H+D) significantly increased antheraxanthin and zeaxanthin contents in both species, especially in *P. minima* (Fig. 5a) and significant increases of the de-epoxidation state of the xanthophyll cycle pigments were detected (Fig. 5b).

In *P. minima*, heat-hardening (H and H+D) led to increases in (1) the total xanthophyll cycle pool size (19% and 46%, respectively), (2) the de-epoxidation state of the xanthophyll cycle pool (27% and 30%, respectively), (3) the carotenoids:chlorophyll ratio (16% and 39%, respectively) and (4) α-tocopherol contents (43% and 65%, respectively) of leaves (Table 2, Fig. 5a–c). α-tocopherol was the only tocopherol isomer found in the leaves of both species. In *S. incanus*, these parameters were hardly affected by H and H+D; only H+D led to a significant increase of the de-epoxidation state of the xanthophyll cycle pool (32%).

3.3.2. Changes in abscisic acid, salicylic acid and jasmonic acid contents

In non-heat-hardened *P. minima* leaves ABA, SA and JA contents (mean values ± SD) were 0.98 ± 0.47, 1.69 ± 0.03 and 3.0 ± 0.80 pmol g^{-1} DW, respectively, all significantly higher than the respective contents in *S. incanus*, which were 0.26 ± 0.03, 0.43 ± 0.07 and 1.52 ± 1.22 pmol g^{-1} DW (Fig. 6). In both species all three hormones showed similar trends in their response to the stress treatments: H leaves contained more ABA, more SA and less JA. Compared to H alone, H+D led to even higher ABA contents in *S. incanus*, but in both species to lower levels of SA, while JA did not change (Fig. 6).

3.3.3. Changes in singlet oxygen scavenging in Primula minima

Singlet oxygen scavenging capacity was measured by the restriction in formation of the $^1O_2$-specific EPR spectrum of TEMPO

Table 1
Maximum heat tolerance of 12 selected alpine plant species before and after a multi-day heat-hardening treatment.

Applied method for testing heat tolerance		[detached leaves]		[HTTS]		
	Day	LT_5	LT_{50}	LT_5	LT_{50}	T_c
Antennaria dioica (L.) Gaertn.[a]	0	47.6	48.2			43.1
	5	49.1	49.7	50.2	54.9	48.5
Daphne striata Tratt.[a]	0	52.2	53.0			43.3
	6	55.6	56.5	51.7	56.2	52.6
Gentiana acaulis L.[a]	0	51.2	51.9			48.8
	5	54.4	55.3	52.5	57.3	48.1
Geum montanum L.[a]	0	46.7	47.2			42.3
	6	51.2	52.3	52.1	56.4	46.0
Heliosperma pusillum (Waldst. and Kit.) Rchb.[c]	0	51.9	52.5			48.0
	4	54.7	55.3	53.9	55.5	50.1
Heliosperma veselskyi Janka[c]	0	45.7	46.1			47.1
	4	54.9	55.5	53.5	55.3	50.3
Minuartia recurva Schinz and Thell.[a]	0	51.0	52.4			45.2
	8	52.1	53.3	45.0	54.1	48.4
Primula glutinosa All.[b]	0	44.3	45.7			41.4
	7	51.9	53.0	50.4	55.1	47.3
Saxifraga aizoides L.[a]	0	44.2	45.2			40.3
	8	47.7	49.0	36.4	47.4	44.1
Silene acaulis ssp. excapa [All.] J. Braun[b]	0	48.5	52.4			45.4
	7	54.2	55.5	35.3	55.2	47.7
Soldanella pusilla Baumg.[b]	0	49.5	50.8			41.3
	7	53.7	55.0	47.4	54.7	46.7
Tanacetum alpinum Sch.Bip.[b]	0	43.6	44.9			42.8
	7	48.3	49.4	44.8	51.1	45.6
Primula minima L.[a]	0	38.8	45.1			43.1
	8	41.9	49.0	52.2	54.9	46.9
Senecio incanus ssp. carniolicus Willd.[a]	0	42.9	45.9			45.9
	8	43.3	49.8	56.8	58.0	48.3

Controlled heat exposure of potted and well-watered plants was performed on Mt. Patscherkofel (1960 m a.s.l.) during summer 2014 in a thermostatically controlled Plexiglas chamber. The temperature regimes were chosen to induce maximum heat-hardening without causing leaf damage. Tissue heat tolerance (LT_5, LT_{50}) of detached leaves was determined before (day 0) and after the 5–8 days lasting heat-hardening process and whole individuals were also measured using the Heat Tolerance Testing System (HTTS) after the heat-hardening. Critical heat thresholds of PS II (T_c) were determined every morning (pre-dawn) to monitor the heat-hardening process. The heat treatment was continued until no further heat-hardening was inducible and maximum heat tolerance was reached. (Data from *P. minima* and *S. incanus* which were investigated during summer 2015 are included at the bottom of the table.)

[a] From Mt. Patscherkofel (2100–2146 m a.s.l.).

[b] From Timmelsjoch (2550–2600 m a.s.l.).

[c] Grown together in the Botanical Garden Innsbruck, 600 m a.s.l. from seeds collected in populations of H. pusillum (Mt. Rudnigkofel, 46.76°N, 12.88°E, 2060 m a. s. l.) and H. veselskyi (Anetwände, 46.77°N, 12.90°E, 790 m a. s. l.) situated in the Lienzer Dolomiten, Austria.

Table 2
Photosynthetic pigment changes in response to heat-hardening in the absence (H) or presence of drought (H+D) compared to non-heat hardened plants (Con). Significant ($P < 0.05$) differences of H or H+D to control and between H and H+D are represented by * and **, respectively.

		Chlorophyll *a*:*b* (mol:mol)	Chlorophyll (μg mg^{-1} DW)	Carotenoids:chlorophylls (mol:mol)
Senecio incanus	**Con**	**3.30 ± 0.09**	**4.81 ± 0.36**	**0.37 ± 0.02**
	H	2.75 ± 0.07*	4.81 ± 0.41	0.35 ± 0.01*
	H+D	2.86 ± 0.02*	4.24 ± 0.34	0.37 ± 0.01
Primula minima	**Con**	**3.66 ± 0.12**	**3.20 ± 0.67**	**0.35 ± 0.01**
	H	3.11 ± 0.06*	2.97 ± 0.30	0.41 ± 0.01*
	H+D	3.14 ± 0.09*	3.05 ± 0.85	0.49 ± 0.01**

(Fig. 7a). Singlet oxygen was produced by photosensitizing Rose Bengal (45 μM) by green LED light (525 nm), thereby avoiding chlorophyll photosensitizing (Fig. 7b). In the presence of leaf extracts the TEMPO spectrum was smaller (Fig. 7c), showing a scavenging of $^1O_2$ by antioxidants. After 4 min of photosensitizing Rose Bengal, the TEMPO signal was significantly smaller in the presence of leaf extracts from H and H+D plants than in controls (Fig. 7d). In summary, plant exposure to H and H+D enhanced the $^1O_2$ scavenging capacity in leaf extracts. An overview of results regarding *P. minima* and *S. incanus* is given in Fig. 8.

3.4. Screening of the maximum heat tolerance of 12 additional alpine plants

The mean maximum LT_{50} of the 12 species grown on the natural sites (Table 1, excluding *H. pusillum* and *H. veselskyi*) was 50.4 °C ± 3.6 when assessing tissue damage of detached leaves visually. As reported for *P. minima* and *S. incanus*, equivalent or higher temperatures (except for *Saxifraga aizoides*, which grew on a shady site) were tolerated when whole plants were treated in the presence of solar irradiation and measured with the HTTS: on average LT_{50} was 54.6 °C ± 2.9 and ranged from 47.4 °C (*S. aizoides*)

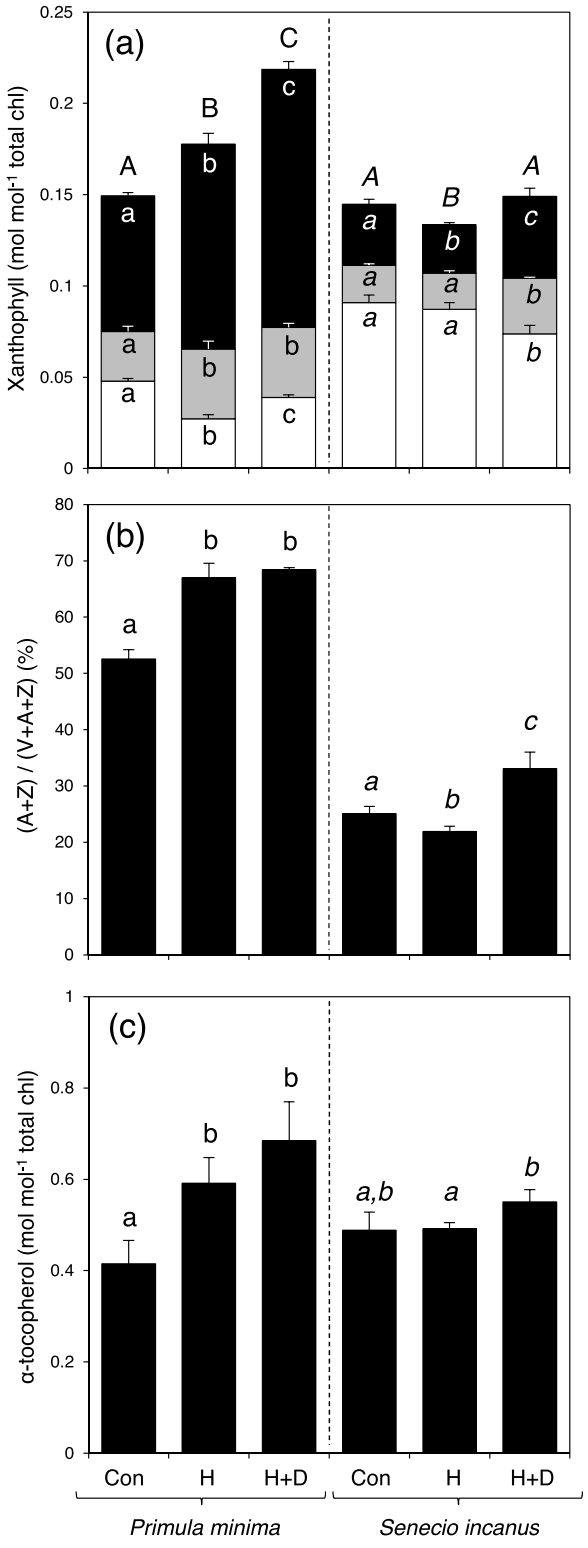

Fig. 5. Changes in xanthophyll cycle carotenoids and α-tocopherol in response to heat-hardening of *Primula minima* and *Senecio incanus*. Potted plants were exposed to increased heat (H) or to increased heat and drought (H+D) and leaves taken for analysis after 8 d of heat-hardening, or from non-heat-hardened plants (Con). **(a)** Violaxanthin, antheraxanthin and zeaxanthin are represented by white, grey and black bars, respectively. Significant differences in the total xanthophyll cycle pool and of individual xanthophylls are represented by capital and lower case letters, respectively. **(b)** De-epoxidation state of the xanthopyll cycle pigments. **(c)** Amounts of α-tocopherol. One-Way Anova was performed for each species individually and significant differences ($P < 0.05$) are represented by different letters, n = 4 ± SD. All amounts were calculated on a molar basis and normalized to total chlorophyll.

to 58.0 °C (*S. incanus*). The two common garden grown species *Heliosperma pusillum* and *H. veselskyi* had insignificantly different maximum LT_{50} when determined on detached leaves (55.3 °C and 55.5 °C, respectively) or by the HTTS (55.5 °C and 55.3 °C, respectively). The corresponding maximum T_c values were 50.1 °C and 50.3 °C, respectively (Table 1).

In summary, PS II inactivation (T_c) under heat stress consistently occurred at lower temperatures than tissue damage (45.6 °C ± 3.0) and heat treatment under solar irradiation (using the HTTS) lessened the heat stress-induced tissue viability loss. Regardless of the method applied, no significant elevational gradient in maximum heat tolerance and PS II inactivation was detectable when comparing species from the two natural growing sites on Mt. Patscherkofel and Timmelsjoch.

4. Discussion

4.1. Physiological aspects of heat-hardening

4.1.1. Leaf temperature and heat-hardening of photosystem II assessed through T_c

Heat-hardening of leaves occurs when a specific leaf temperature threshold is surpassed (Alexandrov, 1977). Too intensive heat stimuli may have negative effects on heat-hardening, as demonstrated for *S. acaulis* (Neuner et al., 2000). Therefore, care was taken to apply temperature regimes that imposed sufficient stress to induce heat hardening without over-stressing the plants. Furthermore, a prerequisite for the comparative analyses of the H and H+D plants was to keep near identical leaf temperatures during the controlled heat-hardening. This was a challenging task due to the permanent risk of overheating of the H+D plants under full sunlight, and because transpiration cooling contributed to changes in leaf temperature by 4–6 K in H plants (Fig. 2f). However, using the heat-hardening chamber it was possible to fine-tune mean leaf temperatures of the H and H+D plants, enabling leaves to have nearly identical temperatures throughout the heat-hardening runs in both species (Fig. 3a and b). Importantly, this enabled the effect of H+D to be separated from the effect of H.

T_c, the critical temperature at which F_0 increases sharply upon continuous heating of dark-acclimated leaf discs, is an indicator of the thermal stability of PS II. In both plant species, T_c increased in the first five days of heat-hardening. Maximum heat tolerance of PS II was reached on day 6 when T_c plateaued (Fig. 3g and h), indicating that the temperature regimes used for H and H+D were adequate to induce heat hardening. T_c appears to correlate with lethal leaf temperatures that induce leaf tissue necrosis (Bilger et al., 1984; Neuner and Pramsohler, 2006; Hüve et al., 2011). Nonetheless, it is not possible to derive tissue LT_{50} directly from T_c because of the enormous scattering of the values (see Bilger et al., 1984). Across the measured plants species, LT_{50} was on average higher than T_c by several degrees K, and the onset of leaf necrosis (LT_5) was initiated at higher temperatures than T_c (Table 1, Suppl. 5).

Prior to heat-hardening, the T_c of *S. incanus* was 45.9 °C and 43.1 °C in *P. minima*, which is a difference of 2.8 K (Fig. 3g and h), indicating that the PS II of *S. incanus* was better heat-adapted already before heat-hardening, as could be expected considering the local climatic differences experienced by the two species (Fig. 1). In *P. minima*, but not in *S. incanus*, T_c was significantly higher in the final phase of heat hardening (days 6–8) under H+D than under H alone (Fig. 3g and h). A drought-induced increase in PS II thermal tolerance was also reported in several studies, i.e. in *Lycopersicon esculentum* and *Solanum nigrum* (Havaux, 1992), seedlings of *Cedrus* spp. (Epron, 1997) and various *Triticum aestivum* cultivars (Lu and Zhang, 1999) and *Aegilops* species (Dulai et al., 2006). Our results strongly support the impact of

drought on PS II thermal tolerance but also indicate possible species-specific differences in the response to H and H+D.

In *P. minima* the SDs for T_c were considerably higher than in *S. incanus*. This may result at least in part from the different morphological features of the species: In *P. minima* the small leaf rosettes were partly closed during the heat treatments and therefore, the interior leaves were shielded by the external leaves from high solar irradiation. This was not the case in *S. incanus*, although irradiation conditions also differed due to the different inclinations of the individual leaves. Leaf rosette closure and anatomical characteristics as e.g. the hairy leaves of *S. incanus* and special water storing tissues and mucopolysaccharides in *P. minima*, may have contributed at least partly to the different responses to H and H+D. Hence, morphological and anatomical features, although often overlooked, should be considered equally important as physiological mechanisms in environmental stress handling.

4.1.2. Evaporative cooling, water loss control and leaf water potential changes during heat-hardening

In the middle of a typical summer day the leaves of *P. minima* and *S. incanus* in the field were clearly cooler than the plant's surroundings, showing the beneficial effects of evaporative cooling (Fig. 2a–d). After exposure to sunlight leaf temperature of H+D plants (compared to H plants) increased by 4–6 K within 90 min, which took longer in *P. minima* than in *S. incanus* (Fig. 2f). This may indicate a species-specific variation in the ability to restrict evaporative cooling in response to drought, in agreement with the time courses of Ψ_{act} as shown in Fig. 3c and d. During heat-hardening Ψ_{act} initially decreased in *P. minima*, particularly during midday (day 0–3), whereas it slightly increased in *S. incanus*, although *S. incanus* was not watered at this point and both species were exposed to similar levels of sunlight and temperature during this period (Fig. 3a and b). In the middle of the heat-hardening time course, the Ψ_{act} of H+D plants was significantly lower in both species compared to the H plants, confirming that the H+D treatment induced significant drought stress. Taken together, these data (Fig. 2f and Fig. 3c and d) indicate that *P. minima* is likely to have a more hydro-labile behaviour compared to *S. incanus*, reflecting the specific adaptations of these species to their habitats.

4.1.3. Photosystem II efficiency (F_v/F_m) during heat hardening

F_v/F_m describes the potential efficiency of PS II in a dark-acclimated state. F_v/F_m is a widely used indicator of plant health and is one of the first indicators to decrease before other physiological parameters change (Murata et al., 2007). F_v/F_m declines when the rate of PS II damage is greater than its repair (for review see Tyystjärvi, 2013; Gururani et al., 2015), also called photoinhibition, and is associated to elevated rates of ROS production (Hideg et al., 1994; Pospíšil and Prasad, 2014). Therefore, it was important to regularly monitor F_v/F_m during heat-hardening alongside a visual assessment of leaves, to allow for minor adjustments of leaf temperature and water potentials so that the plants were not over-stressed. Within 4–5 days of heat-hardening F_v/F_m decreased in *S. incanus*, but was completely restored by the end of treatment (Fig. 3f), indicating acclimation to the H and H+D treatments. In contrast, the F_v/F_m of *P. minima* was initially lower (0.752), indicative of photoinhibition, and progressively decreased further during heat-hardening, although never below 0.65, even in H+D plants (Fig. 3e). However, photoinhibition of PS II can also be considered a protective mechanism of the photosynthetic apparatus by reducing unwanted excitation of PS II (Adir et al., 2003; Adams et al., 2006; Takahashi and Murata, 2008). Following this line of reasoning, the already low F_v/F_m values of non-stressed plants and the lowered values at the final stages of hardening that coincided with maximal heat tolerance of PS II could be seen as a protective strategy. Downregulation of PS II is controlled by different mechanisms (see Trebst, 2003) and serves as an effective protection against over-reducing downstream electron carriers. Overall, changes in F_v/F_m showed that *S. incanus* was better able to remain potential PS II efficiency under H and H+D than *P. minima*.

4.1.4. The impact of drought on maximum tissue heat tolerance

In both species, H+D significantly decreased tissue heat tolerance as determined on the whole plant level by the HTTS. By contrast, when determined on detached leaves, tissue heat tolerance was higher after H+D compared to H. Knowledge of the effects of combined H+D is still poor, and at first sight, the results seem to be contradictory. Rihzsky et al. (2002) demonstrated that the response of tobacco plants to a combination of heat-shock and drought is very different from the response to these stress factors applied individually. The authors suggested the activation of a unique genetic program under heat-shock and drought, including the induction of a senescence-associated transcript (SAG12). From this point of view, as observed on detached leaves, in *P. minima* and *S. incanus* the H+D treatment might have resulted in additive or synergistic effects on the stress response (i.e. increasing heat tolerance). These effects seemed to have been superimposed by antagonistic effects supporting leaf senescence, which could only fully develop when leaves remained attached to the plants. A similar effect of leaf detachment on the measured heat tolerance was recently demonstrated on *L. procumbens* (Buchner et al., 2013). However, caution in the interpretation of these results and verification by further studies is needed, as leaf senescence is a highly complex process that is controlled at multiple levels (see Woo et al., 2013).

4.1.5. Different methods, different results: a matter of what is measured and how

In both species, heat-induced damage of the photosynthetic apparatus preceded leaf viability loss; heat tolerance assessed by PS II activity, either via T_c or F_v/F_m, was significantly lower by several degrees K compared to the visual assessment of tissue necrosis. Furthermore, when whole plants were exposed to H under solar irradiation in the HTTS, heat tolerance was highest (Fig. 4). Light can have protective effects on heat stability of PS II and the photosynthetic apparatus, as has been demonstrated before (Weis, 1982; Havaux et al., 1991), including in alpine plants (Buchner et al., 2015). In accordance with the previous chapter, not only light during heat exposure but also detachment of leaves can affect the measured heat tolerance. It is unclear why LT_{50} values of detached leaves may differ from those taken from intact plants, but it is reasonable to assume that cell death is initiated by leaf excision. However, each method of measuring heat tolerance has strengths and limitations. Chlorophyll fluorescence-based methods are particularly useful to explore the heat tolerance of the primary photosynthetic processes, whereas assays based on cell membrane stability and/or tissue necrosis are relevant for leaf survival. Both measurements provide information of crucial interest to researchers trying to improve the heat tolerance of agricultural plants. We prefer to use the HTTS for testing heat tolerance, because it can be applied *in situ* in the field without transplanting the experimental plants and without leaf excision, producing data with high ecological relevance. Furthermore, for several decades standard exposure duration of 30 min was chosen by most authors studying plant heat tolerance, but this exposure time may not be relevant in the field where heat events can occur for longer (Fig. 1, Suppl. 4). Therefore, in the future it will be valuable to further elaborate on methodological details, particularly regarding the heat dose (Hüve et al., 2011) plants are exposed to.

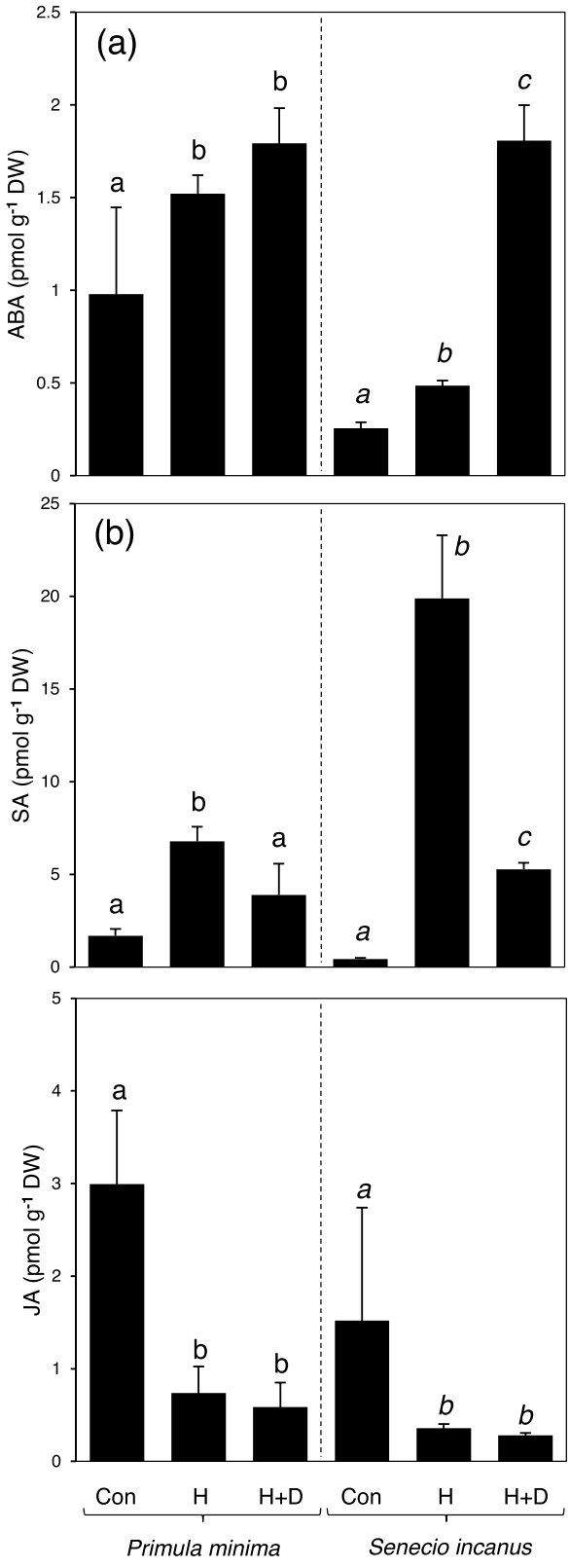

Fig. 6. Changes in leaf hormone amounts in response to heat-hardening of *Primula minima* and *Senecio incanus*. Potted plants were exposed to increased heat (H) or to increased heat and drought (H+D) and leaves taken for analysis after 8 d of heat-hardening, or from non-heat-hardened plants (Con). Amounts of **(a)** abscisic acid (ABA), **(b)** salicylic acid (SA) and **(c)** jasmonic acid (JA) were measured with UHPLC–MS/MS. One-Way Anova was performed for each species and for each hormone individually and significant differences ($P < 0.05$) are represented by different letters, $n = 4 \pm SD$.

4.2. Biochemical aspects of heat-hardening

4.2.1. Plastid pigments and singlet oxygen scavenging

The potential efficiency of PS II was affected by heat hardening and drought, especially in *P. minima* (Fig. 3e). Therefore, we further investigated changes in photosynthetic pigments. The total chlorophyll content was not affected by the heat treatments, but in both species H and H+D treatments significantly decreased the chlorophyll a:b ratio (Table 2), which could have been at least in part due to the difference between the light intensity in the field and in the heat hardening chamber (see PPFD data in Fig. 1 and Fig. 3). Therefore, it cannot be completely ruled out that the results regarding light sensitive parameters and pathways (e.g. F_v/F_m, xanthophyll cycle pigments) may have been partly superimposed to a certain extent by possible effects of the slightly reduced PPFD inside the heat hardening chamber during H and H+D treatments. Nonetheless, in *P. minima* the carotenoids:chlorophyll ratio was significantly higher under H+D than under H alone (Table 2), indicating an increased demand for accessory pigments for protecting the photosynthetic apparatus. In addition, the significant increase in α-tocopherol, xanthophyll cycle pigments and xanthophyll cycle de-epoxidation state upon H and H+D in the leaves of *P. minima* compared to the relatively small changes in *S. incanus* (Fig. 5a–c) also showed a different response of the two species. In nature *P. minima* is less exposed to such high leaf temperatures that *S. incanus* experiences (Fig. 1, Suppl. 4), indicating why heat-hardening was more stressful for *P. minima*, as also reflected by the gradual decline in F_v/F_m (Fig. 3e). Accordingly, the pool of xanthophyll cycle pigments increased in *P. minima*, in particular zeaxanthin (Fig. 5a), which supports NPQ and provides antioxidant or structural protection of the thylakoid membrane. It has been suggested that high mountain plants use various strategies for photoprotection and ROS detoxification, some of which may be species specific (Streb et al., 1997, 1998; Streb and Cornic, 2012; Laureau et al., 2011), and a positive correlation between altitude and leaf levels of α-tocopherol was shown in the alpine *Saxifraga longifolia* (Munné-Bosch et al., 2016). The oxygen-evolving complex of PS II in plants is known to be particularly sensitive to high temperatures (Berry and Björkman, 1980). Photosystem II is damaged by ROS and reactive electrophile species (RES), such as malondialdehyde that is released after $^1O_2$-mediated lipid peroxidation of thylakoid membranes (Yamauchi et al., 2008). Stomatal closure upon drought reduces CO_2 availability for the Calvin cycle, restricting the abundance of electron acceptors, leading to excess light absorption and associated $^1O_2$ production and damage (Roach and Krieger-Liszkay, 2014). Singlet oxygen scavenging capacity was significantly enhanced in *P. minima* leaves exposed to H and H+D (Fig. 7), and positively correlated with the elevated α-tocopherol ($R^2 = 0.99$), β-carotene ($R^2 = 0.82$) and zeaxanthin ($R^2 = 0.97$) levels (Fig. 5a and b). These molecules protect the thylakoid membrane by either directly quenching $^1O_2$ or terminating lipid peroxidation chain reactions (see Munné-Bosch, 2005; Havaux, 2005). In summary, heat hardening could be induced by H and H+D in both species, and in *P. minima*, which is adapted to cooler and wetter habitats than *S. incanus*, this apparently required enhanced protection of thylakoid membranes from oxidative damage.

4.2.2. Changes in plant hormones

Abscisic acid plays a crucial role in adaptation to abiotic stress (Robert-Seilaniantz et al., 2007), in particular to drought, low temperature and osmotic stress (Fujita et al., 2006). One of the many roles of ABA involves the regulation of stomatal closure (Cutler et al., 2010). In *P. minima* ABA levels of the controls were higher than in *S. incanus*. It is worth noting that leaf samples of control, H and H+D plants were all taken at the same time (i.e. after

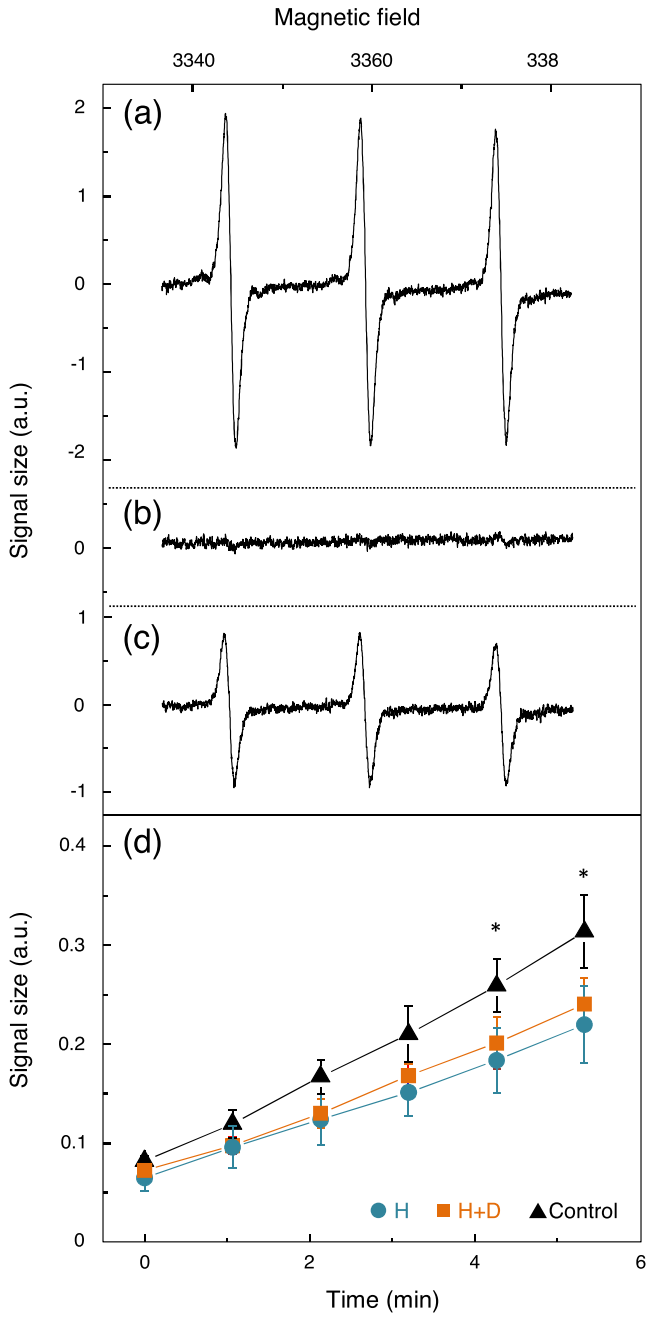

Fig. 7. Changes in quenching of singlet oxygen by leaf extracts of *Primula minima* in response to heat-hardening. Potted plants were exposed to increased heat (H) or to increased heat and drought (H+D) and leaves taken for analysis after 8 d of heat-hardening, or from non-heat-hardened plants (Con). Singlet oxygen ($^1O_2$) was measured by electron paramagnetic resonance (EPR) that was used for detecting TEMPO, the spin-adduct of TEMP after oxidation by $^1O_2$, which was produced by illuminating Rose Bengal by green light. Typical spectra after 10 min of illumination are shown, where signal size represents amounts of $^1O_2$ trapped (**a**) in the presence of Rose Bengal, but absence of leaf extract, (**b**) in the presence of leaf extract, but absence of Rose Bengal and (**c**) in the presence of Rose Bengal and leaf extract. All spectra are scaled identically. (**d**) Time course of spin adduct accumulation in the presence of leaf extracts from control plants (black triangles), H plants (blue squares) or H+D plants (orange circles). Asterisks denote significant differences ($P < 0.05$) between control and H plants; $n = 3 \pm SD$.

8 days). Therefore, the environmental conditions were not exactly identical prior to sampling of the controls of the two species, whereby conditions were cooler and wetter for *S. incanus* due to a slight rainfall on the day of sampling (see grey shading in Fig. 1), in agreement with the lower ABA levels found in *S. incanus* controls. However, in both species, compared to their respective controls, ABA levels were higher in response to H, and highest in leaves from *S. incanus* exposed to H+D. Under H+D ABA increased more in *S. incanus* than in *P. minima* (Fig. 6a), again reflecting the adaptation of *S. incanus* to drier environments coinciding with a capability to efficiently elevate ABA levels required for stomatal closure under drought.

Salicylic and acetylsalicylic acid were shown to be involved in acquiring thermal tolerance (Dat et al., 1998; Lopez-Delgado, 1998), and using *A. thaliana* mutants deficient in hormonal signalling pathways it has been demonstrated that crosstalk exists between ABA and SA in the acquisition of thermal tolerance (Larkindale et al., 2005). Hormonal cross talk can involve both, positive (additive or synergistic) and negative feedback influencing synthesis, transport and signalling of other hormones (Robert-Seilaniantz et al., 2007). Salicylic acid and JA are perhaps best known for their roles in plant response to biotrophic and necrotrophic pathogens, respectively, they can act antagonistically and ABA can be an antagonist of both (Fujita et al., 2006; Spoel and Dong, 2008). In this study, the

H-induced elevation of ABA and SA in the leaves of both species would support a role of both hormones in heat hardening, involving positive feedback between ABA and SA. It is not entirely clear how heat stress is perceived by plants, but one pathway may involve RES produced during heat stress (Clarke et al., 2009), which can bind directly to heat shock factors, causing their trimerization and activation (Higdon et al., 2012). RES are breakdown products of the thylakoid membrane and are a signature of $^1O_2$ production (Farmer and Mueller, 2013; Mano, 2012), linking ROS and RES during heat stress. One heat shock factor associated to heat-stress is WRKY39, which is also upregulated by SA (Li et al., 2010). Jasmonates are hormones derived from α-linolenic acid and produced enzymatically through RES intermediates, such as 12-oxo-phytodienoic acid (OPDA)

(Taki et al., 2005), and a role for α-tocopherol in regulating jasmonate levels has been shown (Munné-Bosch et al., 2007). In this study both H and H+D led to reduced JA levels. Lowered JA levels in leaves of heat-hardened plants have previously been associated with achieving basal thermal tolerance (Clarke et al., 2009). Furthermore, 6–12 h heat-stressed (42 °C) *Oryza sativa* plants also had lowered JA levels (Du et al., 2013), also arguing for a role of JA in plant response to heat.

In both species the highest ABA levels were found under H+D whereas SA levels were lowered in H+D compared to H treatment alone, indicative of negative feedback. Interestingly, several authors reported that under severe stress, plants appear to give preference to protection from abiotic rather than biotic stress (e.g. Fujita et al., 2006; Robert-Seilaniantz et al., 2007), which would agree with a negative feedback of ABA on SA under H+D. Overall, increased $^1O_2$ scavenging alongside enhanced xanthophyll cycle activity and α-tocopherol levels, in particular in leaves of *P. minima*, may have been significant factors promoting heat hardening, orchestrated by hormonal crosstalk involving ABA, SA and JA.

4.3. Future perspectives: the effects of rising temperatures may be exacerbated when combined with other stress factors

Global temperatures are predicted to rise (IPCC, 2013), in Austria at least by +1.4 K during the first half of the 21st century (APCC, 2014). Many authors suggest that high elevation sites will be relatively more affected (reviewed by Rangwala and Miller, 2012). In this paper, we report on the potential effects of a heat wave in combination with drought, which may be relevant to future environmental scenarios that alpine plants may experience. Based on the ecologically most relevant results on LT_{50} gained from

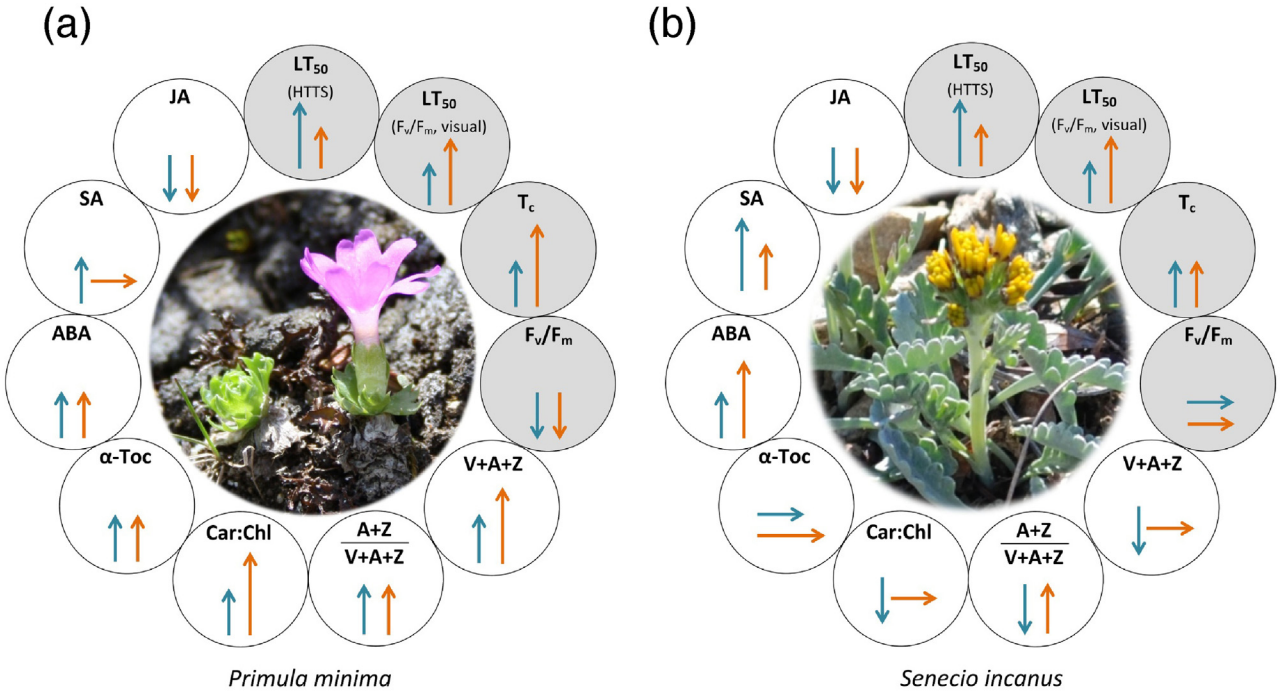

Fig. 8. Schematic illustration of the response of *Primula minima* and *Senecio incanus* leaves to heat-hardening. Potted individuals from **(a)** *Primula minima* and **(b)** *Senecio incanus* plants were exposed to increased heat (H, blue arrows) or to increased heat and drought (H+D, orange arrows) within a controllable heat-hardening chamber on Mt. Patscherkofel (1960 m a.s.l.) for 8 days during summer 2015. Vertical arrows indicate significant ($P < 0.05$) increases (↑) or decreases (↓) of the each parameter as determined at the end of heat hardening (day 8). **Note:** Changes of LT_{50} (heat tolerance), F_v/F_m (potential quantum efficiency of photosystem II) and T_c (heat threshold of photosystem II) are regarded to day 0 before heat-hardening started (grey circles), whereas changes of the other parameters are regarded to leaves from non-heat-hardened plants collected on day 8 (white circles). Arrows with different lengths indicate significant ($P < 0.05$) differences (higher or lower; in accordance with the arrow direction) between the H and H+D treated plants. Horizontal arrows (→) indicate that there is no significant difference with regard to day 0 (LT_{50}, F_v/F_m, T_c) or to non-heat-hardened control plants (other parameters). V violaxanthin, A antheraxanthin, Z zeaxanthin, (A + Z)/(V + A + Z) de-epoxidation state of the xanthophyll cycle pool, Car carotenoid, Chl chlorophyll, α-Toc α-tocopherol, ABA abscisic acid, SA salicylic acid, JA jasmonic acid.

using the HTTS, we demonstrated that H+D combined can diminish tissue heat-hardening capacity.

Transpiration cooling is a key factor in leaf temperature control (Larcher, 2003), making drought a significant contributor to increasing leaf temperatures (Fig. 2e and f). We showed that at the same ambient temperature and irradiation, actual leaf temperatures rose by 4–6 K under drought, far exceeding the predicted increase in mean global temperature. Hence, for predictions of plant performance under elevated temperatures, it will be essential to know whether or not the rising temperatures will be accompanied by drought (see De Boeck et al., 2016). However, the maximum heat tolerance of alpine plants, as determined by the HTTS and under well-watered conditions, was found to be surprisingly high, even in species that were not necessarily known to be extremely heat tolerant, such as *Gentiana acaulis* and *Geum montanum* for which 57.3 °C and 56.4 °C, respectively, were identified as the maximum tissue heat tolerance (LT_{50}).

Heliosperma pusillum and *H. veselskyi* (Table 1) are particularly interesting as they are genetically closely related (Trucchi et al., 2016), but adapted to different habitats, providing the possibility to compare heat tolerance in phylogenetically comparable but ecologically differentially adapted taxa. *Heliosperma pusillum* occurs on open alpine screes with good water availability, whereas *H. veselskyi* grows in the montane belt at shaded sites, below overhanging rocks on dry, shallow soils. According to the contrasting conditions in their habitats, the species differ in morphological and functional traits, e.g. photosynthetic parameters in response to PPFD and leaf temperatures (Bertel et al., 2016). Nonetheless, the *Heliosperma* species used in the present study were grown in a common garden and showed an almost identical maximum heat tolerance, indicating that in both species heat tolerance was adjusted to the conditions within the heat hardening chamber and that their stress response does not seem to be limited by solely genotypic factors (see also Bertel et al., 2016).

Naturally occurring heat damage to leaves can be observed regularly in various alpine species, but these plants apparently possess a high potential for compensating partial damage and the loss of aboveground biomass. In many instances, plants will be able to escape from particularly endangering sites due to the topographically induced mosaics of microclimate conditions, which play a big role in thermal microhabitat differentiation and species distribution in alpine landscapes (see Scherrer and Körner, 2010, 2011). However, on south exposed ruderal sites, and for pioneer seedlings (Ladinig et al., 2015; Marcante et al., 2014) the risk of lethal heat damage will likely increase in the future. As more heat waves (Beniston, 2004; Ballester et al., 2010) and severe drought (Gobiet et al., 2014) are forecast, the loss of species such as *S. incanus* growing on exposed sites may contribute to destabilizing soil and increasing erosion. Furthermore, apart from heat injuries to individual leaves other heat-induced effects such as lowered carbon assimilation (Buchner et al., 2015) and plant migration have been reported for almost all major mountain ranges in Europe (Grabherr et al., 1994; Pauli et al., 2012), the Himalaya (Telwala et al., 2013) and the Andes (Feeley and Silman, 2010). Therefore, for further enhancing our knowledge of the effects of global change on alpine plant communities it is necessary to carefully consider a plethora of aspects such as plant response to stress factors like heat, drought and solar irradiation intensity alone and in combination, as well as interactions at the ecosystem level.

Conflict of interest

The authors declare that they have no conflicts of interest.

Contributions

OB and GN initiated the project. OB built the heat-hardening and heat tolerance testing equipment, designed and supervised the field experiments. JG, SS and MK performed the field experiments, RM conducted infrared thermography and related image analyses. TR measured photosynthetic pigments, α-tocopherol contents, and singlet oxygen scavenging capacity. WS developed the method for the analysis of plant hormones and measured the hormones. Data were analysed by OB, TR, WS, JG, SS and MK. CB contributed experimental plants of *Heliosperma* species and the corresponding text parts. IK and GN advised on experimental design, and provided instruments, lab space and consumables. OB, TR (joint first authorship), IK and GN wrote the manuscript, and all authors proofread the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2016.10.010.

References

APCC, 2014. Österreichischer Sachstandsbericht Klimawandel 2014 (AAR14). Austrian Panel on Climate Change (APCC), Verlag der Österreichischen Akademie der Wissenschaften. APCC, Vienna, Austria, pp. 1096.
Adams III, W.W., Zarter, C.R., Mueh, K.E., Demmig-Adams, B., 2006. Energy dissipation and photoinhibition: a continuum of photoprotection. In: Demming-Adams, B., Adams, W.W., Mattoo, A.K. (Eds.), Photoprotection, Photoinhibition, Gene Regulation, and Environment. Springer, Netherlands, pp. 49–64.
Adir, N., Zer, H., Shochat, S., Ohad, I., 2003. Photoinhibition – a historical perspective. Photosynth. Res. 76, 343–370.
Alexandrov, V.Y., 1977. Cells, molecules and temperature: conformational flexibility of macromolecules and ecological adaptation, 1 st ed. Cells, Molecules and Temperature, Ecological Studies, vol. 21. Springer, Berlin, Germany, pp. 330.
Böhm, R., Auer, I., Brunetti, M., Maugeri, M., Nanni, T., Schöner, W., 2001. Regional temperature variability in the European Alps: 1760–1998 from homogenized instrumental time series. Int. J. Climatol. 21, 1779–1801.
Ballester, J., Rodó, X., Giorgi, F., 2010. Future changes in Central Europe heat waves expected to mostly follow summer mean warming. Clim. Dyn. 35, 1191–1205.
Beniston, M., 2004. The 2003 heat wave in Europe: a shape of things to come? An analysis based on Swiss climatological data and model simulations. Geophys. Res. Lett. 31, L02301.
Berry, J., Björkman, O., 1980. Photosynthetic response and adaptation to temperature in higher plants. Annu. Rev. Plant Phys. 31, 491–543.
Bertel, C., Buchner, O., Schönswetter, P., Frajman, B., Neuner, G., 2016. Environmentally induced and (epi-)genetically based physiological trait differentiation between Heliosperma pusillum and its polytopically evolved ecologically divergent descendent, H. veselskyi (Caryophyllaceae: sileneae). Bot. J. Linn. Soc. 182 (3), 658–669. doi:http://dx.doi.org/10.1111/boj.12467.
Bilger, H.-W., Schreiber, U., Lange, O.L., 1984. Determination of leaf heat resistance: comparative investigation of chlorophyll fluorescence changes and tissue necrosis methods. Oecologia 63, 256–262.
Braun, V., Buchner, O., Neuner, G., 2002. Thermotolerance of photosystem 2 in three alpine plant species under field conditions. Photosynthetica 40, 587–595.
Buchner, O., Neuner, G., 2001. Determination of heat tolerance: a new equipment for field measurements. J. Appl. Bot. - Angewandte Botanik 75, 130–137.
Buchner, O., Neuner, G., 2003. Variability of heat tolerance in alpine plant species measured at different altitudes. Arct. Antarc. Alp. Res. 35, 411–420.
Buchner, O., Karadar, M., Bauer, I., Neuner, G., 2013. A novel system for in situ determination of heat tolerance of plants: first results on alpine dwarf shrubs. Plant Methods 9, 7.
Buchner, O., Stoll, M., Karadar, M., Kranner, I., Neuner, G., 2015. Application of heat stress in situ demonstrates a protective role of irradiation on photosynthetic performance in alpine plants. Plant Cell Environ. 38, 812–826.
Cernusca, A., 1976. Bestandesstruktur, Bioklima und Energiehaushalt von alpinen Zwergstrauchbeständen. Oecol. Plant. 11, 71–102.
Clarke, S.M., Cristescu, S.M., Miersch, O., Harren, F.J.M., Wasternack, C., Mur, L.A.J., 2009. Jasmonates act with salicylic acid to confer basal thermotolerance in Arabidopsis thaliana. New Phytol. 182, 175–187.
Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R., Abrams, S.R., 2010. Abscisic acid: emergence of a core signaling network. Ann. Rev. Plant Biol. 61, 651–679.
Dat, J.F., Foyer, C.H., Scott, I.M., 1998. Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. Plant Physiol. 118, 1455–1461.
De Boeck, H.J., Bassin, S., Verlinden, M., Zeiter, M., Hiltbrunner, E., 2016. Simulated heat waves affected alpine grassland only in combination with drought. New Phytol. 209, 531–541.
De Pinto, M.C., Locato, V., Paradiso, A., De Gara, L., 2015. Role of redox homeostasis in thermotolerance under a climate change scenario. Ann. Bot. -London 115 (4), 487–496. doi:http://dx.doi.org/10.1093/aob/mcv071.
Demmig-Adams, B., Garab, G., Adams III, W.W., Govindjee, 2014. Non-photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria. Springer, New York, pp. 649 (ISBN 978-94-017-9031-4).
Du, H., Liu, H., Xiong, L., 2013. Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. Front. Plant Sci. 4 (397) .
Dulai, S., Molnár, I., Prónay, J., Csernak, A., Tarnai, R., Molnár-Láng, M., 2006. Effects of drought on photosynthetic parameters and heat stability of PSII in wheat and in Aegilops species originating from dry habitats. Acta Biol. Szegediensis 50, 11–17.
Epron, D., 1997. Effects of drought on photosynthesis and on the thermotolerance of photosystem II in seedlings of cedar (Cedrus atlantica and C. libani). J. Exp. Bot. 48, 1835–1841.
Farmer, E.E., Mueller, M.J., 2013. ROS-mediated lipid peroxidation and RES-activated signaling. Ann. Rev. Plant Biol. 64, 429–450.
Feeley, K.J., Silman, M.R., 2010. Land-use and climate change effects on population size and extinction risk of Andean plants. Glob. Change Biol. 16, 3215–3222.
Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., Shinozaki, K., 2006. Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr. Opin. Plant Biol. 9, 436–442.
Gauslaa, Y., 1984. Heat resistance and energy budget in different Scandinavian plants. Holarct. Ecol. 7, 1–78.
Gobiet, A., Kotlarski, S., Beniston, M., Heinrich, G., Rajczak, J., Stoffel, M., 2014. 21st century climate change in the European Alps—a review. Sci. Total Environ. 493, 1138–1151.
Grabherr, G., Mair, A., Stimpfl, H., 1988. Vegetationsprozesse in alpinen Rasen und die Chancen einer echten Renaturierung von Schipisten und anderen Erosionsflächen in alpinen Hochlagen. Jahrbuch 3 der Gesellschaft für Ingenieursbiologie. Sepia, Aachen, Germany, pp. 94–113.
Grabherr, G., Gottfried, M., Pauli, H., 1994. Climate effects on mountain plants. Nature 369 448–448.
Gururani, M.A., Venkatesh, J., Tran, L.S.P., 2015. Regulation of photosynthesis during abiotic stress-induced photoinhibition. Mol. Plant 8, 1304–1320.
Hüve, K., Bichele, I., Rasulov, B., Niinemets, Ü., 2011. When it is too hot for photosynthesis: heat-induced instability of photosynthesis in relation to respiratory burst, cell permeability changes and H2O2 formation. Plant Cell Environ. 34, 113–126.
Havaux, M., 2005. Vitamin E protects against photoinhibition and photooxidative stress in Arabidopsis thaliana. Plant Cell 17, 3451–3469. doi:http://dx.doi.org/10.1105/tpc.105.037036.
Havaux, M., Greppin, H., Strasser, R.J., 1991. Functioning of photosystems I and II in pea leaves exposed to heat stress in the presence or absence of light. Planta 186, 88–89.
Havaux, M., 1992. Stress tolerance of photosystem II in vivo: antagonistic effects of water, heat, and photoinhibition stresses. Plant Physiol. 100, 424–432.
Hideg, E., Spetea, C., Vass, I., 1994. Singlet oxygen production in thylakoid membranes during photoinhibition as detected by EPR spectroscopy. Photosynth. Res. 39, 191–199.
Higdon, A., Diers, A.R., Oh, J.Y., Landar, A., Darley-Usmar, V.M., 2012. Cell signalling by reactive lipid species: new concepts and molecular mechanisms. Biochem. J. 442, 453–464.
IPCC, 2013. In: Stocker, T.F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V. Midgley (Eds.), Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press Cambridge, United Kingdom and New York, USA, pp. 1535.
Königshofer, H., Tromballa, H.W., Löppert, H.G., 2008. Early events in signalling high-temperature stress in tobacco BY2 cells involve alterations in membrane fluidity and enhanced hydrogen peroxide production. Plant Cell Environ. 31, 1771–1780.

Körner, C., Cochrane, P., 1983. Influence of plant physiognomy on leaf temperature on clear midsummer days in the Snowy Mountains south-eastern Australia. Acta Oecol. Oecol. Plant. 4, 117–124.
Körner, C., Larcher, W., 1988. Plant life in cold climates. Symp. Soc. Exp. Biol. 42, 25–57.
Kotak, S., Larkindale, J., Lee, U., Von Koskull-Döring, P., Vierling, E., Scharf, K.-D., 2007. Complexity of the heat stress response in plants. Curr. Opin. Plant Biol. 10, 310–316.
Kranner, I., Minibayeva, F.V., Beckett, R.P., Seal, C.E., 2010. What is stress? Concepts, definitions and applications in seed science. New Phytol. 188, 655–673.
Kreeb, K.-H., 1990. Hitzeresistenz. In: Kreeb, K.-H. (Ed.), Methoden zur Pflanzenökologie und Bioindikation. Gustav Fischer, Jena, Germany, pp. 72–75.
Krieger-Liszkay, A., Trebst, A., 2006. Tocopherol is the scavenger of singlet oxygen produced by the triplet states of chlorophyll in the PSII reaction centre. J. Exp. Bot. 57, 1677–1684.
Ladinig, U., Pramsohler, M., Bauer, I., Zimmermann, S., Neuner, G., Wagner, J., 2015. Is sexual reproduction of high-mountain plants endangered by heat? Oecologia 177, 1195–1210.
Landolt, E., 1992. Unsere Alpenflora, 6th ed. Fischer Stuttgart, Jena, Germany, pp. 318.
Larcher, W., Wagner, J., 2010. Temperatures in the life zones of the Tyrolean Alps. Sitzungsberichte und Anzeiger der mathematisch-naturwissenschaftlichen Klasse, vol. 213. Austrian Academy of Sciences, Vienna, Austria, pp. 31–51.
Larcher, W., 1980. Klimastress im Gebirge - Adaptationstraining und Selektionsfilter für Pflanzen. Florengeschichte im Spiegel Blütenökologischer Erkenntnisse. VS Verlag für Sozialwissenschaften, Wiesbaden, Germany, pp. 49–80.
Larcher, W., 2003. Physiological Plant Ecology, 4th ed. Springer Berlin, Heidelberg, Germany.
Larkindale, J., Knight, M., 2002. Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. Plant Physiol. 128, 682–695.
Larkindale, J., Hall, J.D., Knight, M.R., Vierling, E., 2005. Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant Physiol. 138, 882–897.
Laureau, C., Bligny, R., Streb, P., 2011. The significance of glutathione for photoprotection at contrasting temperatures in the alpine plant species Soldanella alpina and Ranunculus glacialis. Physiol. Plant. 143, 246–260. doi: http://dx.doi.org/10.1111/j.1399-3054.2011.01505.x.
Li, S., Zhou, X., Chen, L., Huang, W., Yu, D., 2010. Functional characterization of Arabidopsis thaliana WRKY39 in heat stress. Mol. Cells 29, 475–483.
Lipiec, J., Doussan, C., Nosalewicz, A., Kondracka, K., 2013. Effect of drought and heat stresses on plant growth and yield: a review. Int. Agrophys. 27, 463–477.
Locato, V., Gadaleta, C., De Gara, L., De Pinto, M.C., 2008. Production of reactive species and modulation of antioxidant network in response to heat shock: a critical balance for cell fate. Plant Cell Environ. 31, 1606–1619.
Lopez-Delgado, H., 1998. Induction of thermotolerance in potato microplants by acetylsalicylic acid and H2O2. J. Exp. Bot. 49, 713–720.
Lu, C., Zhang, J., 1999. Effects of water stress on photosystem II photochemistry and its thermostability in wheat plants. J. Exp. Bot. 50, 1199–1206.
Mano, J., 2012. Reactive carbonyl species: their production from lipid peroxides, action in environmental stress, and the detoxification mechanism. Plant Physiol. Bioch. 59, 90–97.
Marcante, S., Erschbamer, B., Buchner, O., Neuner, G., 2014. Heat tolerance of early developmental stages of glacier foreland species in the growth chamber and in the field. Plant Ecol. 215, 747–758.
Munné-Bosch, S., Peñuelas, J., 2003. Photo- and antioxidative protection, and a role for salicylic acid during drought and recovery in field-grown Phillyrea angustifolia plants. Planta 217, 758–766.
Munné-Bosch, S., Weiler, E.W., Alegre, L., Müller, M., Düchting, P., Falk, J., 2007. α-tocopherol may influence cellular signaling by modulating jasmonic acid levels in plants. Planta 225, 681–691. doi:http://dx.doi.org/10.1007/s00425-006-0375-0.
Munné-Bosch, S., Cotado, A., Morales, M., Fleta-Soriano, E., Villellas, J., Garcia, M.B., 2016. Adaptation of the long-lived monocarpic perennial, Saxifraga longifolia to high altitude. Plant Physiol. 172, 765–775. doi:http://dx.doi.org/10.1104/pp.16.00877.
Munné-Bosch, S., 2005. The role of α-tocopherol in plant stress tolerance. J. Plant Physiol. 162, 743–748.
Murata, N., Takahashi, S., Nishiyama, Y., Allakhverdiev, S., 2007. Photoinhibition of photosystem II under environmental stress. Biochim. Biophys. Acta (BBA)-Bioenerg. 1767, 414–421.
Neuner, G., Buchner, O., 2012. Dynamics of tissue heat tolerance and thermotolerance of PS II in alpine plants. In: Lütz, C. (Ed.), Plants in Alpine Regions. Cell Physiology of Adaption and Survival Strategies. Springer, Vienna, Austria, pp. 61–74.
Neuner, G., Pramsohler, M., 2006. Freezing and high temperature thresholds of photosystem 2 compared to ice nucleation, frost and heat damage in evergreen subalpine plants. Physiol. Plant. 126, 196–204.
Neuner, G., Braun, V., Buchner, O., Taschler, D., 1999. Leaf rosette closure in the alpine rock species Saxifraga paniculata Mill.: significance for survival of drought and heat under high irradiation. Plant Cell Environ. 22, 1539–1548.
Neuner, G., Buchner, O., Braun, V., 2000. Short-term changes in heat tolerance in the Alpine cushion plant Silene acaulis ssp. excapa [All.] J. Braun at different altitudes. Plant Biol. 2, 677–683.
Noctor, G., Mhamdi, A., Foyer, C.H., 2014. The Roles of reactive oxygen metabolism in drought: not so cut and dried. Plant Physiol. 164, 1636–1648.
Oberdorfer, E., 1962. Pflanzensoziologische Exkursionsflore für Süddeutschland, 2nd ed. Ulmer Stuttgart, Germany, pp. 987.
Pauli, H., Gottfried, M., Dullinger, S., Abdaladze, O., Akhalkatsi, M., Alonso, J.L.B., Coldea, G., Dick, J., Erschbamer, B., Calzado, R.F., Ghosn, D., Holten, J.I., Kanka, R., Kazakis, G., Kollar, J., Larsson, P., Moiseev, P., Moiseev, D., Molau, U., Mesa, J.M., Nagy, L., Pelino, G., Puscas, M., Rossi, G., Stanisci, A., Syverhuset, A.O., Theurillat, J.-P., Tomaselli, M., Unterluggauer, P., Villar, L., Vittoz, P., Grabherr, G., 2012. Recent plant diversity changes on Europe's mountain summits. Science 336, 353–355.
Porra, R.J., Thompson, W.A., Kriedemann, P.E., 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta (BBA)-Bioenerg. 975, 384–394.
Pospíšil, P., Prasad, A., 2014. Formation of singlet oxygen and protection against its oxidative damage in Photosystem II under abiotic stress. J. Photochem. Photobiol. B 137, 39–48.
Rangwala, I., Miller, J.R., 2012. Climate change in mountains: a review of elevation-dependent warming and its possible causes. Clim. Change 114, 527–547.
Raven, J.A., 2011. The cost of photoinhibition. Physiol. Plant. 142, 87–104.
Reisigl, H., Pitschmann, H., 1958. Obere Grenzen von Flora und Vegetation in der Nivalstufe der zentralen Ötztaler Alpen (Tirol). Plant Ecol. 8, 93–129.
Remias, D., Lütz-Meindl, U., Lütz, C., 2005. Photosynthesis, pigments and ultrastructure of the alpine snow alga Chlamydomonas nivalis. Eur. J. Phycol. 40, 259–268.
Rizhsky, L., Kiang, H., Mittler, R., 2002. The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiol. 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 Physiol. 134, 1683–1696.
Roach, T., Krieger-Liszkay, A., 2014. Regulation of photosynthetic electron transport and photoinhibition. Curr. Protein Pept. Sci. 15, 351–362.
Robert-Seilaniantz, A., Navarro, L., Bari, R., Jones, J.D., 2007. Pathological hormone imbalances. Curr. Opin. Plant Biol. 10, 372–379.
Salvucci, M.E., Crafts-Brandner, S.J., 2004. Inhibition of photosynthesis by heat stress: the activation state of Rubisco as a limiting factor in photosynthesis. Physiol. Plant. 120, 179–186.
Scherrer, D., Körner, C., 2010. Infra-red thermometry of alpine landscapes challenges climatic warming projections. Glob. Change Biol. 16, 2602–2613.
Scherrer, D., Körner, C., 2011. Topographically controlled thermal-habitat differentiation buffers alpine plant diversity against climate warming. J. Biogeogr. 38, 406–416.
Schreiber, U., Berry, J.A., 1977. Heat-induced changes of chlorophyll fluorescence in intact leaves correlated with damage of the photosynthetic apparatus. Planta 136, 233–238.
Sgobba, A., Paradiso, A., Dipierro, S., De Gara, L., De Pinto, M.C., 2015. Changes in antioxidants are critical in determining cell responses to short- and long-term heat stress. Physiol. Plant. 153, 68–78.
Spoel, S.H., Dong, X., 2008. Making sense of hormone crosstalk during plant immune responses. Cell Host Microbe 3, 348–351.
Streb, P., Cornic, G., 2012. Photosynthesis and antioxidative protection in alpine herbs. In: Lütz, C. (Ed.), Plants in Alpine Regions. Cell Physiology of Adaption and Survival Strategies. Springer, Vienna, Austria, pp. 75–97.
Streb, P., Feierabend, J., Bligny, R., 1997. Resistance to photoinhibition of photosystem II and catalase and antioxidative protection in high mountain plants. Plant. Cell Environ. 20, 1030–1040.
Streb, P., Shang, W., Feierabend, J., Bligny, R., 1998. Divergent strategies of photoprotection in high-mountain plants. Planta 207, 313–324.
Suzuki, N., Mittler, R., 2006. Reactive oxygen species and temperature stresses: a delicate balance between signalling and destruction. Physiol. Plant. 126, 41–51.
Takahashi, S., Murata, N., 2008. How do environmental stresses accelerate photoinhibition? Trends Plant Sci. 13, 178–182.
Taki, N., Sasaki-Sekimoto, Y., Obayashi, T., Kikuta, A., Kobayashi, K., Ainai, T., Yagi, K., Sakurai, N., Suzuki, H., Masuda, T., Takamiya, K.-I., Shibata, D., Kobayashi, Y., Ohta, H., 2005. 12-Oxo-Phytodienoic Acid Triggers Expression of a Distinct Set of Genes and Plays a Role in Wound-Induced Gene Expression in Arabidopsis. Plant Physiol. 139, 1268–1283.
Telwala, Y., Brook, B.W., Manish, K., Pandit, M.K., 2013. Climate-induced elevational range shifts and increase in plant species richness in a Himalayan biodiversity epicentre. PLoS One 8, e57103.
Trebst, A., 2003. Function of β-carotene and tocopherol in photosystem II. Z. Naturforsch. 58c, 609–620.
Trucchi, E., Frajman, B., Haverkamp, T.H.A., Schönswetter, P., Paun, O., 2016. Genomic and metagenomic analyses reveal parallel ecological divergence in Heliosperma pusillum (Caryophyllaceae). bioRxiv 044354. doi:http://dx.doi.org/10.1101/044354 Preprint from http://biorxiv.org/content/early/2016/03/17/044354, (accessed 27.04.16).
Tyystjärvi, E., 2013. Photoinhibition of photosystem II. In: Jeon, K.W. (Ed.), International Review of Cell and Molecular Biology, vol. 300. Academic Press, Elsevier USA, pp. 243–303.
Vile, D., Pervent, M., Belluau, M., Vasseur, F., Bresson, J., Muller, B., Granier, C., Simonneau, T., 2012. Arabidopsis growth under prolonged high temperature and water deficit: independent or interactive effects? Plant Cell Environ. 35, 702–718.

Volkov, R.A., Panchuk, I.I., Mullineaux, P.M., Schöffl, F., 2006. Heat stress-induced H2O2 is required for effective expression of heat shock genes in Arabidopsis. Plant Mol. Biol. 61, 733–746.

Wahid, A., Gelani, S., Ashraf, M., Foolad, M., 2007. Heat tolerance in plants: an overview. Environ. Exp. Bot. 61, 199–223. doi:http://dx.doi.org/10.1016/j.envexpbot.2007.05.011.

Weis, E., 1982. Influence of light on the heat sensitivity of the photosynthetic apparatus in isolated spinach chloroplasts. Plant Physiol. 70, 1530–1534.

Willits, D.H., Peet, M.M., 2001. Measurement of chlorophyll fluorescence as a heat stress indicator in tomato: laboratory and greenhouse comparisons. J. Am. Soc. Hortic. Sci. 126, 188–194.

Woo, H.R., Kim, H.J., Nam, H.G., Lim, P.O., 2013. Plant leaf senescence and death – regulation by multiple layers of control and implications for aging in general. J. Cell Sci. 126, 4823–4833. doi:http://dx.doi.org/10.1242/jcs.109116.

Yamauchi, Y., Furutera, A., Seki, K., Toyoda, Y., Tanaka, K., Sugimoto, Y., 2008. Malondialdehyde generated from peroxidized linolenic acid causes protein modification in heat-stressed plants. Plant Physiol. Biochem. 46, 786–793.

ZAMG, 2014. Annual Climate Report from Zentralanstalt für Meteorologie und Geodynamik. ZAMG, Vienna, Austria https://www.zamg.ac.at/cms/en/climate/climate-overview/current_climate/annual_climate/wetterrueckblick?monat=GJ&jahr=2014, (accessed 25.04.16).

ZAMG, 2015. Annual Climate Report from Zentralanstalt für Meteorologie Und Geodynamik. ZAMG, Vienna, Austria https://www.zamg.ac.at/cms/de/klima/news/sommer-2015-neue-rekorde-bei-temperatur-trockenheit-und-sonnenscheindauer, (accessed 25.04.16).