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Original article

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Abstract

High temperatures during the crop reproductive stage impact seed yield and quality. The changing climate will require consideration of the effects of high temperature events that differ from their intensity, their duration and their frequency over the seed quality-building stages. The impact of these features deserve to be investigated at the light of induced thermo-sensitization which can lead to alleviate expected negative impacts. In our work, maturing seeds of the sulphur-demanding crop, oilseed rape, were exposed to several temperature sequences that varied in intensity, duration and frequency at the onset of seed maturation. Results-measured in seeds that were at the onset of maturation when the temperature stress occurred-indicated that (i) the longer the cumulated duration of the temperature stress, the more negatively impacted the quality criteria with decreased fatty acids (FAs) concentration, increased ω6: ω3 ratio, lower seed membrane integrity and increased seed dormancy and (ii) a mild stress event prior to heat peaks had an alleviating effect on the negative impact of the later heat peaks (priming effect) on seed nitrogen, desiccation tolerance and the phytohormones involved in thermoinhibition. sulphur restriction was positive on FAs, proteins concentrations and negative on breaking dormancy. In addition, sulphur supply interfered with temperature modality, features such that positive impact of sulphur limitation on boosting oxidative response were cancelled with intense late heat peaks. This work provides insights to define thermopriming protocols in relation to the timing of quality building processes, their respective optimal temperature and adequate sulphur supply.

Key words: oilseed rape, seed quality, high temperature, sulphur, repeated stresses, priming, stress memory, thermotolerance.

Running head: Thermotolerance for seed quality in oilseed rape

Main abbreviations: S: sulphur; C: carbon; N: nitrogen; FA: fatty acid; UFA: unsaturated FA; SFA: saturated FA; ABA: abscisic acid; GA3: gibberellic acid; IAA: indole-3-acetic acid; SA: salicylic acid;

SSP: seed storage protein; ROS: reactive oxygen species; TSW: thousand seed weight; DW: dry weight.

Introduction

Evidence for increased frequency of stress events such as heat waves has been observed over recent decades. Based on the last Intergovernmental Panel on Climate Change (IPCC) report (Hoegh-Guldberg et al., 2018), heat waves are expected to become more frequent, to last longer and to increase in intensity during the reproductive phase of economically important crops (Christidis et al., 2015; Trnka et al., 2014). These new climatic patterns have led to attempts to decipher crop behavior and final performance in the light of recurring stresses. While the effects of extreme/mild environmental stresses have been widely investigated from molecular to whole plant levels (Kotak et al., 2007; Ohama et al., 2016; Wahid et al., 2007), far fewer studies have tackled the issue of understanding the effects of their recurrence throughout the crop season. Indeed, the overall magnitude of the plant response to successive stresses might not match the effects induced by individual stressing events because (i) the first stress triggers physiological and metabolic adjustments that bring the plant to a modified status (including phenology) when the stress recurs, which leads to different responses to the second stress and (ii) a mild stress prior to further similar stresses can induce stress memory that lasts the duration of the crop season (i.e. intra-generational memory, Ding, Fromm & Avramova 2012) and is sometimes transmitted to offspring (i.e. transgenerational memory, Molinier, Ries, Zipfel & Hohn 2006; *Wang et al.* 2016; Hatzig, Nuppenau, Snowdon & Schießl 2018; Kinoshita & Seki 2014; Crisp, Ganguly, Eichten, Borevitz & Pogson 2016; Kumar 2018). Stress memory is defined as the process of storage and retrieval of information acquired during an initial exposure to stress (Crisp et al., 2016; "Stress priming, memory, and signalling in plants," 2019). This information acts as a priming process with beneficial effects when the stress recurs and it can lead to earlier, more rapid, intense, and sensitive responses that help plants to acclimate in changing environments (Kinoshita and Seki, 2014). Underlying mechanisms include epigenetic regulation, transcriptional priming, primed conformation of proteins, and/or specific hormonal or metabolic signatures. (Groot et al., 2016; Hatzig et al., 2018; Molinier et al., 2006; Wang et al., 2016)This means that as the climate changes, the effects of repeated

stresses should be harnessed as a crop improvement strategy because this approach has promise for inducing acclimation to heat stress (Wang and Liiang, 2017).

In winter oleaginous crops, the reproductive phase occurs during spring, which might expose flowering, grain filling and grain maturation to high temperature events. Due to its indeterminate growth, winter oilseed rape plants display flowers and growing pods in different proportions throughout the reproductive phase. Consequently, heat stress can impact reproductive organs by limiting number and size, which in turn leads modification to carbon (C) partitioning in favor of already developed organs that have passed the sensitive stage, and this makes analysis of the direct effects of heat stress on the organs more complex (Guilioni et al., 1997). While heat stress at flowering limits pollination (Sage et al., 2015) and/or induces early pod abortion resulting in yield losses in oilseed rape (Morrison and Stewart, 2002; Young et al., 2004), heat stress that occurs during seed filling and maturation affects seed storage compounds quantitatively and qualitatively, leading to seed quality alteration. Few studies have reported the effects on seed quality in oilseed rape of repeated heat stress events that might be erratic and fluctuate as predicted in climate change models in field conditions (Deng & Scarth 1998; Baux *et al.* 2013).

Seed quality encompasses a range of criteria related to nutritional and physiological characteristics (i.e. germination behaviors and storage capacity). In winter oilseed rape, oil content, fatty acid (FA) profiles, and protein content are major nutritional criteria for edible oil and cakes used in human and animal consumption, respectively. Its oil contains higher unsaturated FA (UFA) content than other oil crops (sunflower, soybean), which makes it a healthy edible oil for human consumption (Aguirrezábal et al., 2015). Contrasting variations have been observed in oil content according to the temperature intensity, to the timing of stress exposure and to the pools of seeds analyzed (main stem vs. bulk) (e.g. increased in Brunel-Muguet *et al.* (2015); decreased in Canvin (1965); Aksouh *et al.* (2001); Aksouh-Harradj *et al.* (2006)). High temperatures are known to induce decreases in poly-UFAs in favor of saturated FAs (SFA, mainly C16:0 and C18:0) and mono-UFAs, and increases in the ω6:ω3 ratio (i.e. C18:2/C18:3 ratio), as a result of temperature-triggered impairment of desaturase enzyme activity i.e. oleic and linoleic desaturases (Aksouh-Harradj et al., 2006; Baux et al., 2013, 2008; Brunel-Muguet et

al., 2015; Gauthier et al., 2017; Schulte et al., 2013). By contrast, seed nitrogen (N) and protein concentrations in the oil-free meal is usually negatively correlated with total oil content (Aksouh-Harradj et al., 2006; Aksouh et al., 2001) as observed in other oil crops (in soybean, N concentration Chebrolu *et al.* (2016); protein concentration, Dornbos & Mullen (1992)). Additionally, other seed characteristics related to physiological quality, i.e. seed storage capacity and germination behavior, were investigated but not to any great extent (Brunel-Muguet et al., 2015). A drastic degradation of seed storage capacity has been observed using seed conductivity and the ratio of soluble sugars ([stachyose and raffinose]:sucrose), abscisic acid (ABA) and gibberellic acid (GA3) as proxies (Bailly et al., 2001; Brunel-Muguet et al., 2015) in seeds from long-term heat-stressed mother plants. Other phytohormones were shown to be involved in the control of secondary dormancy, defined as failure in the germination process of mature and non-dormant seeds under adverse conditions (Pekrun et al., 1997). Recent studies have highlighted the role of indole-3-acetic acid (IAA), whose concentrations increase in dormancy-induced seeds (Liu et al., 2019; Shu et al., 2016; Tuan et al., 2019). Although several studies have reported that salicylic acid (SA) enhanced germination in *Arabidopsis* seeds by reducing oxidative damage (Chitnis et al., 2014; Lee and Park, 2010) and inhibited germination because of higher oxidative stress (Xie et al., 2007).

In Brassica species, sulphur (S) nutrition determines yield components and seed quality because of their high S requirements throughout the crop cycle (Brunel-Muguet et al., 2015; D'Hooghe et al., 2014). In addition to its well-known implication in the synthesis and signaling of stress tolerance-controlling phytohormones (Hasanuzzaman et al., 2018), S might be involved in the acquisition of thermotolerance mediated by epigenetic regulation (Bokszczanin et al., 2013). This is based on evidence for S involvement in DNA methylation (through the role of S-adenosylmethionine as a donor of methyl groups, Meng *et al.* 2018), one of the key epigenetic markers that supports stress memory, thus making the analysis of S supply relevant in the context of epigenetic memory.

In our study we focused on the effects of high spring temperatures on seeds at the onset of maturation to deepen our knowledge of this seed quality-determining stage in relation to S nutrition. Our assumptions were that the effects of high temperature at advanced seed filling can greatly vary

depending on whether plants are exposed to a mild heat stress event that primes them to withstand later heat peaks and that S nutrition might impact the ability of the plants to endure heat stress. Overall, our experimental design addresses the following questions: (i) what are the quantitative effects of different high temperature sequences applied at advanced seed filling/onset of seed maturation on seed yield, quality criteria and stress response indicators? (ii) is there any beneficial effect from a mild stress event that occurs prior to later repeated intense heat peaks? (iii) to what extent do the effects of successive high temperature events applied to maturing pods differ from the effect of individual events? (iv) what are the underlying defense pathways triggered by temperature stress? and (v) how does sulphur nutrition impact heat stress responses through acquisition of thermotolerance?

Materials and Methods

Experimental treatments and growth conditions

Seeds of *Brassica napus* L. (cv. Aviso) were germinated in vermiculite in October 2016 under greenhouse conditions. After five weeks the seedlings were transplanted into pots containing perlite and vermiculite (2:1, v/v) for seven weeks and seedlings were grown as described in Poisson *et al.* (2019). Afterwards, seedlings were subjected to a 12-week period of vernalization in a cold chamber (standard model, Froid & Mesures, Beaucouzé, France) maintained at 4°C (night) and 8°C (day) with artificial light during the day (10h day/14h night) and supplied with a 25% Hoagland solution without sulphur, to prevent the plants from building substantial S reserves which would later impact their nutritional status when applying the contrasting sulphur supplies.

Then, the plants were transferred into the greenhouse (Caen, France, 49°11′09 N, 0°21′32 W) and subjected to a thermoperiod of 20°C (day) and 15°C (night) without additional light. The plants were manually provided with two N applications with an NH_4NO_3 solution: 100 kg N/ha at the end of vernalization (Growing Stage 30 (GS30), stem elongation, Lancashire *et al.* 1991) and 50 kg N/ha at early flowering (GS60, bud formation) assuming a plant density of 40 plants.m^{-2}. The two contrasting S supplies i.e. High Sulphur and Low Sulphur were manually applied at the end of vernalization

(GS30) as usually provided in the field. Plants were supplied with a solution of $MgSO_4$ containing 75 kg SO_3/ha (High Sulphur) and 25 kg SO_3/ha (Low Sulphur), which represent, respectively, 100% and 33% of the conventional supply.

Four temperature modalities (Temp-modalities) were applied to plants at stage GS72 (i.e. 20% of the pods having reached their maximum size) for 17 days. The Temp-modalities were designed to assess the effects of a mild temperature event prior to a more intense temperature event including daily heat peaks (Figure 1). In our complete design, we tested five Temp-modalities which included a Temp-control modality that is natural thermoperiod conditions in the greenhouse (Figure 1a). The other four Temp-modalities were the following: (i) the early mild stress modality (Figure 1b) composed of 5 early full days under mild warming [i.e. 25.3°C ±1.8 (day, 16h) /21.7°C ± 0.7 (night, 8h)] and followed by 12 days under natural thermoperiod (mean, maximum and minimum temperatures over the natural thermoperiod being 15.7°C ±2.7, 28.4°C and 11.5°C, respectively), (ii) the 3 late heat peaks modality composed of 14 days under natural thermoperiod followed by 3 days under mild warming with a daily heat peak applied for 5 hours (i.e. 31.4°C ±1.7 between 11 am till 4 pm, Figure 1c), (iii) the 4 late heat peaks modality composed of 10 days under natural thermoperiod followed by 7 days under mild warming with daily heat peaks on days 12, 15, 16 and 17 (Figure 1d), and (iv) the priming modality composed of 5 days under mild warming followed by 5 days under natural thermoperiod and eventually 7 days under mild warming with daily heat peaks on days 12, 15, 16 and 17 (Figure 1e).

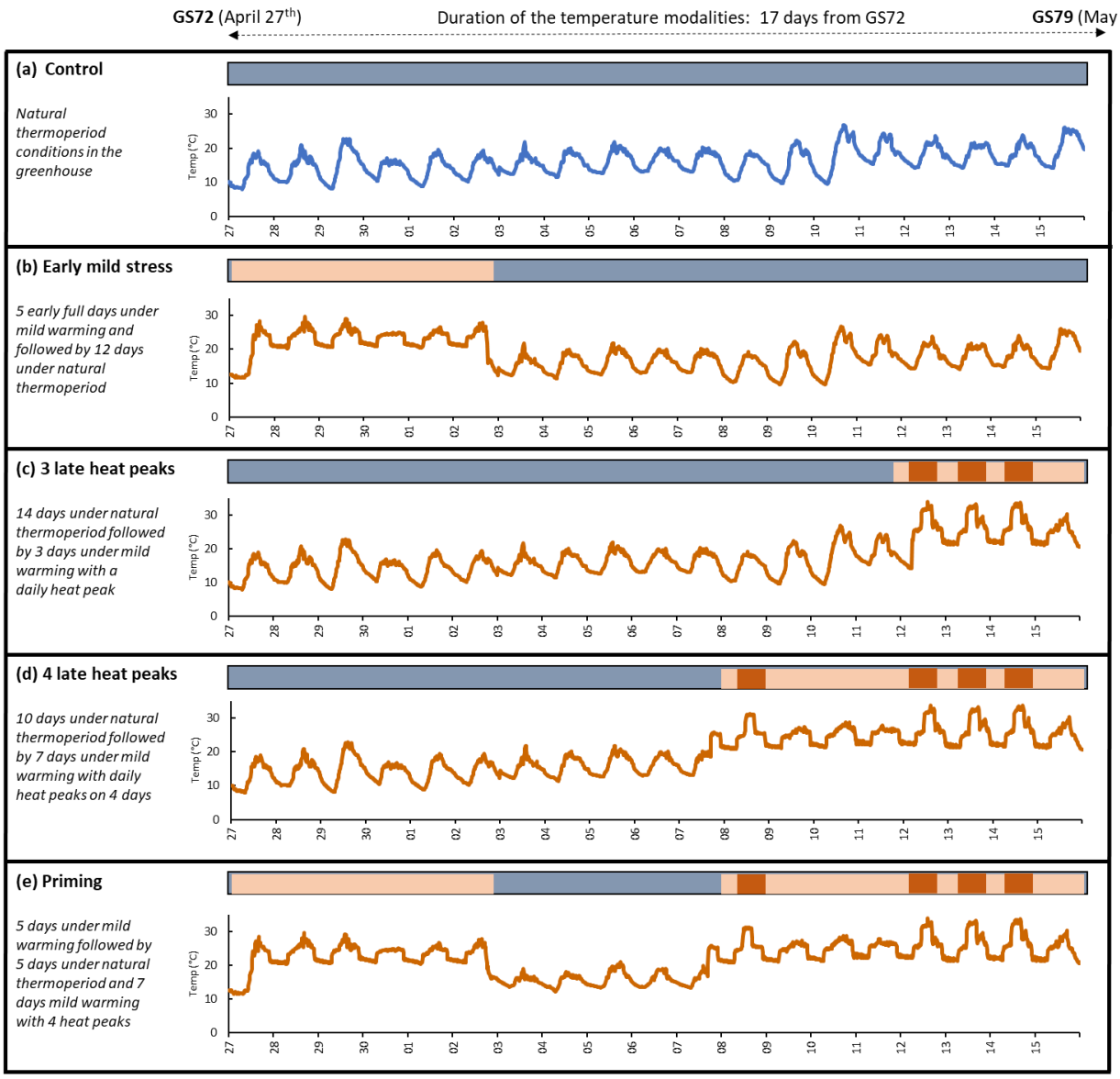


Figure 1: Schematic of the experimental design and recordings of hourly temperatures in the greenhouse for each temperature modality (Temp-modality). Four different temperature protocols and the Temp-control were applied under High Sulphur and Low Sulphur conditions (10 treatments in total). Graphics are organized by level of sequence complexity: (a) control modality, (b) early mild stress modality, (c) 3 late heat peaks, (d) 4 late heat peaks and (e) priming modality.

For each temperature modality and sulphur condition, four replicates were grown (i.e. 40 plants in total) in the greenhouse under natural thermoperiod and transferred when required in a separate greenhouse unit, where the higher temperature conditions (mild warming and heat peaks) were applied

and monitored, according to the experimental design (Figure 1). The temperature intensities were chosen according to previous studies that used similar ranges to mimic mild and intense temperature treatments under controlled conditions (Aksouh-Harradj et al., 2006). Temperatures were recorded hourly with temperature probes (105T Campbell, Campbell Scientific Ltd., Leicestershire, UK). Throughout the 17 days, the incident Photosynthetically Active Radiation (PARi) values was recorded (every 15 minutes) in the greenhouse and processed to calculate the daily PARi values (Supplemental Data, Table S1). No substantial difference in daily PARi were observed between the greenhouse units with the natural thermoperiod conditions and the higher temperature conditions (mild warming and heat peaks).

Because mixed-age pods were present throughout the temperature sequences, on the day before the beginning of the temperature modalities exposure we have labeled each branch of the plants to identify the two categories of pods: (i) pods whose length was above 5 cm ($pods_{L\geq5cm}$) and (ii) pods whose length was shorter than 5 cm ($pods_{L<5cm}$). Indeed, preliminary experiments (Supplemental Data, Table S2) indicated that $pods_{L\geq5cm}$ (or longer) contain seeds that have reached at least half their final fresh weight (about 55%), which coincides with advanced seed-filling development and the onset of seed maturation (Borisjuk et al., 2013). When the pods started desiccating, they were carefully and individually wrapped with plastic pouches to avoid seed dispersal and the mixing of seeds between the pod categories.

Seed yield and components

At maturity, the seeds from the two categories of pods were weighed after freeze-drying for dry weight (DW) measurements. To determine the individual seed weight (Thousand Seed Weight (TSW)), we weighed and photographed seeds from both pod categories so as to score their number using image analysis algorithms (ImageJ Software, Schindelin et al. 2012).

Biochemical characteristics of seeds from $pods_{L\geq5cm}$

In the following sections, biochemical characteristics of each individual plant (n=4, and n=3 only for hormones) were measured solely on seeds from $pods_{L\geq 5cm}$.

Seed carbon, nitrogen and sulphur concentrations

Seeds of each individual plant were pooled and dried for 48h at 50°C. The dried seeds were ground manually and the resulting powder (around 3 mg per sample) was placed into tin capsules for analysis. The percentage of total carbon, nitrogen, and sulphur per mg DW of the seeds were determined with a C/N/S analyzer (EA3000, Euro Vector, Milan, Italy) linked to a continuous flow isotope mass spectrometer (IRMS, Isoprime, GV Instrument, Manchester, UK).

Seed fatty acid concentration

Oil and fatty acid profile contents (C16:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1 and C22:1) were determined as described in Marchand *et al.* (2016). Approximately 200 mg of seeds from each plant were suspended in 1 mL of methanol/toluene/H_2SO_4 solution (100:20:2.5; v/v) containing C17:0 as internal standard (5 µg mL^{-1}), overnight at 85◦C for transmethylation. After cooling, 500 µL of hexane was added and the hexane phase containing the resulting fatty acid methyl esters (FAMES) was recovered for gas chromatography analysis combined with flame ionization detection (GC-FID). The FAMES (1 µL) were injected into an Agilent 7890 gas chromatograph equipped with a Carbowax column (15m by 0.53mm, 1.2 m) (Alltech Associates, Deerfield, IL) and FID system. FAMES were identified by comparing their retention times with those of commercial standards (Sigma, St. Louis, MO) and quantified using ChemStation (Agilent) to calculate the peak areas.

Seed storage protein concentration

Protein analysis was performed in two steps: (i) 20 g of ground seeds from plants of each treatment was previously stored at -20ºC before protein extraction and (ii) the Bradford assay. The Bradford assay is based on the use of a standard range with several dilutions of the reference protein (Bovine Serum Albumin, BSA). Thus, after dilution (by 10) the samples were placed in a microplate and the

measure is made at 570 nm after addition of the Bradford reagent. The calibration straight line allowed the total protein concentrations to be calculated by considering the mass and volume of the sample. The detailed steps for extraction and the Bradford assays are described for leaves in Akmouche *et al.* (2019).

Seed soluble sugar concentrations

Soluble sugars were extracted from 50 mg lyophilized and ground seeds, with 1 mL methanol/water (80:20, v/v) and 40 µL melicitose used as the internal sugar standard. Glucose, fructose, sucrose, raffinose, and stachyose contents were quantified using High Performance Liquid Chromatography (HPLC) on a cation exchange column (Sugar-PAK, 300 X 6.5 mm, Millipore Waters, Milford, MA, USA) eluted at 0.5 mL min^{-1} and 85 °C with 0.1 mM Ca-EDTA in water and quantified using a refractive index detector (2410 Differential Refractometer, Millipore Waters, Waters Corporation, MA, USA), according to Brunel-Muguet *et al.* (2015). The [raffinose+stachyose]:sucrose ratio was used as an indicator of seed drying tolerance i.e. the higher the value, the more tolerant the seed is to desiccation (Bailly et al., 2001).

Analysis of stress signaling and seed dormancy-related phytohormones

For each treatment, 50 mg of ground seeds were used to quantify 2-*cis*, 4-*trans*-abscisic acid, gibberellic acid, indole-3-acetic acid and salicylic acid. Previously freeze-dried samples were mixed with 500 µL of extraction solvent [2-propanol/H_2O/ concentrated HCl (2:1:0.002, v/v/v)] and then analyzed by HPLC-MS as described in (Pan et al., 2010). Because the dynamics of ABA and GA3 controls the balance between dormancy and germination, the ABA:GA3 ratio was used as a proxy for seed dormancy under stress condition (Debeaujon and Koornneef, 2000; Finkelstein, 2013).

Seed conductivity measurements

Conductivity measurements were performed according to Brunel-Muguet *et al.* (2015). For each treatment, measurements with the electrolytes were made with 20 pre-weighed seeds. At room

temperature (20ºC), the seeds were placed in 5 mL of ultrapure water for 16 hours. Conductivity was measured with a portable electrochemical analyzer (Consort C931, UK).

Determination of seed total phenolic content and antioxidant capacity

Prior to the extraction, seeds from plants of each treatment were oven-dried at 60ºC for at least 48 hours and then ground. The extraction procedure was adapted from Szydłowska-Czerniak, Amarowicz & Szłyk (2010) in terms of solvent, extraction time and sample:solvent ratio. Seeds were extracted with methanol:H_2O (50:50, v/v) at a ratio of 20:1 (mg/mL) by using mechanical stirring for 1 h at 25 ºC. Total phenolic content was spectrophotometrically determined with a modified Folin-Ciocalteu method (González-Centeno et al., 2015) and was expressed as the mean of six determinations of sinapic acid in mg equivalents per DW g of seeds. To measure the antioxidant capacity, seed extracts were diluted at a ratio 1/4 with methanol:H_2O (50:50, v/v) according to the methodology previously described by González-Centeno *et al.* (2012). ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] assay values were expressed as mg of Trolox equivalent antioxidant capacity per g of seeds DW.

Statistical analyses

Two-way ANOVAs to test temperature (Temp) stress and S nutrition effects, both considered as independent factors, were performed on the measured variables. The Temp-modalities, sulphur condition and Temp x Sulphur interaction effects were analyzed using R software (version 4.0.2). Prior to the ANOVAs, residues independency and normality, and homogeneity of variances were previously tested (Dubin-Watson, Shapiro-Wilk, Bartlett and Levene tests respectively). ANOVAs tables presented the means for each Temp-modality (gathering the values of both sulphur supplies) and for each sulphur supplies (gathering the values of the five Temp-conditions including the Temp-control) (Tables 1, 2 and 3). Mean comparison tests were performed differently according to whether interaction effects were detected or not. When no interaction effects were observed, Tukey tests were performed amongst the five temperature modalities (Tables 1, 2 and 3). Otherwise, when interaction

effects were observed, comparison amongst the 10 treatments (crossing the five temperature modalities and the two sulphur conditions) were performed as one-way ANOVA and considering 10 independent treatments for the Tukey tests (Figures 2, 3 and 4). The last table in Supplemental data (Table S3) summarizes the mean ± se of the all measured variables for each of the 10 treatments in order to provide reference values in a given combination "sulphur supply/Temperature modality".

Results

Plant growth and yield components

Table 1 displays yield and yield components i.e. seed number and TSW for the overall seeds (Total) and the two categories of seeds collected separately (from $pods_{L\geq5cm}$ and $pods_{L<5cm}$). No significant Temp x Sulphur interaction effect was observed for any of the yield related-measured variables. The total seed yield (including seeds from both categories of pods) ranged from 8.2 to 9.8 g $plant^{-1}$, with no significant sulphur effect. Decreases in the total seed yield were observed for all the four Temp-modalities, being significant only in the priming modality (-16.3% relative to Temp-control) . These decreases were due mostly to the seed yield from $pods_{L<5cm,}$ (though not significant) since seed yield from $pods_{L\geq5cm}$ remained almost unchanged compared to the Temp-control plants. The total seed numbers were not impacted by the Temp-modality but a slight sulphur effect was observed ($p<0.05$), with Low Sulphur values being 5% lower than High Sulphur values on average. The TSWs from $pods_{L<5cm}$ ranged from 3.0 g (3 late heat peaks modality) to 3.4 g (Temp-control), whereas the minimum and maximum values from $pods_{L\geq5cm}$ were 3.2 g (priming modality) and 3.7 g (3 late heat peaks modality). Under both sulphur supplies, no Temp-modality effects on the TSW were observed in either pod category. These results confirmed that (i) seeds from $pods_{L\geq5cm}$ had reached the onset of seed maturation and (ii) seeds developing in $pods_{L<5cm}$ or seeds from fertilized flowers that developed after the temperature sequences all benefited from increased sink strength, due to young pod abortion or reductions in late flowering.

Nutritional seed quality criteria

Seed C, N and S concentrations

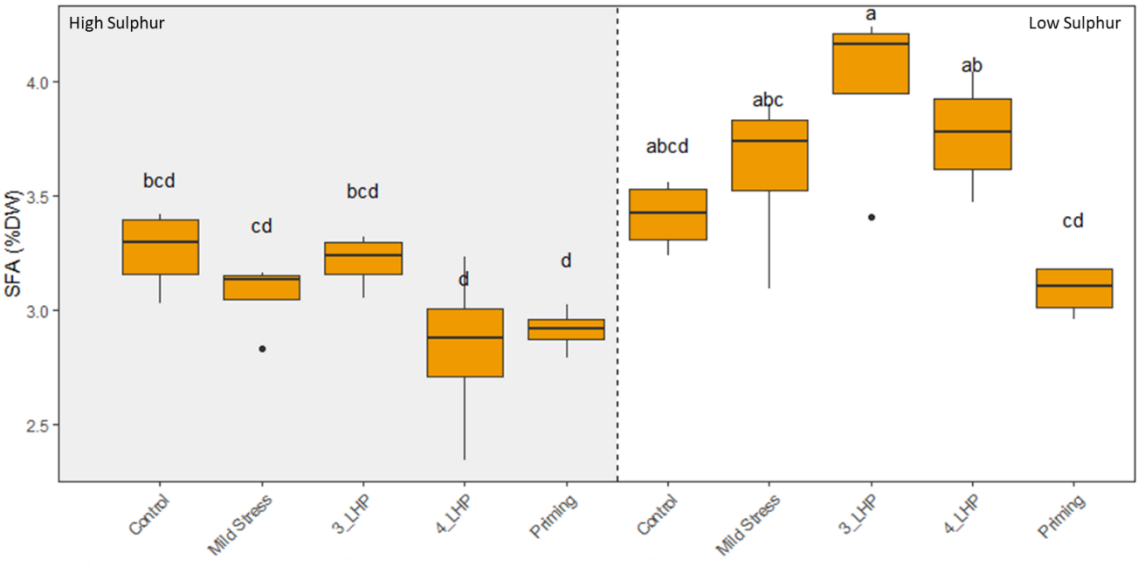
Table 2 displays C, N and S seed concentrations. Similar to yield components, no Temp x Sulphur interaction effect was observed. The seed C concentration was impacted by Temp-modalities ($p<0.001$) but no sulphur effect was observed. The priming modality displayed the lowest value (-5.4% from Temp-control) whereas the Temp-control displayed the highest value. Similar to C, the seed N concentration was only affected by Temp-modalities ($p<0.001$). Under both sulphur conditions, extreme N values were observed for the priming modality, which had the highest ranking (+21.9 % compared to Temp-control). These observations showed that the early mild stress had a positive effect over the late heat peaks, which suggested an alleviating effect. As expected, the seed S concentration was significantly impacted by the sulphur supply ($p<0.001$), with Low Sulphur values being 32% lower than High Sulphur values on average. While no significant differences were observed among the Temp-modalities, the S in the priming modality was slightly higher than in Temp-control (+19.9%).

Fatty Acids concentrations and profiles

Total fatty acids, saturated FAs and unsaturated FAs concentrations and the ω6:ω3 ratios are displayed in Table 2 and in Figure 2. For total FAs concentration, no Temp x Sulphur interaction effect was observed on FAs concentration. Effects of Temp-modalities were observed ($p<0.01$) with values ranging from 33% to 41% of DW for priming and 3 late heat peaks modalities respectively (Table 2). Values under Low Sulphur were significantly higher ($p<0.001$) on average (41 %DW) than values under High Sulphur (35 %DW). Temp-modality effect was observed in both SFAs and UFAs concentrations, with a significant effect of Temp x Sulphur interaction only observed on SFAs ($p<0.01$) (Table 2, Figure 2). The SFAs (including C16:0, C18:0, C20:0, C22:0) concentrations ranged from 3.0% to 3.6%DW and the UFAs (including C16:1, C18:1, C18:2, C18:3, C20:1) concentrations ranged from 30% to 38%DW. For both SFAs and UFAs concentrations, the priming modality and the 3 late heat peaks displayed the lowest and highest values respectively.

A highly significant sulphur effect was observed in both SFAs and UFAs concentrations, with values under Low Sulphur (3.6 %DW of SFAs and 38% DW of UFAs) being significantly higher on average than values under High Sulphur (3.1 %DW of SFAs and 32% DW of UFAs) ($p<0.001$) (Table 2, Figure 2). Overall, the priming modality had the greatest impact on decreasing FAs concentrations, as a result of the lowest decreases in SFAs and UFAs concentrations, and the early mild stress event did not alleviate the negative effects of later heat peaks on total FAs concentration. In addition, the 4 late heat peaks sequence had a greater impact than the 3 late heat peaks sequence on total FAs, SFAs and UFAs meaning the more intense events, the more impacting.

The ω6:ω3 ratio (i.e. C18:2/C18:3 ratio) is commonly used as an indicator of the edible quality of vegetable oils. This ratio was significantly impacted by the Temp-modalities, but no significant Sulphur nor Temp x Sulphur interaction effects were observed (Table 2). The highest and lowest ratios were observed on the priming and early mild stress modalities respectively (+9.3 and -8.8% compared to the Temp-control). In addition, the temperature effect on 3 late heat peaks modality was less negative than the early mild stress modality (Table 2). These results indicated that the number of desaturations decreased with greater duration of stress exposure (priming modality) and intensity and earliness of the heat stress event (4 late heat peaks modality) under both sulphur conditions.



Variable	Temp effect	Sulphur effect	Temp x Sulphur
SFAs	0.001 ***	0.000 **	0.009 **

Figure 2: Boxplot and ANOVA table for saturated FAs (SFAs) measured on seeds from pods $_{L \geq 5cm}$ under the 10 treatments (crossing temperature modalities and sulphur conditions). SFAs displayed highly significant interaction (Temp x Sulphur) effects. Letters indicate the ranking amongst the 10 treatments. P-values and levels of significance are given for Temp, Sulphur and Temp x Sulphur interaction effects. Levels of significance: ns non-significant, *p<0.05**, *p<0.01***, *p<0.001****. SFAs: saturated fatty acids; 3_LHP: 3 late heat peaks modality; 4_LHP: 4 late heat peaks modality.

Seed storage protein concentrations

The seed storage protein (SSP) concentration ranged from 123 mg/g DW to 168 mg/g DW, with values under High Sulphur condition being significantly lower on average (106 mg/g DW) than values under Low Sulphur condition (185 mg/g DW) (Table 2, *p<0.001*). In contrast, no significant effects of Temp-modalities on SSP were observed although the lowest values were observed under the late heat peaks modalities (Table 2). The increase under the early mild stress modality might result from reductions in the number of growing sinks due to pod abortion, reduced seed filling and/or impaired pollination.

Physiological seed quality-related criteria

The soluble sugar composition as an indicator of desiccation tolerance acquisition

Table 3 presents the concentration of the main soluble sugar in seeds i.e. sucrose, raffinose and stachyose. For the three main soluble sugars, only temperature effects were observed ($p<0.001$). All the Temp-modalities led to decreased soluble sugar concentrations under both ulphur conditions. Sucrose, raffinose and stachyose concentrations were the highest in the Temp-control and the lowest in the priming and early mild stress modalities. Under the priming modality, sucrose, raffinose and stachyose concentrations were respectively 54%, 55% and 52% lower than the Temp-control. Similarly, under the early mild stress modality these concentrations were respectively 59%, 55% and 56% lower than under the Temp-control. These results indicated that the early mild stress had a negative impact on the sugar soluble concentrations. The [raffinose+stachyose]:sucrose ratio, used as a proxy of seed desiccation tolerance, ranged from 0.35 (4 late heat peaks modality) to 0.46 (early mild stress modality) without significant Sulphur effect (Table 3). These results indicate that whatever the sulphur supply, the 4 late heat peaks modality was the most detrimental to acquisition of seed desiccation tolerance. However, these data also highlighted that the prior event of mild stress alleviated the negative effects of late heat peaks since the value under the priming modality was higher than under the early mild stress modality.

Seed conductivity as indicator of membrane permeability

Seed conductivity values ranged from 0.8 µS/mg DW (Temp-control) to 2.9 µS/mg DW (priming modality), with a highly significant temperature effect ($p<0.001$, Table 3). No effect of Sulphur nor Temp x Sulphur interaction effects were observed. Extreme values were observed in Temp-control (the lowest) and in priming modality (the highest, +262% compared to Temp-control). The other three Temp-modalities (early mild stress, 3 and 4 late heat peaks modalities) led to similar values than under the Temp-control. These results indicate that seed conductivity is highly responsive to the duration of the stressing period, because seeds from temperature modalities displaying the shortest numbers of days where temperature was higher than the control temperature, were the least negatively impacted.

Phytohormone changes as indicators of temperature-induced seed dormancy

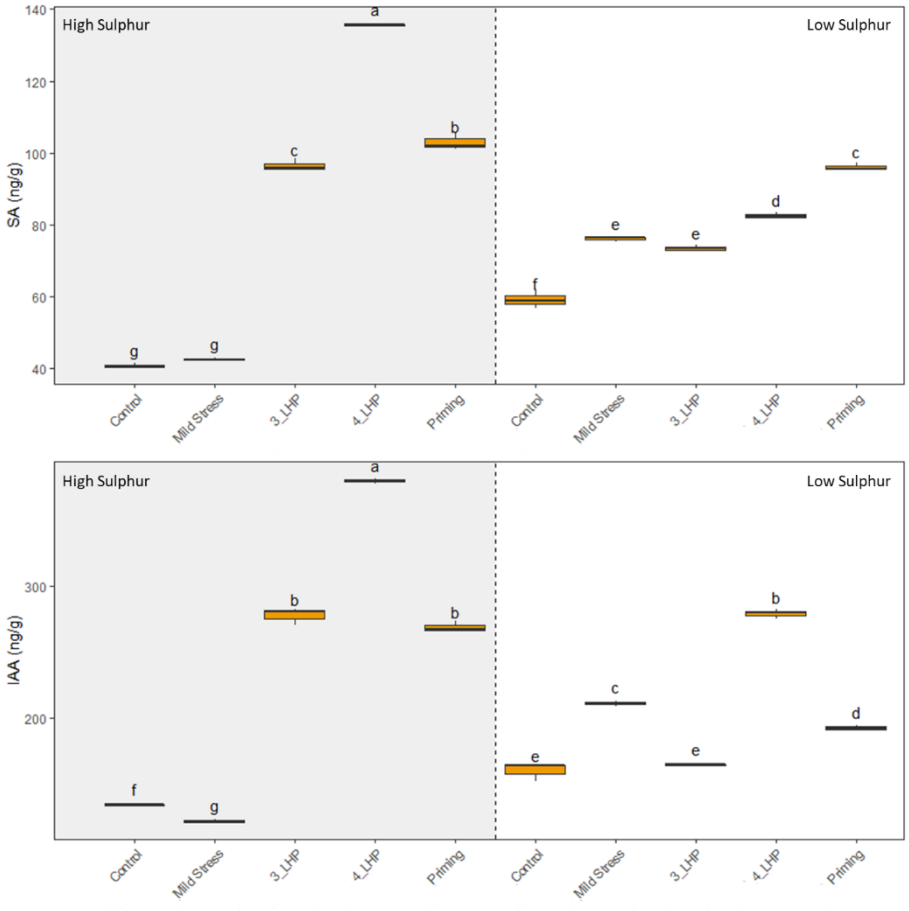
Figure 3 displays the results of the main hormones involved with dormancy in seeds (i.e. IAA, SA and ABA:GA3 ratio). Temp-modality effects, as well as Sulphur and Temp x Sulphur interaction effects were observed for the three variables with highly significance ($p<0.001$). The ABA:GA3 ratio ranged from 0.51 (early mild stress modality) to 0.70 (4 late heat peaks modality), with significant lower values in High Sulphur (0.47 on average) than in Low Sulphur condition (0.78 on average, that is 66% higher). The interaction effects pointed out that according to the sulphur condition, the Temp-modality effect was different i.e. while the early mild stress modality and the 3 late heat peaks modality under high sulphur were ranked the same, these Temp-modalities under Low Sulphur were highly contrasting (with values being much higher under the 3 late heat peaks modality) (Figure 3).

A high ABA:GA3 ratio indicates increased secondary seed dormancy, which is induced by thermoinhibition. As expected, under high temperature this ratio increased, but under High Sulphur condition, the modality that displayed the more days with temperature above the control temperature (priming modality) was the most negatively impacted (highest value), whereas under Low Sulphur condition, the late heat peaks events (4 or 3) induced the highest ratios. In addition, under Low Sulphur condition, the early mild stress tended to alleviate the negative effects of late heat peaks by lowering the ratio (lower values in priming and early mild stress modalities than in late heat peaks modalities).

Seed concentrations of IAA and SA were measured to investigate their variation under the different temperature sequences and sulphur supplies. Consistent with the ABA:GA3 ratio, high concentrations of IAA and SA were observed under high temperatures. IAA and SA ranged from 147 ng/g DW to 330 ng/g DW and 50 ng/g DW to 109 ng/g DW respectively, with Temp-control displaying the lowest values and 4 late heat peaks modality displaying the highest values for both measured variables (Figure 3). Both hormones concentrations were significantly lower in Low Sulphur than in High Sulphur condition, with High Sulphur values in IAA being 17% higher than Low Sulphur and values in SA being 9% higher than Low Sulphur. By contrast to the ABA:GA3 ratio, Sulphur limitation

decreased IAA concentrations except under Temp-control and under the early mild stress modalities with values being respectively 16% and 73% higher in Low Sulphur than in High Sulphur (Figure 3). Under High Sulphur condition, the early mild stress event prior to the 4 late heat peaks (priming modality) led to alleviate the strong increase in IAA concentration observed in the late heat peaks solely (4 late heat peaks modality), thus suggesting an effective priming effect. The interaction effects pointed out that according to the sulphur condition, the Temp-modality effect was different i.e. while the priming modality led to higher value than under the early mild stress modality under High Sulphur, it led to lower value than the early mild stress modality under Low Sulphur (Figure 3).

As observed for IAA concentrations, SA concentrations decreased under Low Sulphur except under the Temp-control and under the early mild stress modalities. Similar to IAA concentrations, the early mild stress event prior to the 4 late heat peaks (Priming modality) led to alleviate the strong increase in SA concentration observed under the 4 late heat peaks solely (4 late heat peaks modality), although this was only observed under the High Sulphur condition.



Variable	Temp effect	Sulphur effect	Temp x Sulphur
SA	0.000 ***	0.000 ***	0.000 ***
IAA	0.000 ***	0.000 ***	0.000 ***
ABA:GA3	0.000 ***	0.000 ***	0.000 ***

Figure 3: Boxplots and ANOVA table for seed physiological quality variables measured on seeds from pods $_{L\geq 5cm}$ under the 10 treatments (crossing temperature modalities and sulphur conditions). Hormones (SA, IAA) concentration and ABA:GA3 ratio displayed highly significant interaction

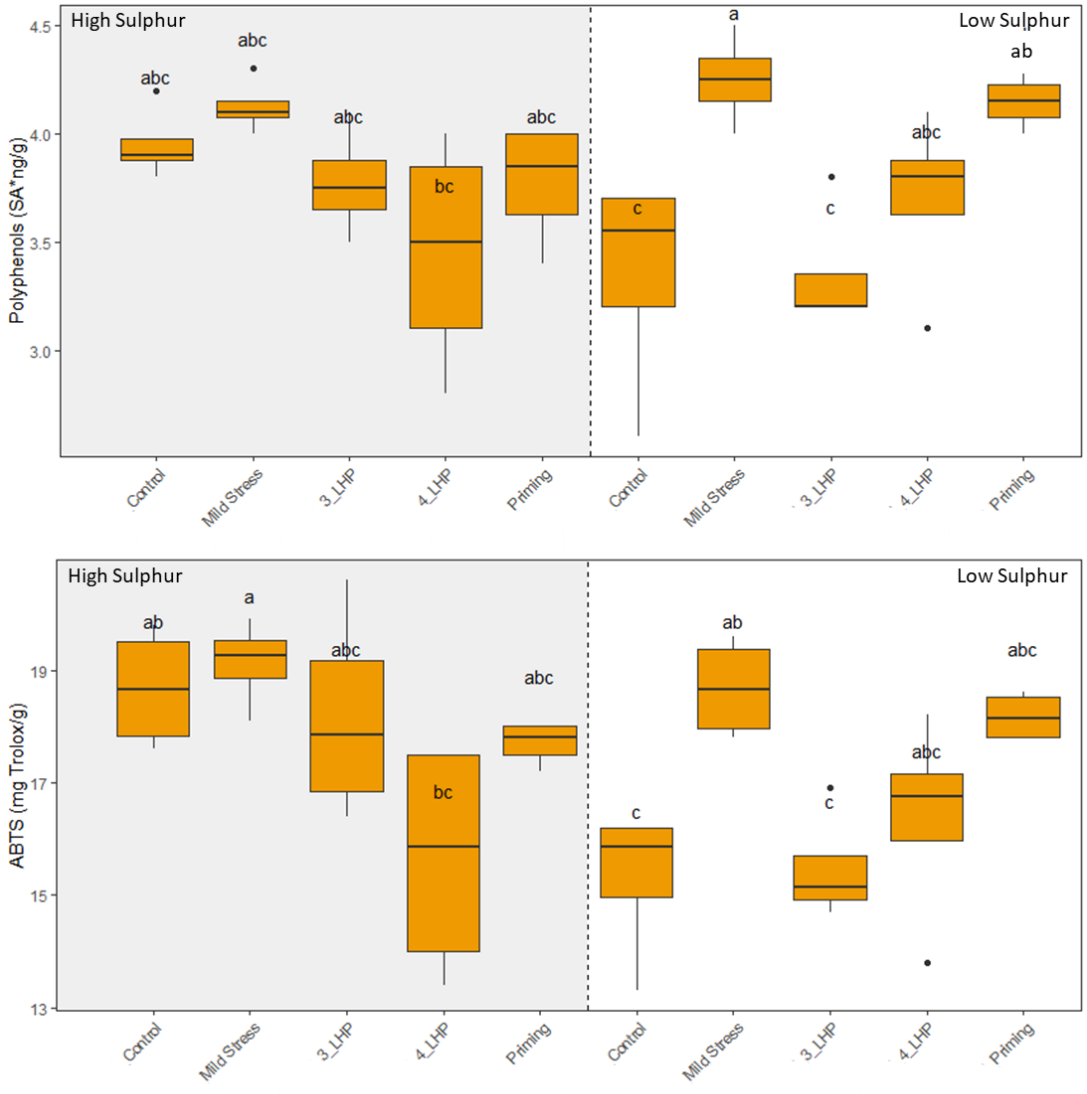
(Temp x Sulphur) effects. For each variable, letters indicate the ranking amongst the 10 treatments. P-values and levels of significance are given for Temp, Sulphur and Temp x Sulphur interaction effects. Levels of significance: ns non-significant, *p<0.05*, p<0.01**, p<0.001***. 3_LHP: 3 late heat peaks modality; 4_LHP: 4 late heat peaks modality; SA: salicylic acid; IAA: indole-3-acetic acid; ABA: abscisic acid; GA3: gibberellic acid.

Total phenolic content and antioxidant capacity in seeds as stress response indicators

Figure 4 displays phenolic concentrations and the antioxidant activity (ABST values) measured in mature seeds from $pods_{L\geq 5cm}$. Phenolic concentrations ranged from 3.6 to 4.2 mg SA/g seed DW with no significant effect of S supply. By contrast, significant Temp-modality (*p < 0.01*) and Temp x Sulphur interaction (*p< 0.05*) effects were observed (Figure 4). Under High Sulphur condition, extreme seed phenolic concentrations were observed under the early mild stress modality (+2% compared to Temp-control in High Sulphur) and under the 4 late heat peaks modality (-12% compared to Temp-control in High Sulphur) whereas under Low Sulphur, extreme values were observed under the early mild stress modality (+26% compared to Temp-control in Low Sulphur) and 3 late heat peaks modality (similar value to the Temp-control in Low Sulphur). These results illustrate the Temp x Sulphur interactions and the benefits of Sulphur restriction in specific temperature modalities to boost phenolic concentrations.

The antioxidant capacity measured with the ABTS assays revealed Temp-modality effects (*p<0.01*), Sulphur effects (*p<0.05*) and Temp x Sulphur interaction effects (*p<0.05*). Temp-modality rankings were similar to those observed for seed phenolic concentrations with extreme values being observed in the early mild stress modality (highest values) and late heat peaks modalities (3 or 4, lowest values) (Figure 4). Overall, under both Sulphur conditions, increased antioxidant capacities were observed in the early mild stress modality while decreased antioxidant capacities (or levels similar to the Temp-control) were observed when the stress occurred later, and despite the number of heat peaks. In addition, while lower oxidative response than the Temp-control were observed in the priming modality under High Sulphur conditions, a higher response was observed under Low Sulphur (Figure 4). The

results also indicated that the early mild stress event prior to the 4 late heat peaks (priming modality) were beneficial to the sharp decrease in ABTS values observed under the 4 late heat peaks alone, both Sulphur conditions. Regarding the effects of Sulphur conditions, limitation led to decreased antioxidant capacity in the seeds with mean values being 17.9 mg Trolox/g under High Sulphur and 16.8 mg Trolox/g under Low Sulphur (i.e. -6%) which suggest a crucial role for the Sulphur supply in favoring oxidative responses, not only under the Temp-control condition but also in the 3 late heat peaks modality (Figure 4). Eventually, the Temp x Sulphur interaction effects were observed because Sulphur conditions modified the ranking of the temperature modalities compared the Temp-control, i.e. under High Sulphur ABTS values were lower in the priming and 4 late heat peaks modalities than the Temp-control but higher under Low Sulphur. Taking into account the values of all the treatments, antioxidant capacities of the seeds were positively correlated to both phenolic concentration ($r=0.93$, $p<0.001$) and the [raffinose+stachyose]:sucrose ratio ($r=0.64$, $p<0.05$) (Supplemental Data, Figure S1).



Variable	Temp effect	Sulphur effect	Temp x Sulphur
Polyphenols	0.002 **	0.603	0,021 *
ABTS	0.002 **	0.016 *	0.010 *

Figure 4: Boxplots and ANOVA table for phenolic concentrations and ABTS values measured on seeds from pods$_{L\geq 5cm}$ under the 10 treatments (crossing temperature modalities and sulphur conditions). Both variables displayed highly significant interaction (Temp x Sulphur) effects. For each variable, letters indicate the ranking amongst the 10 treatments. P-values and levels of significance are given for Temp, Sulphur and Temp x Sulphur interaction effects. Levels of significance: ns non-significant, *p<0.05**, *p<0.01***, *p<0.001****. SA*: synaptic acid, ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

Discussion

Yield components were only impacted in reproductive plant parts developed after the stress

Yield components were not drastically impacted by the high temperature sequences applied at GS72 (Table 1). No effect of temperature was observed on yield of $pods_{L\geq5cm}$, meaning that these seeds experienced a normal filling period. Therefore, the effect of the heat sequences on total seed yield ($p<0.05$) was the consequence of a yield reduction in seeds from $pods_{L<5cm}$, which resulted from reduced seed number and individual seed weight, even though neither component reduction was significant. Indeed, abortions of young pods during the Temp-modality exposure and/or failure of pollination in the flowers present during this period might have occurred (Young et al., 2004). The reproductive adaptation might also have reduced the incidence of late flowering (specific to indeterminate species) to the benefit of filling seeds in already developed pods. These observations are consistent with prior studies in pea (another indeterminate growth species) that demonstrated heat-stress modification of seed distribution along the main stem, which led to larger quantities of seeds on the basal parts than on the upper parts where reproductive organs are younger. These results explained the maintenance of seed yield from basal reproductive parts due to a higher allocation rate of carbohydrates and excluded the direct effects of heat stress on developing seeds (Guilioni et al., 2003). The non-significant effect of sulphur on seed yield components, other than in the total seed number, indicates that the Low Sulphur supply in our study satisfied growth rate and yield requirements.

Priming effects on quality criteria are dependent on the timing of the stress events

Our initial objective was to pinpoint any beneficial effect from a mild stress event that occurs prior to repeated later intense heat peaks. This could be accounted for by a beneficial heat stress memory generated by induced thermo-sensitization. Our work highlights that the alleviating effects of the early mild stress event prior to later heat peaks are not observed for all the measured quality variables. Our results pointed out that these alleviating effects are likely determined by (i) whether the expected

priming event occurs concomitantly with biosynthesis of seed storage compounds, and (ii) whether the temperature is adequate to enhance the targeted process.

Figure 5 presented the Temp-modality effects on yield components and nutritional and physiological quality criteria measured in maturing seeds, for the Temp-modalities (early mild stress, 3 or 4 late heat peaks and priming modality) and the sulphur limitation. The priming modality significantly decreased seed C, total FAs, SFAs and UFAs concentrations under both Sulphur conditions. These observations suggest that the more the duration of the heat stress sequence (i.e. sequence that records the more days with temperature higher than the control temperature), the most detrimental for maintaining C concentration and hence FAs synthesis, since the priming modality (that cumulated the highest number of days with temperature above the Temp-control modality) induced a greater decrease than the early mild stress or the late heat peaks alone. These results correlate with earlier studies (Aksouh-Harradj et al., 2006; Aksouh et al., 2001) of intense temperature exposure during seed maturation, but contrast with others that highlighted increased total FAs concentrations under a long mild stressing event (Brunel-Muguet et al., 2015).

Disruption of FAs accumulation in oilseed rape was pointed out under intense heat stress across a range of temperatures similar to those imposed under our heat-peak conditions. This was shown to be the consequence of photosynthesis inhibition and downregulation of BnWRI1 gene expression, which is a key regulator of FA biosynthesis (Huang et al., 2019; Ruuska et al., 2002). Our results also revealed higher total FAs, saturated FAs and unsaturated FAs concentrations under Low Sulphur condition (Table 2, Figure 2), which contrasted with prior results (Brunel-Muguet et al., 2015). These differences can be explained in the current work by the lower Sulphur supply and the different approaches to seed sampling and analysis (on maturing seeds exposed to the heat stress modalities). The negative effects of high temperature – mainly of duration of high temperature sequence (Figure 5) - on total FAs and unsaturated FAs also indicated that (i) FA biosynthesis was concomitant with the 17-days high-temperature treatment starting approximately 3 weeks after the onset of flowering (Figure 1), with FAs levels rising during the late storage stage (i.e. 20 after pollination (Borisjuk et al., 2013; Niu et al., 2009)) and (ii) the later the heat peaks, the lower the effects. The increased ω6:ω3

ratio under the priming and 4 late heat peaks modalities is also detrimental to oil quality, and is known to result from impaired functioning of the oleic and linoleic desaturases (Aksouh-Harradj et al., 2006). As usually observed, the accumulation of lipids and proteins (herein linked to seed N concentration) are negatively correlated because they are competitive processes that have spatial and temporal overlaps (Borisjuk et al., 2013; Grami and Stefansson, 1977). Therefore, consistently, while the priming modality decreased the total FA concentration, the seed N concentration remained similar to Temp-control under both Sulphur conditions (Table 2). A decrease in seed storage protein concentrations under the late heat peaks modalities were observed, although not significant, suggesting that the high concentrations observed in the priming modality could be interpreted as a priming effect that helped to overcome further negative effects of the late heat peaks. This observation supports the hypothesis of compensatory effects between the pods that were maturing and the ones that were still developing during the 17-days temperature sequence. Indeed, seed N accumulation was likely to be impaired in filling seeds, and this resulted in N reallocation towards filled and maturing seeds. In contrast, while the seed storage protein concentrations were not significantly increased under high temperature, they increased under Low Sulphur condition as previously observed (Brunel-Muguet et al., 2015).

Ultimately, seed physiological characteristics were also highly dependent on the features of the high temperature sequences (duration, intensity, frequency), which shapes the dynamics of biosynthesis of storage compounds involved in seed dormancy and stress tolerance. Seed storability and desiccation tolerance can be estimated by two proxies i.e. membrane conductivity and the [raffinose+stachyose]:sucrose ratio. Our results highlighted strong negative effects of high temperature on seed conductivity, indicating degradation of membrane permeability (Table 3). However, the [raffinose+stachyose]:sucrose ratio remained unchanged in the priming and in the early mild stress modalities, which suggests acquisition of desiccation tolerance irrespective of Sulphur supply. The beneficial effects of the early mild stress on desiccation tolerance were maintained over the late heat peaks which points out its alleviating effect.

Several phytohormones control seed dormancy, as indicated by the ABA:GA3 ratio, which has been shown to vary according to stresses imposed on the parent plant during seed development (Brunel-Muguet et al., 2015). A high ABA:GA3 ratio indicates higher seed dormancy, which is expected for efficient seed storage before favorable environmental conditions permit the seeds to germinate. The priming modality and the late heat peaks modalities had the greatest impact on the ABA:GA3 ratio (Figures 3 and 5). Along with the non-significant effect of the early mild stress modality, these observations indicate that the intensity and timing of the stress exposure are determining on the ABA:GA3 ratio. In this example, the temperature of the mild stress was not deleterious on the ABA:GA3 ratio, or this early sequence occurs before the hormone syntheses, thus leading to no observed effects. These observations also pointed out that the temperature of the late heat peaks was deleterious on the ABA:GA3 ratio and that these heat peaks occurred when these hormones were synthetized. Therefore, our results raised questions not only about the compound-specific biosynthesis/maturation temperatures but also the synchronization between these compound specific-processes and the temperature event applied in the aim to induce an effective thermo-sensitization effects.

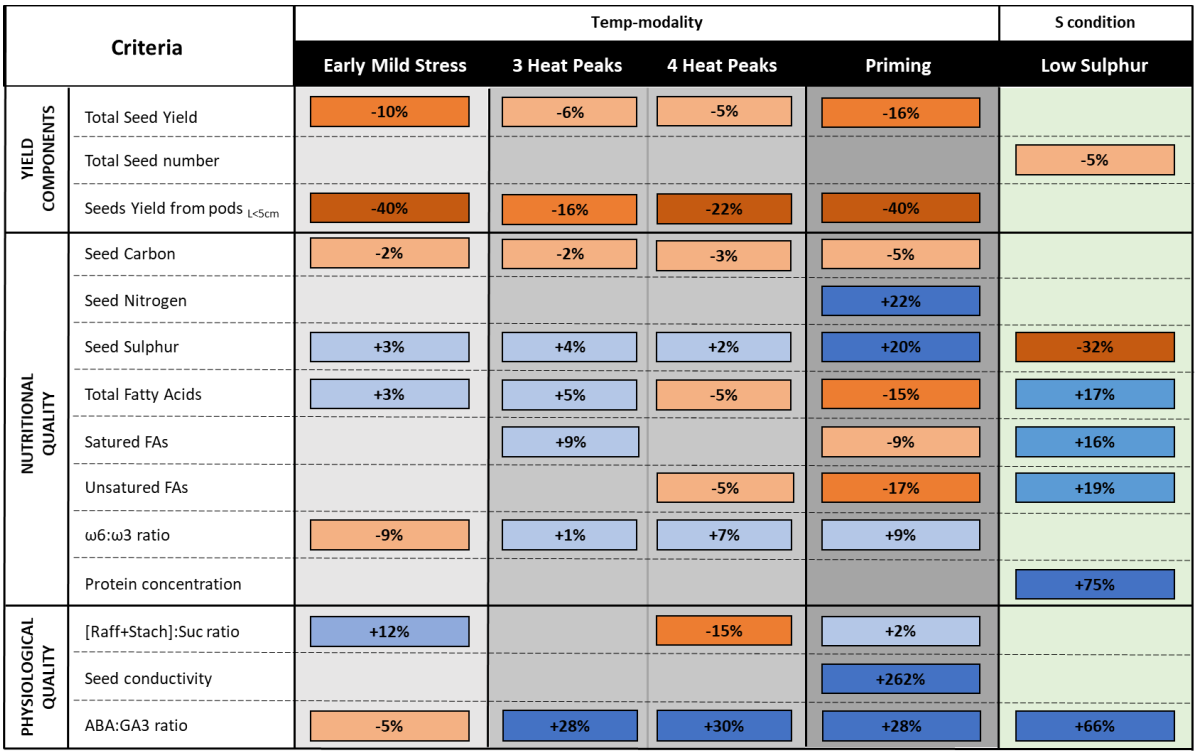


Figure 5: Summary of the effects of the four temperature sequences (early mild stress, 3 and 4 late heat peaks and priming modality) and the Sulphur conditions on yield components, nutritional and physiological quality criteria. Indicators of quality were measured on seeds from pods$_{L\geq5cm}$ at the beginning of stress exposure. The effects are given in reference to the ANOVAs in Tables 1, 2, 3 and Figures 2 and 3. As illustrated in the legend, colors indicate the trends by level of increase or decrease to the Temp-control modality. Numbers in boxes display the relative difference between the Temp-modality and the Temp-control (for temperature modalities) and between Low Sulphur and High Sulphur (for Sulphur conditions). FAs: fatty acids, Raff: raffinose, Stach: stachyose, Suc: sucrose, ABA: abscisic acid, GA3: gibberellic acid.

Modulation of antioxidant capacities and thermotolerance acquisition as stress defense strategy

Heat stress is known to trigger oxidative bursts that lead to a wide spectrum of responses including enzymatic and non-enzymatic components such as polyphenols, hormones and sugars, which have been shown to scavenge reactive oxygen species (ROS) (Nishizawa et al., 2008; Serrano et al., 2019;

Soares et al., 2019; Soengas et al., 2018). In our study, measurements of the antioxidant capacity indicated complex results that differed according to the S supply (Figure 4). In the High Sulphur condition, the greatest increase were observed under the early mild stress modality and not under the priming nor the late heat peaks modalities, likely to phenolic concentrations. These observations contrasted with prior studies which indicated enhancement of subcellular antioxidant activities using other antioxidant systems as proxies (such as enzymes superoxide dismutase, glutathione reductase, and peroxidase in mitochondria and chloroplasts of wheat leaves) under multiple heat priming sequences prior to later high temperature events (Wang et al., 2014).

Our findings also pointed out that(Wang et al., 2014) the measured concentrations of raffinose and stachyose, which have been reported to protect against oxidative damage (Nishizawa et al., 2008), were not increased under early mild stress modality (irrespective of the Sulphur conditions), and so it was not possible to ascertain their role in the antioxidant defense pathways in our conditions (Table 3). But as discussed above, this soluble sugar-related results should be interpreted carefully regarding their timing and optimal biosynthesis within the maturing seeds (Baud et al., 2002; Leprince et al., 2017). Indeed, our results might suggest that the temperature events (whether mild or intense) were above the temperature threshold of the stepwise transfer reactions involving raffinose synthase and stachyose synthase, leading to impairment of sugar biosynthesis and thus the sugar-mediated oxidative response (Gangl et al., 2015).

Phytohormones such as IAA and SA have been demonstrated to interact with ROS during stress tolerance as stress-signaling cues (Balfagón et al., 2019; Bielach et al., 2017; Clarke et al., 2004; Prerostova et al., 2020; Sharma and Laxmi, 2016) and have also been linked to stress thermotolerance (Bokszczanin et al., 2013; Clarke et al., 2009; Shu et al., 2016; Tuan et al., 2019) because increases in the levels of these compounds under high temperature are associated with greater stress-induced dormancy and thermoinhibition (Toh et al., 2012). (Bokszczanin et al., 2013; Clarke et al., 2009)

In our study, we observed drastic increases in IAA and SA in mature seeds collected from pods exposed to the longest cumulated high temperature (priming modality) or late heat peaks at the onset of maturation under High Sulphur conditions and to a lesser extent under Low Sulphur conditions

(Figure 3). The 4 late heat peak modality induced the highest increase in IAA and SA, while the early mild stress modality remained similar to the control under both Sulphur conditions. These observations pointed out that the early mild stress event prior to 4 ate heat peaks allowed their negative effects on IAA and SA concentrations to be alleviated thus leading to lower thermoinhibition. Our results also highlighted that the late 3 heat peaks modality had less effect than the late 4 heat peaks on IAA and SA, which suggests that the shorter the period of intense stressing, the lower the impact on the seed hormones concentration. (Bokszczanin et al., 2013; Clarke et al., 2009)

Impact of S availability on thermotolerance acquisition

Firstly, the level of sulphur limitation used in our experiment was mild enough not to impact seed yield and components as only a slight negative effect on the total seed number was observed (Figure 5). The effects of sulphur restriction were observed on nutritional quality criteria i.e. FAs-related variables, seed storage proteins and physiological quality criteria i.e. ABA:GA3 ratio, which were all increased under sulphur limitation. Interactions effects were only observed for saturated FAs and the ABA:GA3 ratio. They highlighted that late heat peaks or early mild stress events led to increased unsaturated FAs concentrations under Low Sulphur but not under High Sulphur (Figure 2). The ABA:GA3 ratio was only increased under Low Sulphur with the late heat peaks (Figure 3).

The variables related to thermoinhibition (i.e. phytohormones IAA and SA) and oxidative responses were globally decreased by sulphur limitation (Figures 3 and 4). Nevertheless, the sense of variation or the extent of the decreases varied according to the temperature modality. Indeed, Temp x Sulphur interaction effects were observed for IAA, SA concentrations and the ABA:GA3 ratio, phenolic concentrations and ABTS values. Our results pinpointed increased antioxidant capacities with early mild stress event (observed for the priming modality and the mild stress modality) under Low Sulphur compared to the natural thermoperiod whereas similar or slightly lower values to the natural thermoperiod were observed with the early mild stress event under High Sulphur (Figure 4). For phytohormones IAA and SA, the temperature modalities globally increased the concentrations under the two Sulphur conditions although this trend was more pronounced under High Sulphur than under

Low Sulphur. Nevertheless, only under Low Sulphur, the early mild stress event alone trigger increased concentrations compared to Temp-control. Sulphur nutrition has been reported to improve antioxidant defenses due to S-containing antioxidant compounds such as the redox-active cysteine residues of glutathione and thioredoxin (Mukwevho et al., 2014) and also specific phytohormones (Bashir et al., 2015; Hasanuzzaman et al., 2018; Xia et al., 2015). IAA biosynthesis relies on the effectiveness of S metabolism via adequate levels of glutathione (Kopriva et al., 2016) and is improved when S is limiting (Nikiforova et al., 2003). SA is also known to interact with S during SA homeostasis regulation (Baek et al., 2010). When comparing both Sulphur conditions under the natural thermoperiod, our results are in line with these findings as a slight increase was observed under Low Sulphur. Our study also highlighted how the intensity, the timing and the duration of heat stress can restrict the boosting effects of sulphur restriction.

Conclusion

Our study analyzed the effects of different high temperature protocols applied at the onset of seed maturation on yield components, seed nutritional and physiological quality under two Sulphur supplies. The initial working hypothesis was to highlight inducing thermotolerance protocols based on stress memory acquisition. Our results pinpointed that the effects of duration, timing and intensity of the stressing events that designed the different temperature protocols were determining as they led to different impacts on the measured quality criteria. Our results showed that thermo-sensitization protocols must require: (i) the optimal temperature to promote a targeted process and (ii) the synchronization between the expected thermopriming event and the underlying biosynthesis and maturation processes specific to the targeted storage compound. Our experiments were also designed to observe whether sulphur nutrition (which is essential to Brassica species) interfered with expected thermo-sensitization effects. The level of sulphur limitation in our assay was mild enough to impact a few nutritional (fatty acids, seed storage protein concentration) and physiological (IAA, SA, ABA:GA3 ratio) quality criteria, as well as the antioxidant capacity. This raises the question of the direct role of sulphur in the cascade of events leading to the hormones biosynthesis and the indirect

role of sulphur in stress memory mediated by epigenetic regulation. In contrast, sulphur restriction optimized seed oil concentrations in stress-exposed maturing seeds, especially when the stress was delayed, thus highlighting the need to satisfy sulphur requirements in certain climatic contexts.

Overall, we foresee the need for trade-offs to optimize quality criteria that are dependent on features of temperature such as intensity, frequency and timing of application. High temperature acclimation strategies should also include sulphur supply which restricted levels can either improve seed quality criteria (e.g. total FAs, saturated and unsaturated FAs) in temperature-stressed maturing seed, lessen the positive effects of a thermopriming profile (e.g. indicators of desiccation tolerance) or amplify the oxidative responses to high temperature stress when applied at sensitive stages during the maturation process.

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Tables

	Total			Pods L < 5cm			Pods L ≥ 5cm		
Factor\Variables	Yield	Seed number	TSW	Yield	Seed number	TSW	Yield	Seed number	TSW
Temp-modality									
Control	9.8 a	2784 ns	3.3 ns	3.2 ns	956 ns	3.4 ns	6.6 ns	1829 ns	3.6 ns
Early mild stress	8.8 ab	2565 ns	3.1 ns	1.9 ns	615 ns	3.1 ns	6.9 ns	1950 ns	3.6 ns
3 late heat peaks	9.2 ab	2723 ns	2.9 ns	2.7 ns	927 ns	3.0 ns	6.5 ns	1796 ns	3.7 ns
4 late heat peaks	9.3 ab	2724 ns	3.1 ns	2.5 ns	785 ns	3.1 ns	6.8 ns	1939 ns	3.5 ns
Priming	8.2 b	2497 ns	3.2 ns	1.9 ns	545 ns	3.2 ns	6.2 ns	1952 ns	3.2 ns
se	*0.4*	*80*	*0.3*	*0.5*	*0.5*	*0.3*	*0.6*	*158*	*0.2*
Sulphur									
High Sulphur	9.1 ns	2730 a	3.1 ns	2.6 ns	809 ns	3.1 ns	6.5 ns	1921 ns	3.4 ns
Low Sulphur	9.0 ns	2587 b	3.1 ns	2.3 ns	722 ns	3.2 ns	6.7 ns	1865 ns	3.6 ns
se	*0.3*	*52*	*0.2*	*0.3*	*0.3*	*0.2*	*0.4*	*97.0*	*0.1*
Temp effect	**0.047** *	0.068	0.874	0.309	0.082	0.886	0.944	0.912	0.393
Sulphur effect	0.769	**0.048** *	0.981	0.514	0.424	0.745	0.732	0.684	0.372
Temp x Sulphur	0.342	0.573	0.090	0.195	0.061	0.244	0.526	0.130	0.822

Table 1: Yield components distinguishing the two pools of pods (i.e. $pods_{L< 5cm}$ and $pods_{L\geq 5cm}$ at the beginning of the Temp-modality application). Results are presented by factor (Temp-modality, Sulphur condition). For a given variable, different letters (Tukey multiple comparisons test) indicate the ranking among Temp-modalities (including Temp-control) or between the two Sulphur conditions. P-values and levels of significance are given for Temp, Sulphur and Temp x Sulphur interaction effects. Levels of significance: ns non-significant, *p<0.05**, *p<0.01*****, *p<0.001*******. SE: standard error, TSW: Thousand Seed Weight.

	Seed C. N and S concentrations (%DW)			Oil contents and fatty acids (%DW)				Protein (mg/g DW)
	Carbon	Nitrogen	Sulphur	Fatty acids	SFA	UFA	ω6:ω3 ratio	SSP
Temp-modality								
Control	59.2 a	3.2 b	0.311 ns	39 a	3.3 -	36 a	2.05 ab	150 ns
Early mild stress	58.2 ab	3.5 b	0.321 ns	40 a	3.3 -	37 a	1.87 b	168 ns
3 late heat peaks	58.2 ab	3.2 b	0.323 ns	41 a	3.6 -	38 a	2.08 ab	136 ns
4 late heat peaks	57.2 bc	3.4 b	0.316 ns	37 ab	3.3 -	34 ab	2.20 a	123 ns
Priming	56.0 c	3.9 a	0.373 ns	33 b	3.0 -	30 b	2.24 a	150 ns
se	*0,5*	*0,1*	*0,027*	*2*	*0.1*	*2*	*0,06*	*21*
Sulphur								
High Sulphur	57,7 ns	3,5 ns	0,392 a	35 b	3.1 -	32 b	2,05 ns	106 b
Low Sulphur	57,8 ns	3,4 ns	0,266 b	41 a	4.6 -	38 a	2,12 ns	185 a
se	*0,4*	*0,1*	*0,009*	*1*	*0.1*	*1*	*0,05*	*10*
Temp effect	**0,001** **	**0,000** ***	**0,013** *	**0,002** **	**0.001** **	**0.003** **	**0,001** **	0,278
Sulphur effect	0,829	0,550	**0,000** ***	**0,000** ***	**0.000** ***	**0.000** ***	0,225	**0,000** ***
Temp x Sulphur	0,613	0,574	0,296	0,112	**0.009** **	0.129	0,387	0,233

Table 2: Nutritional seed quality criteria measured on seeds from pods$_{L\geq 5cm}$. Results are presented by factor (Temp-modality, Sulphur condition). For a given measured variable, different letters (Tukey multiple comparisons test) indicate the ranking among Temp-modalities (including Temp-control) or between the two Sulphur conditions. As SFAs are variables with highly significant interaction (Temp x Sulphur) effects, results for each individual treatment are displayed in Figure 2. P-values and levels of significance are given for Temp, Sulphur and Temp x Sulphur interaction effects. Levels of significance: ns non-significant, *p<0.05*, p<0.01**, p<0.001****. SE: standard error, DW: dry weight, SSP: Seed Storage Protein.

	Soluble sugar				Conductivity
Factor\Variables	Sucrose (%DW)	Raffinose (%DW)	Stachyose (%DW)	[Raffinose+stachyose] : sucrose	Seed conductivity (µS/mg)
Temp-modality					
Control	7.4 a	0.47 a	2.5 a	0.41 b	0.8 b
Early mild stress	3.0 c	0.21 c	1.1 b	0.46 ab	1.0 b
3 late heat peaks	6.1 ab	0.38 ab	2.1 a	0.41 b	1.0 b
4 late heat peaks	4.6 bc	0.26 bc	1.4 b	0.35 c	1.0 b
Priming	3.4 c	0.21 c	1.2 b	0.42 ab	2.9 a
se	*0.5*	*0.03*	*0.1*	*0.01*	*0.2*
Sulphur					
High Sulphur	4.9 ns	0.32 ns	1.7 ns	0.42 ns	1.4 ns
Low Sulphur	4.8 ns	0.29 ns	1.6 ns	0.40 ns	1.3 ns
se	*0.5*	*0.03*	*0.2*	*0.01*	*0.2*
Temp effect	**0.000** ***	**0.000** ***	**0.000** ***	**0.000** ***	**0.000** ***
Sulphur effect	0.850	0.850	0.620	0.054	0.336
Temp x Sulphur	0.247	0.383	0.431	0.058	0.658

Table 3: Seed physiological quality values measured on seeds from $pods_{L\geq 5cm}$. Results are presented by factor (Temp-modality, Sulphur condition). For a given measured variable, different letters (Tukey multiple comparisons test) indicate the ranking among Temp-modalities (including Temp-control) or between the two Sulphur conditions. P-values and levels of significance are given for Temp, Sulphur and Temp x Sulphur interaction effects. Levels of significance: ns non-significant, *p<0.05**, *p<0.01***, *p<0.001****. SE: standard error, DW: dry weight.

Supplemental Data

Period covering the 17 day of heat treatment	Daily PARi in the heated unit (MJ/day)	Daily PARi in the control unit (MJ/day)	Ratio
April 27th	5.1	7.4	1.4
April 28 th	5.2	7.2	1.4
April 29 th	3.3	3.7	1.1
April 30 th	5.0	5.9	1.2
May 1st	5.5	7.1	1.3
May 2nd	6.7	7.1	1.0
May 3rd	5.3	5.3	1.0
May 4 th	5.8	5.8	1.0
May 5 th	3.5	3.4	1.0
May 6 th	6.5	7.5	1.1
May 7 th	4.4	5.0	1.1
May 8 th	4.4	10.1	2.3
May 9 th	4.8	9.8	2.0
May 10 th	4.8	4.5	0.9
May 11 th	5.7	5.5	1.0
May 12 th	6.7	5.4	0.8
May 13 th	6.1	9.4	1.5
May 14 th	5.1	7.4	1.4
May 15th	5.2	7.2	1.4

Table S1: Daily incident Photosynthetically Active Radiation values (PARi, MJ/day) in the heated unit and in the control unit throughout the 17 days of Temp- modality application (from the 27th of April until the 15th of May).

Harvest date	Harvest 1	Harvest 2
Mean Seed FW	3.31 (0.76)	5.98 (0.99)
p-value	**0.02***	

Table S2: Mean individual seed (*n*) fresh weight (FW, mg/seed) at the beginning (Harvest 1, *n*=212) and at the end of Temp-modality exposure (Harvest 2, *n*=250) over 17 days. FWs are given for Temp-control plants. Standard deviation into brackets. Level of significance of the ANOVA test for the date effect: $p<0.05$*.

Sulphur condition	High Sulphur					Low Sulphur							
Temp-modality	**Control**	**Early Mild Stress**	**3 Late Heat Peaks**	**4 Late Heat Peaks**	**Priming**	**Control**	**Early Mild Stress**	**3 Late Heat Peaks**	**4 Late Heat Peaks**	**Priming**	**Temp effect**	**Sulphur effect**	**Temp x Sulphur**
Yield components (per plant)													
Total Seed Yield (g)	9.92 (0.57)	8.82 (0.82)	9.55 (0.74)	9.61 (0.55)	7.52 (0.91)	9.70 (1.35)	8.71 (0.64)	8.77 (1.61)	8.95 (0.46)	8.78 (1.87)	**0.04***	0.77	0.34
Total Seed Number	2774 (203)	2672 (184)	2802 (249)	2882 (192)	2519 (168)	2794 (328)	2457 (253)	2643 (183)	2565 (121)	2474 (124)	0.07	**0.04***	0.57
Thousand Seed Weight (g)	3.53 (0.29)	3.21 (0.34)	3.42 (0.34)	3.35 (0.28)	2.85 (0.65)	3.36 (0.46)	3.40 (0.34)	3.17 (0.62)	3.31 (0.15)	3.54 (1.51)	0.87	0.98	0.09
Seed C. N and S concentrations (%DW)													
Carbon	59.8 (0.7)	58.1 (0.9)	57.9 (1.7)	57.3 (1.6)	55.4 (1.3)	58.7 (1.8)	58.3 (1.2)	58.4 (1.9)	57.0 (1.0)	56.6 (1.8)	**0.00*** **	0.83	0.61
Nitrogen	3.36 (0.23)	3.44 (0.22)	3.22 (0.34)	3.32 (0.24)	3.97 (0.19)	3.11 (0.50)	3.51 (0.09)	3.18 (0.20)	3.46 (0.25)	3.79 (0.33)	**0.00*** **	0.55	0.57
Sulfur	0.39 (0.05)	0.38 (0.05)	0.39 (0.05)	0.40 (0.04)	0.42 (0.02)	0.24 (0.04)	0.27 (0.03)	0.26 (0.01)	0.24 (0.01)	0.33 (0.04)	**0.01***	**0.00*** **	0.30
Oil contents and fatty acids (%DW)													
Total Fatty Acid	39.3 (4.5)	36.4 (1.1)	37.3 (1.1)	31.2 (4.8)	30.1 (3.2)	39.2 (8.1)	44.5 (1.0)	45.1 (4.1)	42.6 (5.0)	35.0 (1.9)	**0.00** **	**0.00*** **	0.11
ω6:ω3 ratio	2.03 (0.12)	1.83 (0.10)	2.05 (0.11)	2.08 (0.20)	2.29 (0.21)	2.07 (0.29)	1.91 (0.08)	2.11 (0.14)	2.33 (0.21)	2.19 (0.09)	**0.00** **	0.22	0.38
Protein concentration (mg/g DW)													
Seed Storage Protein	91.0 (15.2)	149.9 (55.6)	79.9 (48.7)	93.2 (13.0)	118.3 (16.6)	208.3 (48.4)	186.3 (66.7)	192.5 (36.2)	153.7 (38.2)	182.6 (33.7)	0.28	**0.00 *** **	0.23
Soluble sugars concentrations (%DW)													
Sucrose	6.52 (0.80)	2.95 (0.25)	6.42 (2.17)	5.04 (1.76)	3.69 (0.82)	8.28 (0.84)	2.99 (0.88)	5.80 (2.10)	4.14 (1.06)	3.02 (0.38)	**0.00*** **	0.85	0.25
Raffinose	0.44 (0.08)	0.21 (0.02)	0.41 (0.13)	0.30 (0.13)	0.25 (0.07)	0.50 (0.05)	0.21 (0.05)	0.35 (0.15)	0.21 (0.05)	0.17 (0.02)	**0.00*** **	0.85	0.38
Stachyose	2.35 (0.38)	1.15 (0.15)	2.09 (0.52)	1.51 (0.58)	1.40 (0.41)	2.71 (0.25)	1.14 (0.32)	2.11 (0.76)	1.21 (0.31)	1.00 (0.10)	**0.00*** **	0.62	0.43
[raff+stach]:sucr	0.43 (0.02)	0.46 (0.02)	0.40 (0.03)	0.35 (0.03)	0.44 (0.04)	0.39 (0.02)	0.45 (0.03)	0.42 (0.02)	0.34 (0.01)	0.39 (0.02)	**0.00*** **	0.05	0.06
Seed conductivity (µS/mg DW)													
Seed Conductivity	0.87 (0.45)	1.28 (0.73)	1.19 (0.55)	0.97 (0.50)	2.86 (1.57)	0.78 (0.16)	0.66 (0.13)	0.81 (0.27)	1.08 (0.78)	3.01 (0.93)	**0.00*** **	0.34	0.66
Hormone concentrations (ng/g DW)													
Indole-3-Acetic Acid	134.3 (1.2)	121.7 (1.1)	278.2 (6.6)	380.3 (1.8)	268.9 (4.3)	160.1 (6.9)	211.5 (1.9)	164.6 (1.2)	279.5 (3.6)	192.7 (1.9)	**0.00*** **	**0.00*** **	**0.00*** **
Salicylic Acid	40.8 (0.6)	42.6 (0.3)	96.6 (1.6)	135.6 (0.5)	103.1 (2.5)	59.3 (2.6)	76.1 (0.7)	73.5 (0.9)	82.5 (0.9)	96.1 (1.1)	**0.00*** **	**0.00*** **	**0.00*** **
ABA:GA3 ratio	0.28 (0.01)	0.41 (0.00)	0.42 (0.01)	0.49 (0.01)	0.74 (0.01)	0.79 (0.03)	0.60 (0.01)	0.95 (0.00)	0.91 (0.01)	0.63 (0.02)	**0.00*** **	**0.00*** **	**0.00*** **
Phenolic content and antioxidant capacity													
Polyphenols	3.96 (0.15)	4.12 (0.18)	3.79 (0.23)	3.44 (0.49)	3.76 (0.30)	3.34 (0.48)	4.26 (0.20)	3.35 (0.26)	3.71 (0.36)	4.11 (0.15)	**0.00** **	0.60	**0.01***
ABST	18.70 (1.04)	19.14 (0.87)	18.18 (1.93)	15.66 (1.97)	17.72 (0.76)	15.29 (1.31)	18.68 (1.91)	15.47 (0.96)	16.40 (1.75)	18.17 (0.84)	**0.00** **	**0.01***	**0.00** **

Table S3: Summary of the mean value ± se of the all measured variables for each of the 10 treatments in order to provide reference values in a given combination "Sulphur supply/Temperature modality". Standard deviation into brackets. P-values and levels of significance are given for Temp, Sulphur and Temp x Sulphur interaction effects. Levels of significance: ns non-significant, p<0.05*, p<0.01**, p<0.001***. DW: dry weight.

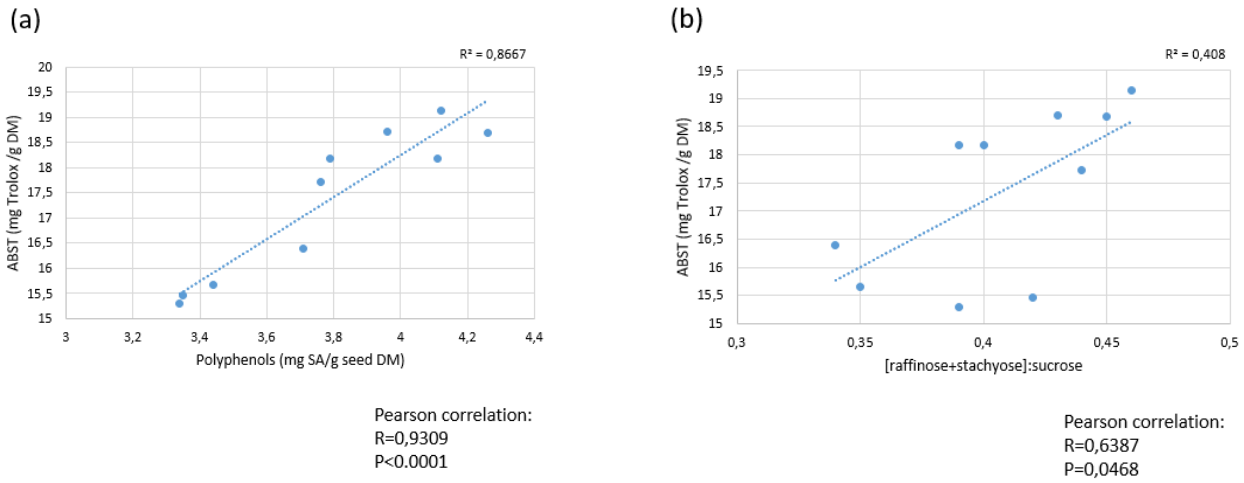

Figure S1: Illustration of the correlation between ABTS value related to (a) polyphenols and (b) soluble sugars ratio. SA: synaptic acid, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).