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HAL Authorization

1 Original article

2 **High temperature patterns at the onset of seed maturation determine seed yield and quality in**
3 **oilseed rape (*Brassica napus* L.) in relation to sulphur nutrition**

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25

26

1 **Abstract**

2 High temperatures during the crop reproductive stage impact seed yield and quality. The changing
3 climate will require consideration of the effects of high temperature events that differ from their
4 intensity, their duration and their frequency over the seed quality-building stages. The impact of these
5 features deserve to be investigated at the light of induced thermo-sensitization which can lead to
6 alleviate expected negative impacts. In our work, maturing seeds of the sulphur-demanding crop,
7 oilseed rape, were exposed to several temperature sequences that varied in intensity, duration and
8 frequency at the onset of seed maturation. Results-measured in seeds that were at the onset of
9 maturation when the temperature stress occurred-indicated that (i) the longer the cumulated duration of
10 the temperature stress, the more negatively impacted the quality criteria with decreased fatty acids
11 (FAs) concentration, increased $\omega 6$: $\omega 3$ ratio, lower seed membrane integrity and increased seed
12 dormancy and (ii) a mild stress event prior to heat peaks had an alleviating effect on the negative
13 impact of the later heat peaks (priming effect) on seed nitrogen, desiccation tolerance and the
14 phytohormones involved in thermoinhibition. sulphur restriction was positive on FAs, proteins
15 concentrations and negative on breaking dormancy. In addition, sulphur supply interfered with
16 temperature modality, features such that positive impact of sulphur limitation on boosting oxidative
17 response were cancelled with intense late heat peaks. This work provides insights to define
18 thermopriming protocols in relation to the timing of quality building processes, their respective
19 optimal temperature and adequate sulphur supply.

20

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

23

24 **Running head:** Thermotolerance for seed quality in oilseed rape

25

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

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

3 **Introduction**

4 Evidence for increased frequency of stress events such as heat waves has been observed over recent
5 decades. Based on the last Intergovernmental Panel on Climate Change (IPCC) report (Hoegh-
6 Guldberg et al., 2018), heat waves are expected to become more frequent, to last longer and to increase
7 in intensity during the reproductive phase of economically important crops (Christidis et al., 2015;
8 Trnka et al., 2014). These new climatic patterns have led to attempts to decipher crop behavior and
9 final performance in the light of recurring stresses. While the effects of extreme/mild environmental
10 stresses have been widely investigated from molecular to whole plant levels (Kotak et al., 2007;
11 Ohama et al., 2016; Wahid et al., 2007), far fewer studies have tackled the issue of understanding the
12 effects of their recurrence throughout the crop season. Indeed, the overall magnitude of the plant
13 response to successive stresses might not match the effects induced by individual stressing events
14 because (i) the first stress triggers physiological and metabolic adjustments that bring the plant to a
15 modified status (including phenology) when the stress recurs, which leads to different responses to the
16 second stress and (ii) a mild stress prior to further similar stresses can induce stress memory that lasts
17 the duration of the crop season (i.e. intra-generational memory, Ding, Fromm & Avramova 2012) and
18 is sometimes transmitted to offspring (i.e. transgenerational memory, Molinier, Ries, Zipfel & Hohn
19 2006; Wang *et al.* 2016; Hatzig, Nuppenau, Snowdon & Schiebl 2018; Kinoshita & Seki 2014; Crisp,
20 Ganguly, Eichten, Borevitz & Pogson 2016; Kumar 2018). Stress memory is defined as the process of
21 storage and retrieval of information acquired during an initial exposure to stress (Crisp et al., 2016;
22 “Stress priming, memory, and signalling in plants,” 2019). This information acts as a priming process
23 with beneficial effects when the stress recurs and it can lead to earlier, more rapid, intense, and
24 sensitive responses that help plants to acclimate in changing environments (Kinoshita and Seki, 2014).
25 Underlying mechanisms include epigenetic regulation, transcriptional priming, primed conformation
26 of proteins, and/or specific hormonal or metabolic signatures. (Groot et al., 2016; Hatzig et al., 2018;
27 Molinier et al., 2006; Wang et al., 2016) This means that as the climate changes, the effects of repeated

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

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

16 Seed quality encompasses a range of criteria related to nutritional and physiological characteristics
17 (i.e. germination behaviors and storage capacity). In winter oilseed rape, oil content, fatty acid (FA)
18 profiles, and protein content are major nutritional criteria for edible oil and cakes used in human and
19 animal consumption, respectively. Its oil contains higher unsaturated FA (UFA) content than other oil
20 crops (sunflower, soybean), which makes it a healthy edible oil for human consumption (Aguirrezábal
21 et al., 2015). Contrasting variations have been observed in oil content according to the temperature
22 intensity, to the timing of stress exposure and to the pools of seeds analyzed (main stem vs. bulk) (e.g.
23 increased in Brunel-Muguet *et al.* (2015); decreased in Canvin (1965); Aksouh *et al.* (2001); Aksouh-
24 Harradj *et al.* (2006)). High temperatures are known to induce decreases in poly-UFAs in favor of
25 saturated FAs (SFA, mainly C16:0 and C18:0) and mono-UFAs, and increases in the $\omega 6:\omega 3$ ratio (i.e.
26 C18:2/C18:3 ratio), as a result of temperature-triggered impairment of desaturase enzyme activity i.e.
27 oleic and linoleic desaturases (Aksouh-Harradj et al., 2006; Baux et al., 2013, 2008; Brunel-Muguet et

1 al., 2015; Gauthier et al., 2017; Schulte et al., 2013). By contrast, seed nitrogen (N) and protein
2 concentrations in the oil-free meal is usually negatively correlated with total oil content (Aksouh-
3 Harradj et al., 2006; Aksouh et al., 2001) as observed in other oil crops (in soybean, N concentration
4 Chebrolu *et al.* (2016); protein concentration, Dornbos & Mullen (1992)). Additionally, other seed
5 characteristics related to physiological quality, i.e. seed storage capacity and germination behavior,
6 were investigated but not to any great extent (Brunel-Muguet et al., 2015). A drastic degradation of
7 seed storage capacity has been observed using seed conductivity and the ratio of soluble sugars
8 ([stachyose and raffinose]:sucrose), abscisic acid (ABA) and gibberellic acid (GA3) as proxies (Bailly
9 et al., 2001; Brunel-Muguet et al., 2015) in seeds from long-term heat-stressed mother plants. Other
10 phytohormones were shown to be involved in the control of secondary dormancy, defined as failure in
11 the germination process of mature and non-dormant seeds under adverse conditions (Pekrun et al.,
12 1997). Recent studies have highlighted the role of indole-3-acetic acid (IAA), whose concentrations
13 increase in dormancy-induced seeds (Liu et al., 2019; Shu et al., 2016; Tuan et al., 2019). Although
14 several studies have reported that salicylic acid (SA) enhanced germination in *Arabidopsis* seeds by
15 reducing oxidative damage (Chitnis et al., 2014; Lee and Park, 2010) and inhibited germination
16 because of higher oxidative stress (Xie et al., 2007).

17 In Brassica species, sulphur (S) nutrition determines yield components and seed quality because of
18 their high S requirements throughout the crop cycle (Brunel-Muguet et al., 2015; D'Hooghe et al.,
19 2014). In addition to its well-known implication in the synthesis and signaling of stress tolerance-
20 controlling phytohormones (Hasanuzzaman et al., 2018), S might be involved in the acquisition of
21 thermotolerance mediated by epigenetic regulation (Bokszczanin et al., 2013). This is based on
22 evidence for S involvement in DNA methylation (through the role of S-adenosylmethionine as a donor
23 of methyl groups, Meng *et al.* 2018), one of the key epigenetic markers that supports stress memory,
24 thus making the analysis of S supply relevant in the context of epigenetic memory.

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

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

11

12 **Materials and Methods**

13 *Experimental treatments and growth conditions*

14 Seeds of *Brassica napus* L. (cv. Aviso) were germinated in vermiculite in October 2016 under
15 greenhouse conditions. After five weeks the seedlings were transplanted into pots containing perlite
16 and vermiculite (2:1, v/v) for seven weeks and seedlings were grown as described in Poisson *et al.*
17 (2019). Afterwards, seedlings were subjected to a 12-week period of vernalization in a cold chamber
18 (standard model, Froid & Mesures, Beaucouzé, France) maintained at 4°C (night) and 8°C (day) with
19 artificial light during the day (10h day/14h night) and supplied with a 25% Hoagland solution without
20 sulphur, to prevent the plants from building substantial S reserves which would later impact their
21 nutritional status when applying the contrasting sulphur supplies.

22 Then, the plants were transferred into the greenhouse (Caen, France, 49°11'09 N, 0°21'32 W) and
23 subjected to a thermoperiod of 20°C (day) and 15°C (night) without additional light. The plants were
24 manually provided with two N applications with an NH₄NO₃ solution: 100 kg N/ha at the end of
25 vernalization (Growing Stage 30 (GS30), stem elongation, Lancashire *et al.* 1991) and 50 kg N/ha at
26 early flowering (GS60, bud formation) assuming a plant density of 40 plants.m⁻². The two contrasting
27 S supplies i.e. High Sulphur and Low Sulphur were manually applied at the end of vernalization

1 (GS30) as usually provided in the field. Plants were supplied with a solution of MgSO_4 containing 75
2 kg SO_3/ha (High Sulphur) and 25 kg SO_3/ha (Low Sulphur), which represent, respectively, 100% and
3 33% of the conventional supply.

4 Four temperature modalities (Temp-modalities) were applied to plants at stage GS72 (i.e. 20% of the
5 pods having reached their maximum size) for 17 days. The Temp-modalities were designed to assess
6 the effects of a mild temperature event prior to a more intense temperature event including daily heat
7 peaks (Figure 1). In our complete design, we tested five Temp-modalities which included a Temp-
8 control modality that is natural thermoperiod conditions in the greenhouse (Figure 1a). The other four
9 Temp-modalities were the following: (i) the early mild stress modality (Figure 1b) composed of 5
10 early full days under mild warming [i.e. $25.3^\circ\text{C} \pm 1.8$ (day, 16h) / $21.7^\circ\text{C} \pm 0.7$ (night, 8h)] and
11 followed by 12 days under natural thermoperiod (mean, maximum and minimum temperatures over
12 the natural thermoperiod being $15.7^\circ\text{C} \pm 2.7$, 28.4°C and 11.5°C , respectively), (ii) the 3 late heat
13 peaks modality composed of 14 days under natural thermoperiod followed by 3 days under mild
14 warming with a daily heat peak applied for 5 hours (i.e. $31.4^\circ\text{C} \pm 1.7$ between 11 am till 4 pm, Figure
15 1c), (iii) the 4 late heat peaks modality composed of 10 days under natural thermoperiod followed by 7
16 days under mild warming with daily heat peaks on days 12, 15, 16 and 17 (Figure 1d), and (iv) the
17 priming modality composed of 5 days under mild warming followed by 5 days under natural
18 thermoperiod and eventually 7 days under mild warming with daily heat peaks on days 12, 15, 16 and
19 17 (Figure 1e).

20

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

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

19

20 ***Seed yield and components***

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

25

26 ***Biochemical characteristics of seeds from pods_{L \geq 5cm}***

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

3

4 *Seed carbon, nitrogen and sulphur concentrations*

5 Seeds of each individual plant were pooled and dried for 48h at 50°C. The dried seeds were ground
6 manually and the resulting powder (around 3 mg per sample) was placed into tin capsules for analysis.

7 The percentage of total carbon, nitrogen, and sulphur per mg DW of the seeds were determined with a
8 C/N/S analyzer (EA3000, Euro Vector, Milan, Italy) linked to a continuous flow isotope mass
9 spectrometer (IRMS, Isoprime, GV Instrument, Manchester, UK).

10

11 *Seed fatty acid concentration*

12 Oil and fatty acid profile contents (C16:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1 and C22:1) were
13 determined as described in Marchand *et al.* (2016). Approximately 200 mg of seeds from each plant
14 were suspended in 1 mL of methanol/toluene/H₂SO₄ solution (100:20:2.5; v/v) containing C17:0 as
15 internal standard (5 $\mu\text{g mL}^{-1}$), overnight at 85°C for transmethylation. After cooling, 500 μL of hexane
16 was added and the hexane phase containing the resulting fatty acid methyl esters (FAMES) was
17 recovered for gas chromatography analysis combined with flame ionization detection (GC-FID). The
18 FAMES (1 μL) were injected into an Agilent 7890 gas chromatograph equipped with a Carbowax
19 column (15m by 0.53mm, 1.2 m) (Alltech Associates, Deerfield, IL) and FID system. FAMES were
20 identified by comparing their retention times with those of commercial standards (Sigma, St. Louis,
21 MO) and quantified using ChemStation (Agilent) to calculate the peak areas.

22

23 *Seed storage protein concentration*

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

1 measure is made at 570 nm after addition of the Bradford reagent. The calibration straight line allowed
2 the total protein concentrations to be calculated by considering the mass and volume of the sample.
3 The detailed steps for extraction and the Bradford assays are described for leaves in Akmouche *et al.*
4 (2019).

5

6 *Seed soluble sugar concentrations*

7 Soluble sugars were extracted from 50 mg lyophilized and ground seeds, with 1 mL methanol/water
8 (80:20, v/v) and 40 μ L melicitose used as the internal sugar standard. Glucose, fructose, sucrose,
9 raffinose, and stachyose contents were quantified using High Performance Liquid Chromatography
10 (HPLC) on a cation exchange column (Sugar-PAK, 300 X 6.5 mm, Millipore Waters, Milford, MA,
11 USA) eluted at 0.5 mL min⁻¹ and 85 °C with 0.1 mM Ca-EDTA in water and quantified using a
12 refractive index detector (2410 Differential Refractometer, Millipore Waters, Waters Corporation,
13 MA, USA), according to Brunel-Muguet *et al.* (2015). The [raffinose+stachyose]:sucrose ratio was
14 used as an indicator of seed drying tolerance i.e. the higher the value, the more tolerant the seed is to
15 desiccation (Bailly *et al.*, 2001).

16

17 *Analysis of stress signaling and seed dormancy-related phytohormones*

18 For each treatment, 50 mg of ground seeds were used to quantify 2-*cis*, 4-*trans*-abscisic acid,
19 gibberellic acid, indole-3-acetic acid and salicylic acid. Previously freeze-dried samples were mixed
20 with 500 μ L of extraction solvent [2-propanol/H₂O/ concentrated HCl (2:1:0.002, v/v/v)] and then
21 analyzed by HPLC-MS as described in (Pan *et al.*, 2010). Because the dynamics of ABA and GA3
22 controls the balance between dormancy and germination, the ABA:GA3 ratio was used as a proxy for
23 seed dormancy under stress condition (Debeaujon and Koornneef, 2000; Finkelstein, 2013).

24

25 *Seed conductivity measurements*

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

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

3

4 *Determination of seed total phenolic content and antioxidant capacity*

5 Prior to the extraction, seeds from plants of each treatment were oven-dried at 60°C for at least 48
6 hours and then ground. The extraction procedure was adapted from Szydłowska-Czerniak, Amarowicz
7 & Szłyk (2010) in terms of solvent, extraction time and sample:solvent ratio. Seeds were extracted
8 with methanol:H₂O (50:50, v/v) at a ratio of 20:1 (mg/mL) by using mechanical stirring for 1 h at 25
9 °C. Total phenolic content was spectrophotometrically determined with a modified Folin-Ciocalteu
10 method (González-Centeno et al., 2015) and was expressed as the mean of six determinations of
11 sinapic acid in mg equivalents per DW g of seeds. To measure the antioxidant capacity, seed extracts
12 were diluted at a ratio 1/4 with methanol:H₂O (50:50, v/v) according to the methodology previously
13 described by González-Centeno *et al.* (2012). ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic
14 acid)] assay values were expressed as mg of Trolox equivalent antioxidant capacity per g of seeds
15 DW.

16

17 *Statistical analyses*

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

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

6

7 **Results**

8 *Plant growth and yield components*

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

26

27 *Nutritional seed quality criteria*

1 *Seed C, N and S concentrations*

2 Table 2 displays C, N and S seed concentrations. Similar to yield components, no Temp x Sulphur
3 interaction effect was observed. The seed C concentration was impacted by Temp-modalities
4 ($p < 0.001$) but no sulphur effect was observed. The priming modality displayed the lowest value (-
5 5.4% from Temp-control) whereas the Temp-control displayed the highest value. Similar to C, the
6 seed N concentration was only affected by Temp-modalities ($p < 0.001$). Under both sulphur
7 conditions, extreme N values were observed for the priming modality, which had the highest ranking
8 (+21.9 % compared to Temp-control). These observations showed that the early mild stress had a
9 positive effect over the late heat peaks, which suggested an alleviating effect. As expected, the seed S
10 concentration was significantly impacted by the sulphur supply ($p < 0.001$), with Low Sulphur values
11 being 32% lower than High Sulphur values on average. While no significant differences were
12 observed among the Temp-modalities, the S in the priming modality was slightly higher than in Temp-
13 control (+19.9%).

14

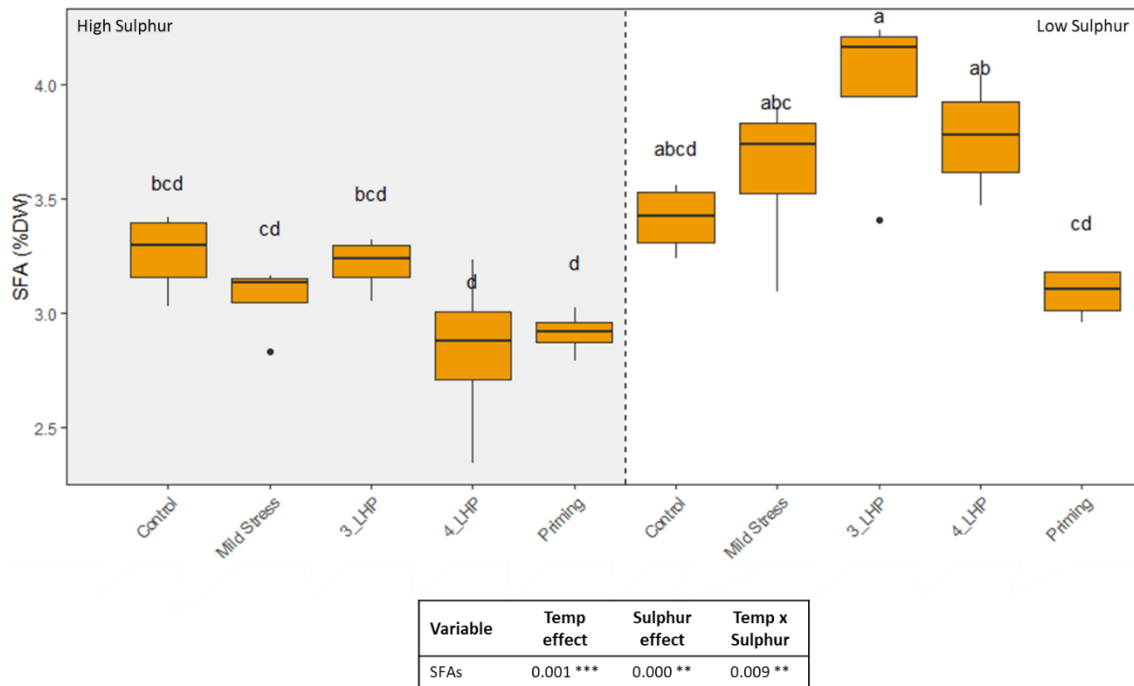
15 *Fatty Acids concentrations and profiles*

16 Total fatty acids, saturated FAs and unsaturated FAs concentrations and the $\omega 6:\omega 3$ ratios are displayed
17 in Table 2 and in Figure 2. For total FAs concentration, no Temp x Sulphur interaction effect was
18 observed on FAs concentration. Effects of Temp-modalities were observed ($p < 0.01$) with values
19 ranging from 33% to 41% of DW for priming and 3 late heat peaks modalities respectively (Table 2).
20 Values under Low Sulphur were significantly higher ($p < 0.001$) on average (41 %DW) than values
21 under High Sulphur (35 %DW). Temp-modality effect was observed in both SFAs and UFAs
22 concentrations, with a significant effect of Temp x Sulphur interaction only observed on SFAs
23 ($p < 0.01$) (Table 2, Figure 2). The SFAs (including C16:0, C18:0, C20:0, C22:0) concentrations ranged
24 from 3.0% to 3.6%DW and the UFAs (including C16:1, C18:1, C18:2, C18:3, C20:1) concentrations
25 ranged from 30% to 38%DW. For both SFAs and UFAs concentrations, the priming modality and the
26 3 late heat peaks displayed the lowest and highest values respectively.

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

9 The $\omega 6:\omega 3$ ratio (i.e. C18:2/C18:3 ratio) is commonly used as an indicator of the edible quality of
10 vegetable oils. This ratio was significantly impacted by the Temp-modalities, but no significant
11 Sulphur nor Temp x Sulphur interaction effects were observed (Table 2). The highest and lowest ratios
12 were observed on the priming and early mild stress modalities respectively (+9.3 and -8.8% compared
13 to the Temp-control). In addition, the temperature effect on 3 late heat peaks modality was less
14 negative than the early mild stress modality (Table 2). These results indicated that the number of
15 desaturations decreased with greater duration of stress exposure (priming modality) and intensity and
16 earliness of the heat stress event (4 late heat peaks modality) under both sulphur conditions.

17



1

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

8

9 *Seed storage protein concentrations*

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

17

1 ***Physiological seed quality-related criteria***

2 *The soluble sugar composition as an indicator of desiccation tolerance acquisition*

3 Table 3 presents the concentration of the main soluble sugar in seeds i.e. sucrose, raffinose and
4 stachyose. For the three main soluble sugars, only temperature effects were observed ($p < 0.001$). All
5 the Temp-modalities led to decreased soluble sugar concentrations under both sulphur conditions.
6 Sucrose, raffinose and stachyose concentrations were the highest in the Temp-control and the lowest
7 in the priming and early mild stress modalities. Under the priming modality, sucrose, raffinose and
8 stachyose concentrations were respectively 54%, 55% and 52% lower than the Temp-control.
9 Similarly, under the early mild stress modality these concentrations were respectively 59%, 55% and
10 56% lower than under the Temp-control. These results indicated that the early mild stress had a
11 negative impact on the sugar soluble concentrations. The [raffinose+stachyose]:sucrose ratio, used as a
12 proxy of seed desiccation tolerance, ranged from 0.35 (4 late heat peaks modality) to 0.46 (early mild
13 stress modality) without significant Sulphur effect (Table 3). These results indicate that whatever the
14 sulphur supply, the 4 late heat peaks modality was the most detrimental to acquisition of seed
15 desiccation tolerance. However, these data also highlighted that the prior event of mild stress
16 alleviated the negative effects of late heat peaks since the value under the priming modality was higher
17 than under the early mild stress modality.

18

19 *Seed conductivity as indicator of membrane permeability*

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

1

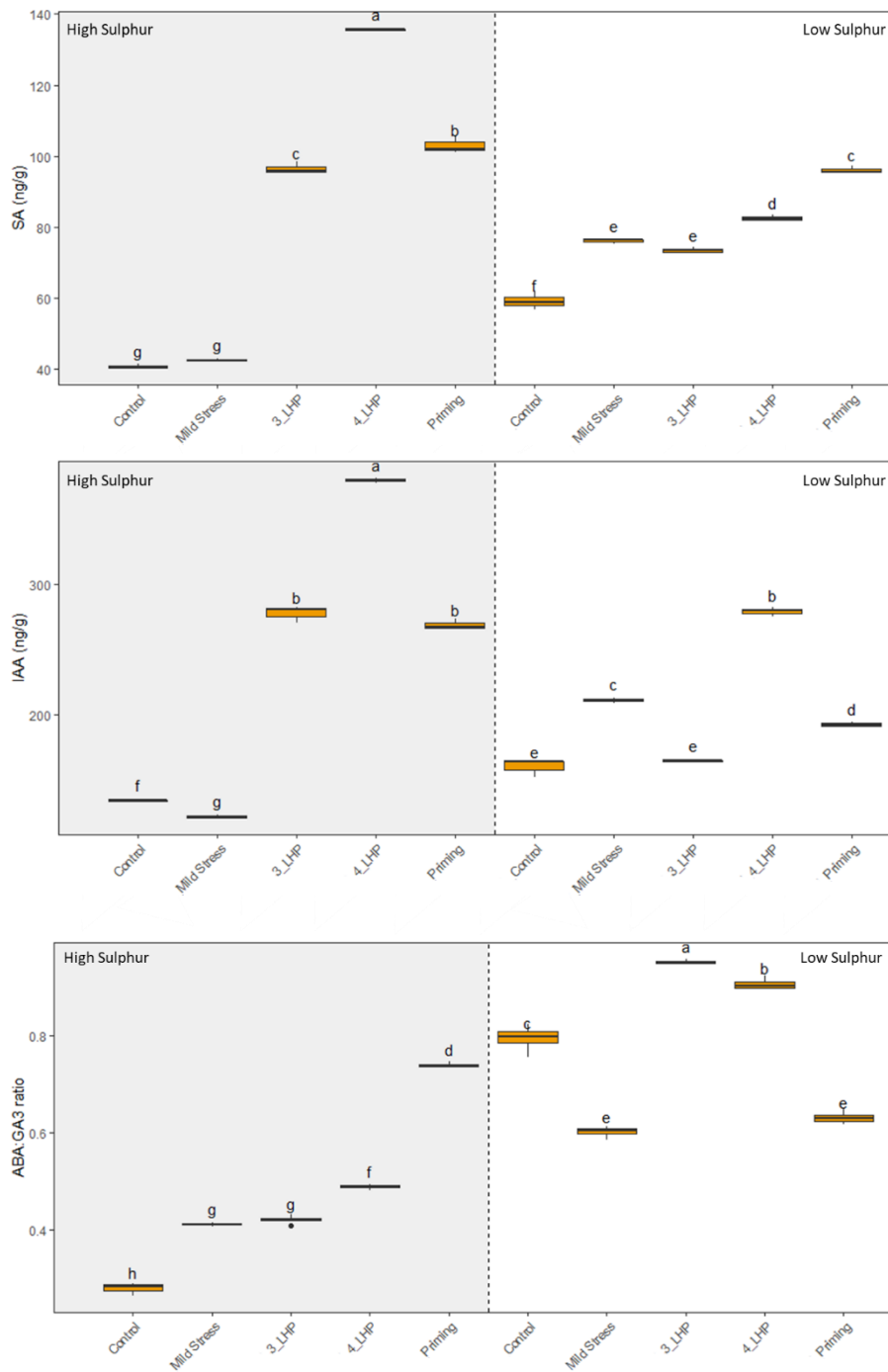
2 *Phytohormone changes as indicators of temperature-induced seed dormancy*

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

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

20 Seed concentrations of IAA and SA were measured to investigate their variation under the different
21 temperature sequences and sulphur supplies. Consistent with the ABA:GA3 ratio, high concentrations
22 of IAA and SA were observed under high temperatures. IAA and SA ranged from 147 ng/g DW to
23 330 ng/g DW and 50 ng/g DW to 109 ng/g DW respectively, with Temp-control displaying the lowest
24 values and 4 late heat peaks modality displaying the highest values for both measured variables
25 (Figure 3). Both hormones concentrations were significantly lower in Low Sulphur than in High
26 Sulphur condition, with High Sulphur values in IAA being 17% higher than Low Sulphur and values
27 in SA being 9% higher than Low Sulphur. By contrast to the ABA:GA3 ratio, Sulphur limitation

1 decreased IAA concentrations except under Temp-control and under the early mild stress modalities
2 with values being respectively 16% and 73% higher in Low Sulphur than in High Sulphur (Figure 3).
3 Under High Sulphur condition, the early mild stress event prior to the 4 late heat peaks (priming
4 modality) led to alleviate the strong increase in IAA concentration observed in the late heat peaks
5 solely (4 late heat peaks modality), thus suggesting an effective priming effect. The interaction effects
6 pointed out that according to the sulphur condition, the Temp-modality effect was different i.e. while
7 the priming modality led to higher value than under the early mild stress modality under High Sulphur,
8 it led to lower value than the early mild stress modality under Low Sulphur (Figure 3).
9 As observed for IAA concentrations, SA concentrations decreased under Low Sulphur except under
10 the Temp-control and under the early mild stress modalities. Similar to IAA concentrations, the early
11 mild stress event prior to the 4 late heat peaks (Priming modality) led to alleviate the strong increase in
12 SA concentration observed under the 4 late heat peaks solely (4 late heat peaks modality), although
13 this was only observed under the High Sulphur condition.



1

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 ***

2

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

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

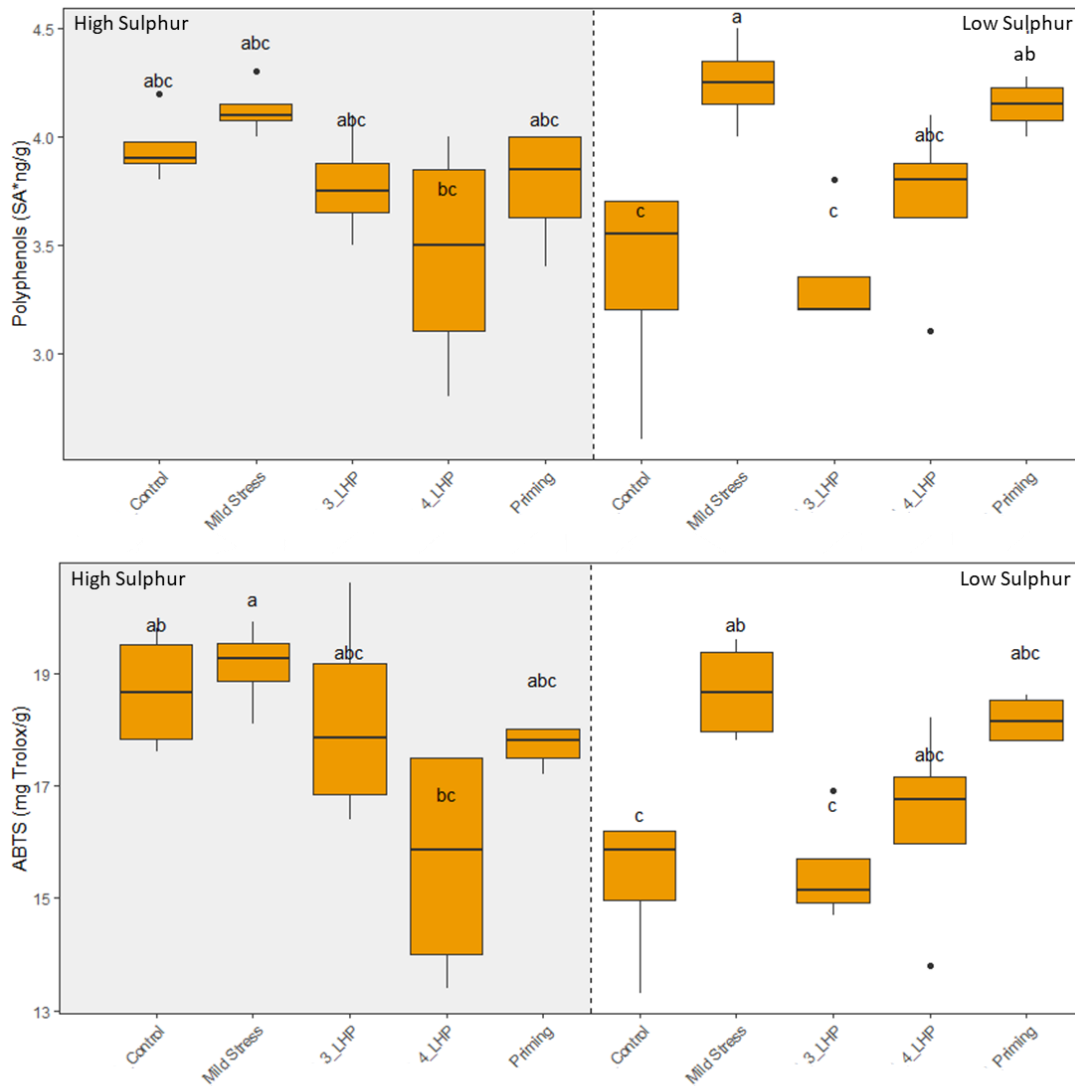
6

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

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

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

1 results also indicated that the early mild stress event prior to the 4 late heat peaks (priming modality)
2 were beneficial to the sharp decrease in ABTS values observed under the 4 late heat peaks alone, both
3 Sulphur conditions. Regarding the effects of Sulphur conditions, limitation led to decreased
4 antioxidant capacity in the seeds with mean values being 17.9 mg Trolox/g under High Sulphur and
5 16.8 mg Trolox/g under Low Sulphur (i.e. -6%) which suggest a crucial role for the Sulphur supply in
6 favoring oxidative responses, not only under the Temp-control condition but also in the 3 late heat
7 peaks modality (Figure 4). Eventually, the Temp x Sulphur interaction effects were observed because
8 Sulphur conditions modified the ranking of the temperature modalities compared the Temp-control,
9 i.e. under High Sulphur ABTS values were lower in the priming and 4 late heat peaks modalities than
10 the Temp-control but higher under Low Sulphur. Taking into account the values of all the treatments,
11 antioxidant capacities of the seeds were positively correlated to both phenolic concentration ($r=0.93$,
12 $p<0.001$) and the [raffinose+stachyose]:sucrose ratio ($r=0.64$, $p<0.05$) (Supplemental Data, Figure
13 S1).



1

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

1

2 **Discussion**

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

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

20

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

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

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

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

15 Disruption of FAs accumulation in oilseed rape was pointed out under intense heat stress across a
16 range of temperatures similar to those imposed under our heat-peak conditions. This was shown to be
17 the consequence of photosynthesis inhibition and downregulation of BnWRI1 gene expression, which
18 is a key regulator of FA biosynthesis (Huang et al., 2019; Ruuska et al., 2002). Our results also
19 revealed higher total FAs, saturated FAs and unsaturated FAs concentrations under Low Sulphur
20 condition (Table 2, Figure 2), which contrasted with prior results (Brunel-Muguet et al., 2015). These
21 differences can be explained in the current work by the lower Sulphur supply and the different
22 approaches to seed sampling and analysis (on maturing seeds exposed to the heat stress modalities).

23 The negative effects of high temperature – mainly of duration of high temperature sequence (Figure 5)
24 - on total FAs and unsaturated FAs also indicated that (i) FA biosynthesis was concomitant with the
25 17-days high-temperature treatment starting approximately 3 weeks after the onset of flowering
26 (Figure 1), with FAs levels rising during the late storage stage (i.e. 20 after pollination (Borisjuk et al.,
27 2013; Niu et al., 2009)) and (ii) the later the heat peaks, the lower the effects. The increased $\omega 6:\omega 3$

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

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

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

Criteria		Temp-modality				S condition
		Early Mild Stress	3 Heat Peaks	4 Heat Peaks	Priming	Low Sulphur
YIELD COMPONENTS	Total Seed Yield	-10%	-6%	-5%	-16%	
	Total Seed number					-5%
	Seeds Yield from pods $L < 5cm$	-40%	-16%	-22%	-40%	
NUTRITIONAL QUALITY	Seed Carbon	-2%	-2%	-3%	-5%	
	Seed Nitrogen				+22%	
	Seed Sulphur	+3%	+4%	+2%	+20%	-32%
	Total Fatty Acids	+3%	+5%	-5%	-15%	+17%
	Saturated FAs		+9%		-9%	+16%
	Unsaturated FAs			-5%	-17%	+19%
	$\omega 6:\omega 3$ ratio	-9%	+1%	+7%	+9%	
	Protein concentration					+75%
PHYSIOLOGICAL QUALITY	[Raff+Stach]:Suc ratio	+12%		-15%	+2%	
	Seed conductivity				+262%	
	ABA:GA3 ratio	-5%	+28%	+30%	+28%	+66%



1

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

11

12 *Modulation of antioxidant capacities and thermotolerance acquisition as stress defense strategy*

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

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

9 Our findings also pointed out that(Wang et al., 2014) the measured concentrations of raffinose and
10 stachyose, which have been reported to protect against oxidative damage (Nishizawa et al., 2008),
11 were not increased under early mild stress modality (irrespective of the Sulphur conditions), and so it
12 was not possible to ascertain their role in the antioxidant defense pathways in our conditions (Table 3).
13 But as discussed above, this soluble sugar-related results should be interpreted carefully regarding
14 their timing and optimal biosynthesis within the maturing seeds (Baud et al., 2002; Leprince et al.,
15 2017). Indeed, our results might suggest that the temperature events (whether mild or intense) were
16 above the temperature threshold of the stepwise transfer reactions involving raffinose synthase and
17 stachyose synthase, leading to impairment of sugar biosynthesis and thus the sugar-mediated oxidative
18 response (Gangl et al., 2015).

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

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

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

8

9 *Impact of S availability on thermotolerance acquisition*

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

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

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

12

13 **Conclusion**

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

1 role of sulphur in stress memory mediated by epigenetic regulation. In contrast, sulphur restriction
2 optimized seed oil concentrations in stress-exposed maturing seeds, especially when the stress was
3 delayed, thus highlighting the need to satisfy sulphur requirements in certain climatic contexts.
4 Overall, we foresee the need for trade-offs to optimize quality criteria that are dependent on features of
5 temperature such as intensity, frequency and timing of application. High temperature acclimation
6 strategies should also include sulphur supply which restricted levels can either improve seed quality
7 criteria (e.g. total FAs, saturated and unsaturated FAs) in temperature-stressed maturing seed, lessen
8 the positive effects of a thermoprimer profile (e.g. indicators of desiccation tolerance) or amplify the
9 oxidative responses to high temperature stress when applied at sensitive stages during the maturation
10 process.

11

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33

34

1 **Tables**

Factor\Variables	Total			Pods L < 5cm			Pods L ≥ 5cm		
	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
<i>se</i>	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
<i>se</i>	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

2

3 **Table 1:** Yield components distinguishing the two pools of pods (i.e. pods_{L<5cm} and pods_{L≥5cm} at the beginning of the Temp-modality application). Results are
4 presented by factor (Temp-modality, Sulphur condition). For a given variable, different letters (Tukey multiple comparisons test) indicate the ranking among
5 Temp-modalities (including Temp-control) or between the two Sulphur conditions. P-values and levels of significance are given for Temp, Sulphur and Temp
6 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.

7

	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
<i>se</i>	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
<i>se</i>	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

1

2 **Table 2:** Nutritional seed quality criteria measured on seeds from pods_{L≥ 5cm}. Results are presented by factor (Temp-modality, Sulphur condition). For a given
3 measured variable, different letters (Tukey multiple comparisons test) indicate the ranking among Temp-modalities (including Temp-control) or between the
4 two Sulphur conditions. As SFAs are variables with highly significant interaction (Temp x Sulphur) effects, results for each individual treatment are displayed
5 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,
6 $p < 0.05$ *, $p < 0.01$ ***, $p < 0.001$ ***. SE: standard error, DW: dry weight, SSP: Seed Storage Protein.

7

Factor\Variables	Soluble sugar				Conductivity
	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
<i>se</i>	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
<i>se</i>	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

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

1 **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 27 th	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 1 st	5.5	7.1	1.3
May 2 nd	6.7	7.1	1.0
May 3 rd	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 15 th	5.2	7.2	1.4

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

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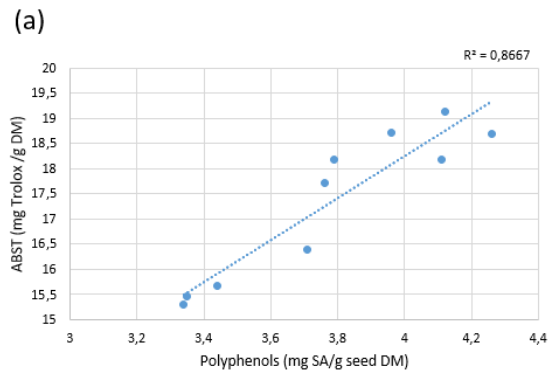
Harvest date	Harvest 1	Harvest 2
Mean Seed FW	3.31 (0.76)	5.98 (0.99)
p-value	0.02*	

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: $p < 0.05^*$.

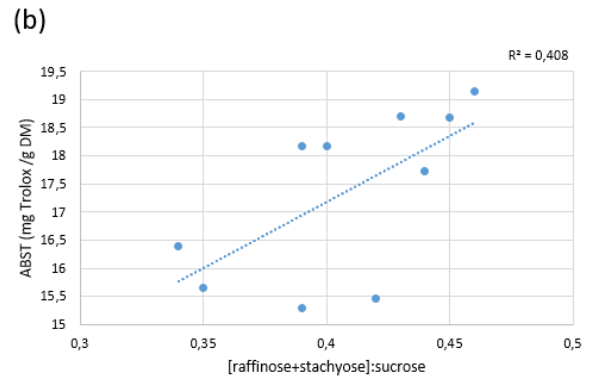
Sulphur condition	High Sulphur					Low Sulphur					Temp effect	Sulphur effect	Temp x Sulphur
	Control	Early Mild Stress	3 Late Heat Peaks	4 Late Heat Peaks	Priming	Control	Early Mild Stress	3 Late Heat Peaks	4 Late Heat Peaks	Priming			
Yield components (per plant)													
Total Seed Yield (g)	9.92 (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
$\omega 6:\omega 3$ 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**

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



Pearson correlation:
 $R=0,9309$
 $P<0.0001$



Pearson correlation:
 $R=0,6387$
 $P=0,0468$

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3 **Figure S1:** Illustration of the correlation between ABTS value related to (a) polyphenols and (b)

4 soluble sugars ratio. SA: synaptic acid, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).