 doi:10.20944/preprints202102.0461.v1

Salinity and short-term exposure to high temperature altered dead pericarp properties and diminished yield of the crop plant *Brassica juncea*

Bupur Swetha[1]†, Jeevan R. Singiri[1]†, Nurit Novoplansky[1], Rohith Grandhi[1], Jansirani Srinivasan[1], Janardan Khadka[1], Noga Sikron-Persi[1], Ivan Galis[2], Gideon Grafi[1]*

[1]French Associates Institute for Agriculture and Biotechnology of Drylands, Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Midreshet Ben Gurion 84990, Israel. [2]Institute of Plant Science and Resources, Okayama University, Kurashiki, Okayama, 710-0046, Japan.

†Equal contribution

*Corresponding author: Gideon Grafi, email: ggrafi@bgu.ac.il

 doi:10.20944/preprints202102.0461.v1

Abstract

Climate change is expected to increase the frequency and severity of abiotic stresses that lead to loss of crop yield. We investigated the effect of salinity (S), short episodes of high temperature (HS) and combination of S+HS at the reproductive phase on dead pericarps properties and yield of the crop plant *Brassica juncea*. Three intervals of HS resulted in massive seed abortion; seeds from salt-treated plants germinated poorly. Pericarp extracts of salt-treated plants reduced seed germination of *B. juncea*; all pericarp extracts completely inhibited seed germination of tomato and *Arabidopsis*; removal of pericarp extracts restored seed germination. HS reduced all metabolites accumulated in dead pericarps, except for upregulation of isomaltose and cellobiose. Salt induced alteration in metabolite levels including increase in proline, reduction in TCA intermediates and changes in phytohormone levels. Proteome analysis revealed hundreds of proteins stored in dead pericarps whose levels and composition were altered under salt stress. The integration of metabolic and proteomic data showed that changes in metabolites were highly correlated with changes in proteins involved in their biosynthetic pathways. Thus, besides providing a physical shield for seed/embryo protection dead pericarps store beneficial substances whose levels, composition and biological function are altered under stress, further highlighting the elaborated function of dead organs enclosing embryos in seed biology and ecology. The detrimental effect of HS on crop production might have implications for global food security in the face of climate change.

Keywords: Dead pericarps; salinity; short episodes of high temperature; stress response; reproductive phase; seed abortion; phytohormones, *Brassica juncea*

 doi:10.20944/preprints202102.0461.v1

1. Introduction

In virtually, all agricultural regions, abiotic stresses such as drought, salinity, and temperature extremes reduce average yields for most major crop plants by more than 50%, presenting a huge barrier to feeding an ever-growing world population [1-3]. With the expected changes in global climate, environmental stresses are likely to increase in severity leading to serious effects on crop yields [4]. Moreover, soil salinization affects an estimated 1 to 3 million hectares in the enlarged EU and is considered a major degradation process endangering the potential use of European soils [5]. The average global annual temperature has increased by 1.1^{o}C higher than the average temperature at the preindustrial era, and it is predicted to keep rising by 3-5^{o}C by the end of this century, due to accumulation of greenhouse gases in the atmosphere. Yet, climate change not only impact the average annual temperature, but also increases incidents of extreme climate events, including heat waves and hot spell [6-8]. Hot spells are the most critical factor affecting crop yield particularly when occur in combination with other stresses and during flowering and seed development [8,9]. Thus, exposure of mother plants to stress conditions during vegetative and reproductive stages has a great impact on progeny seed properties [10-12]. Recent work demonstrated that maternal environment not only affecting the embryo properties but also the properties of the maternally derived dead organs enclosing the embryo (DOEE) [13-15]. DOEEs are emerging as important components of the dispersal unit (DU) that have been evolved in plant species probably in conjunction with their habitats to carry out multiple functions to nurture the embryos and to ensure offspring success in their ecological niche [16]. Thus, besides providing a protective shield for the embryo and dispersal accessories, DOEEs also function as a long-term storage for proteins such as hydrolases, reactive oxygen species (ROS) detoxifying enzymes and cell wall modifying enzymes as well as regulatory substances that control plant growth and

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development, microbial growth as well as germination of heterologous species [16]. The expected changes in DOEEs properties as a result of maternal environment could have an impact on seed viability and persistence, germination and seedling establishment and consequently on plant population dynamics and diversity.

Crop plants are essentially highly sensitive to abiotic stresses that cause significant yield losses worldwide [3, 17,18]. Multiple studies related to the effect of heat shock on plant performance were performed under long-term exposure (often >24 h) to high temperatures (37-45^{o}C), though in recent years the effect of short episodes of high temperature (heat waves/hot spells) are getting more attention [8,19]. Here, we sought to examine the effect of salinity and short-term exposure to high temperature during the reproductive phase on progeny seed production and DOEE properties of a crop plant. To this end, we selected the crop plant *Brassica juncea* (L.) Czern & Coss. (Brassicaceae) that together with other *Brassica* species represent an important source of vegetable oil worldwide [20,21]. Like other *Brassica* and leguminous crop plants, *B. juncea* fruits are indehiscent, that is the fruit remains intact and is not splitting open at maturity. We employed multiple methodologies, including proteomics and metabolomics, to investigate the effect of maternal environment on seed progeny production and fate with a special focus on the properties of dead pericarps. Our data demonstrated that DOEE properties have been retained in crop plants similarly to wild species and highlighted the elaborated function of *B. juncea* dead pericarp as a storage entity for beneficial substances (metabolites and proteins), whose levels and composition are significantly altered in response to salinity and particularly, in response to short episodes of high temperatures.

2. Materials and Methods

2.1. Plant growth conditions and exposure to stress

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Brassica juncea (yellow mustard) seeds purchased from the local market were sown in standard gardening soil composed of peat and perlite (2:1 ratio) in small pots. Mustard seedlings were transplanted (at 18 days after sowing) into 1L-pots having red sandy soil supplemented with 4 g/L slow release fertilizer (Green Multigan 20%N, 11% P_2O_5, 16% K_2O and trace elements). Briefly, plants were irrigated with water for one month until the onset of flowering. At which time half of the plants were exposed to salt stress of 50 mM NaCl for one month until the beginning of fruit appearance. At this stage, 10 pots from each treatment (Cont and Salt) were transplanted into 10L-buckets having standard gardening soil and NaCl concentration was gradually increasing, in a 5-day manner, to 75, 100, 150 up to 200 mM. After reaching the highest salt concentration, half of the water and salt irrigated-plants were subjected to 3 intervals of heat shock treatment (37ºC, 3 h each) in a course of 4 days. The irrigation with 200 mM NaCl was continued for another week and then all pots were irrigated only with water until fruit matured and dried out, and the mustard pods were harvested for further analysis. A schematic experimental flow chart is shown in supplementary Fig. S1.

2.2. Proteome analysis

For proteome analysis, 10 mg of ground pericarps derived from control and salt-treated plants were placed in 2 ml tube with 100 µl PBS and incubated at 4 ˚C for 1 h with gentle rotation, then centrifuged at 4ºC at high speed (16,000 x g) for 10 min. 50 µl of supernatants were collected, lyophilized and stored at -20˚C until used for comparative, quantitative proteome analysis.

Proteome analysis of pericarps (three replicates) was performed by the proteomic services of The Smoler Protein Research Center at the Technion, Israel. Note, each replicate contains randomly selected pericarps from a pool of 16 plants. Raw data was processed with MaxQuant at the Smoler Proteomics Center of the Technion. Quantification and

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normalization was performed using the LFQ method. Subsequent bioinformatic analysis was carried out at the Bioinformatics Core Facility, Ben-Gurion University, using R and Partek Genomics Suite. Proteins marked as "contaminant", "reverse" and "only identified by site" were filtered out. In an additional filtering step, only proteins in which at least one of the groups (Control, Salt) had at least 2 non-zero replicates were retained. LFQ intensities were Log_2 transformed, and zero intensities were imputed (replaced) by random numbers derived from a normal distribution in the low expression range (width = 0.2, downshift = 1.6). Imputation was repeated 10 times to avoid relying too heavily on fabricated numbers. Each of the 10 imputed datasets was submitted to hypothesis testing for differential protein expression using Limma [22]. The statistical model tested the contrast between salt proteins and control proteins. A protein was considered differentially expressed (DE) if it had nominal p-value < 0.05 and absolute fold change (in linear scale) > 1.3 in at least 8 of the 10 imputed datasets. Subsequently, a more stringent cutoff was used, requiring an FDR-adjusted p-value< 0.05 in at least 8 of the 10 imputed datasets.

2.3. Primary metabolites analysis

Quantification of primary metabolites were performed using GC–MS method essentially as described [23]. Briefly, powdered lyophilized pericarps (70 mg) were extracted with 1 ml of a precooled mix containing methanol, chloroform and MiliQ water (2.5:1:1 v/v, respectively) supplemented with ribitol as the internal standard (4.5 µg/ml) and vortexed thoroughly. Following incubation for 10 min at 25ºC on an orbital shaker, samples were sonicated for 10 min in ultra-sonication bath at room temperature and centrifuged at high speed (10 min, 16,000 x g). The supernatant was collected, added 300 µl of miliQ water and 300 µl of chloroform, vortexed for 10 seconds and centrifuged for 5 min at high speed and the upper phase was collected and aliquots were lyophilized and subjected to derivatization.

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Derivatization was performed by adding 40 µl methoxyamine hydrochloride (20 mg/ml in pyridine) to the dry sample and incubation for 2 h at 37°C on a shaker platform. Samples were added 70 µl MSTFA and 7 µl of alkane mix and incubated with constant shaking for 30 min at 37°C. Samples were subjected for Gas chromatography- mass spectrometry (GC-MS) analysis (Agilent Ltd) as described in [23,24]. Separation was carried out on a Thermo Scientific DSQ II GC/MS using a FactorFour Capillary VF-5ms column. Acquired chromatograms and mass spectra were evaluated using Xcalibur (version 2.0.7) software and metabolites were identified and annotated using the Mass Spectral and Retention Time Index libraries available from the Max-Planck Institute for Plant Physiology, Golm, Germany (http://csbdb.mpimp-golm.mpg.de/csbdb/ gmd/msri/gmd_msri.html). The level of metabolites was calculated by normalizing the intensity of the peak of each metabolite to the ribitol standard. PCA, ANOVA, Student's t tests, and hierarchical clustering analysis were performed using the Metaboanalyst 4.0 [25] (Xia and Wishart, 2016).

2.4. Plant hormone analysis

Phytohormone content (abscisic acid, ABA; indoleacetic acid, IAA; isopentenyladenine, iP; trans-zeatin, tZ; jasmonic acid, JA; jasmonoyl-isoleucine, JA-Ile; gibberellin A_1, GA_1; gibberellin A_4, GA_4; and salicylic acid, SA) was determined by previously reported method [26] with specified modifications. In brief, dry pericarps were homogenized to fine powder and 50 mg of sample was suspended in 4 ml of extraction buffer [1% (v/v) acetic acid in acetonitrile/water (4:1)] with a mixture of stable isotope-labeled internal standards (IS) [26]. Suspended samples were extracted for 1h at 4°C and centrifuged at 3 000 x *g* for 10 min at 4°C. Supernatants were collected, pellets washed with addition of 4 ml extraction buffer without IS and centrifuged as before. Acetonitrile from combined supernatants was evaporated in vacuum concentrator and samples in 1% aqueous solution were purified by solid phase

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extraction using Oasis-HLB, -MCX, and -WAX cartridges (Waters Corp., Milford, MA, USA) to obtain acidic (ABA, IAA, JA, JA-Ile, SA, GA1, GA4) and basic (tZ, iP) fractions. In contrast to previous method, iP and tZ were eluted from Oasis MCX cartridge by NH_4OH/water/acetonitrile (1:8:10) after washing with 1.2% (v/v) NH_4OH solution. While SA was earlier collected from an aliquot of Oasis MCX eluate, it was now eluted in subsequent step from Oasis WAX cartridge by 3% (v/v) formic acid in acetonitrile after recovery of ABA, IAA, JA, JA-Ile, GA_1 and GA_4 by 1% (v/v) acetic acid in acetonitrile/water (4:1). After evaporation and volume reduction of each fraction, samples were analyzed on Agilent 1260–6410 Triple Quad LC/MS system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a Capcell Pak ADME-HR S2 column (Osaka Soda Co. Ltd., Osaka, Japan). In addition to column type, gradient of the mobile phases was changed from 3% to 55% in 22 min at a flow rate of 0.4 ml min^{-1} for ABA, IAA, JA, JA-Ile, GA_1 and GA_4; for SA, 3% to 98% in 8 min at a flow rate of 0.4 ml min^{-1} was used in the modified method. Gradient conditions for iP and tZ remained unchanged. Mass-to-charge ratio (*m/z*) transitions of analytes were used as described [27]. Contents of plant hormones were calculated by comparison to respective IS peaks and normalization by dry weight for each sample.

2.5. Nutrient Analysis

Fresh powdered pericarp (30 mg) or 30 seeds of *Brassica juncea* from untreated (control) and stress-treated plants (salt, HS, S+HS) were incubated with 600 µl Milli-Q for 14 hours on an orbital shaker at 4°C. After incubation samples were centrifuged at high speed (16,000 x g) and the supernatant was collected, filtered through 0.22 µm spin filter and 200 µl of each sample were diluted with 5.8 ml of Milli-Q water and subjected to nutrient analysis by the inductively coupled plasma-optical emission spectroscopy (ICP-OES) using ICP-720-ES (Varian Inc., USA). Ca, Mg, Cl, Na, P, S, K were released upon hydration from Mustard

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pericarp and also from seed release was determined by IC was determined by ICS-5000 instrument (Dionex, Thermo Fisher Scientific). Data were analyzed by Chromeleon 6.8 chromatography data system (Dionex, Thermo Fisher Scientific).

2.6. Germination assays

Germination of *B. juncea* seeds were performed in 4-8 replicates each containing 20 seeds either on a red sandy soil or on a blot paper supplemented with water. Germination was inspected daily in a course of 4 days and the final percentage of germination was recorded. The effect of extracts obtained from *B. juncea* pericarps on germination of *B. juncea* seeds was performed in a Petri dish on a blot paper supplemented with water or with control and salt-treated pericarp extracts. Germination was initially performed in the dark at 22°C, inspected daily and photographed.

2.7. Bacterial growth assay

The assay was performed essentially as described [28]. Briefly, *Escherichia coli* (ATCC 10978) were grown overnight on LB medium at 37°C, the culture was diluted, transferred to 25% LB broth and grown at 37 °C to 0.03–0.05 optical density (OD595; Epoch, Biotek, Winooski, VT, USA). To a 150 µL aliquot of the culture 50 µL of LB (control 1) PBS (control 2), 50 µl of PBS + 25% Hoagland solution, kanamycin (final concentration 50 µg/ml), caryopsis extracts (50 µl) or with 50 µl filtered (through 0.2 µm) husk extract (three replicates per treatment) in a flat-bottom 96-well microtiter plate. Plates were incubated in the dark using a spectrophotometer (Synergy 4, Biotek, USA) and reads (OD595) were taken in intervals of 30 min in a course of 12 h. The average OD for each blank replicate at a given time point was

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subtracted from the OD of each replicate treatment at the corresponding time point and standard errors were calculated for each treatment at every time point.

2.8. Statistical analysis

Unpaired t test was performed using the GraphPad QuickCalcs Web site: https://www.graphpad.com/quickcalcs/ttest1/?Format=C (accessed November 2019) or using the Microsoft Excel platform. For comparison of multiple groups we used one-way ANOVA calculator, with Tukey HSD (https://www.socscistatistics.com/tests/anova/default2.aspx; accessed November 13, 2018). All assays were repeated at least three times and representative results are shown.

3. Results

3.1. Exposure to salinity and heat stress has a dramatic impact on progeny seed production of Brassica juncea

The crop plant *B. juncea* was grown in a net house and subjected gradually, at the time flowering commenced, to increasing concentration of salt (final concentration 200 mM; supplementary Fig. S1). Thereafter, half of control and salt-treated plants were exposed to three heat shock (HS) intervals, each for 3 h at 37°C in a course of 4 days, after which all plants were irrigated with water until fruits matured and dried out. The response of plants to HS treatment was confirmed by immunoblotting with antibody to small heat shock protein 17.6 (sHSP17.6) showing strong upregulation in leaves of HS-treated plants (Fig. 1A). The average weights of a fruit and of a seed from salt-treated plants were significantly reduced (~2-fold) (Fig. 1B, 1C). The most prominent effect was the complete abortion of seeds derived from plants subjected during flowering and seed filling to HS (Fig. 1D). Seed produced on salt-treated plants were

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poorly germinated either on a blot paper (~7.5%) or gardening soil (5%) compared to seeds from control plants (93.75% and 82.5% on blot paper and soil, respectively).

Notably, plants grown in 10-L pots showed the same trend but at a lower magnitude. Accordingly, no significant differences were observed in average fruit and seed weights between control and salt-treated plants (Fig. S2A, S2B); a more pronounced effect observed in average fruit weight of HS-treated plants (Fig. S2A). Almost no abortion was observed for control plants, 21% for salt and 78% and 66% for HS and S+HS plants, respectively (Fig. S2C). Interestingly, in HS-treated plants, the abortion was fruit related, that is either all seeds within a fruit were viable or aborted. This suggests that some HS-treated fruits had probably completed their development before exposure to HS, seeds were normal in size and shape and germinated properly (Fig. S2D). Notably, under 10-L growth condition, seeds derived from salt-treated plants showed about 50% germination compared to seeds from control plants (Fig. S2D).

3.2. The effect of pericarp extracts on seed germination

All further analyses were performed with plants grown in small pots as they appeared to be more homogenous. Like many other crop plants, *B. juncea* fruits are indehiscent avoiding seed dispersal at maturity and enabling efficient harvest of the seeds by farmers. We wanted to examine the effect of maternal growth conditions on pericarp properties and its capability to control germination of *B. juncea* seeds. To this end, seeds of *B. juncea* were germinated on red sandy soil in the presence of pericarp extracts derived from control, salt (S), HS and S+HS treated plants and germination was inspected daily up to 4 days after sowing. The results showed that the most notable effect on germination was exerted by pericarps derived from salt and S+HS-treated plants showing a significant reduction in germination rate as well as in final

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germination after 96 h compared to germination in water or in control and HS pericarp extracts (Fig. 2).

Further germination assays revealed that pericarps from control and stress-treated plants possess allelopathic substances that strongly inhibited seed germination of *Arabidopsis* and tomato. However, germination was almost fully recovered after washing out pericarp extracts (supplementary Fig. S3).

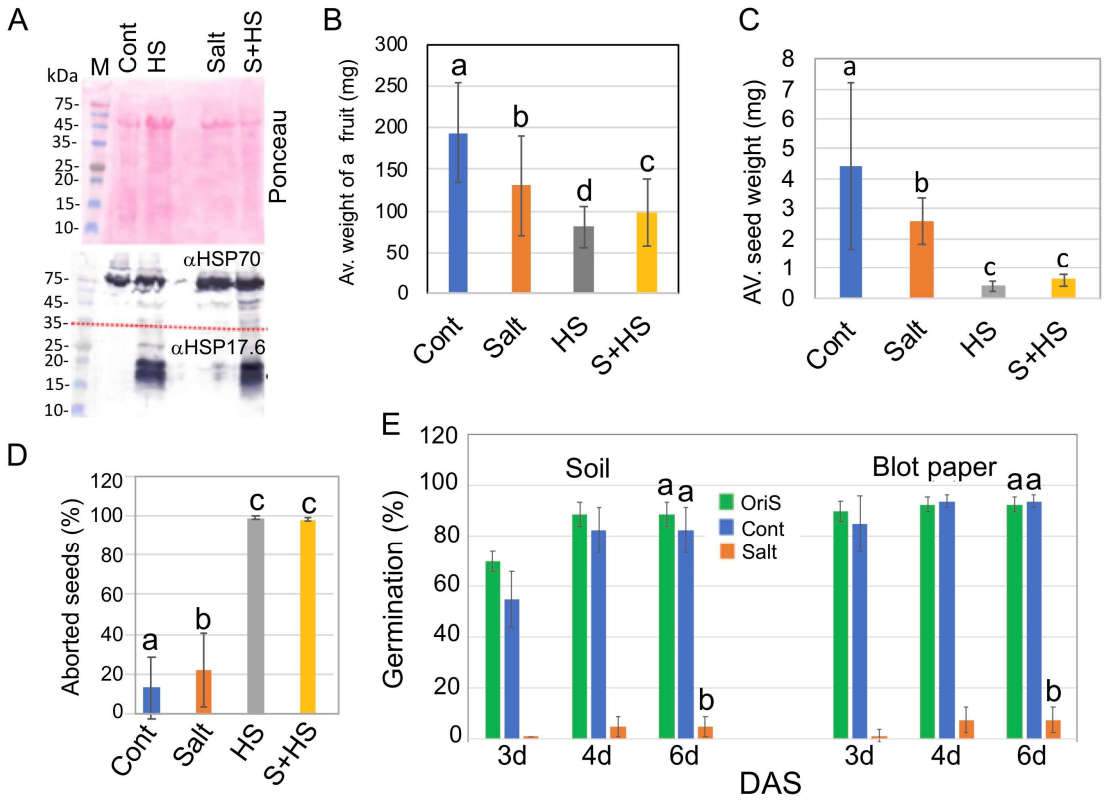

Fig. 1. Effect of maternal environment on seed performance and fate of *B. junceae*. (A) Heat shock induces expression of small HSPs. Proteins extracted from leaves of control (Cont) or heat shock (HS), Salt and S+HS-treated plants were subjected to immunoblotting (lower panel) using anti HSP70 (αHSP70) and anti-HSP17.6 (αHSP17.6). Upper panel is the Ponceau staining of the membrane. Note the membrane was cut into two parts (broken line), the upper containing proteins above 35 kDa was probed with αHSP70 and the lower part with αHSP17.6. M, protein molecular weight markers given in kDa. Significant reduction in average weight of a fruit (B) and a seed (C) following exposure to stress conditions. (D) Percentage of aborted

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seeds. Note the complete abortion of seeds under heat shock (HS) treatments. (E) Germination of seeds derived from slat-treated plants is significantly reduced. Germination was performed on red sandy soil (Soil) or on a blot paper. DAS, days after sowing. Vertical bars represent the standard deviation. Different letters indicate statistically significant differences between treatments ($P < 0.05$). Statistical analysis was performed by One-Way ANOVA Calculator plus Tukey HSD (Social Science Statistic).

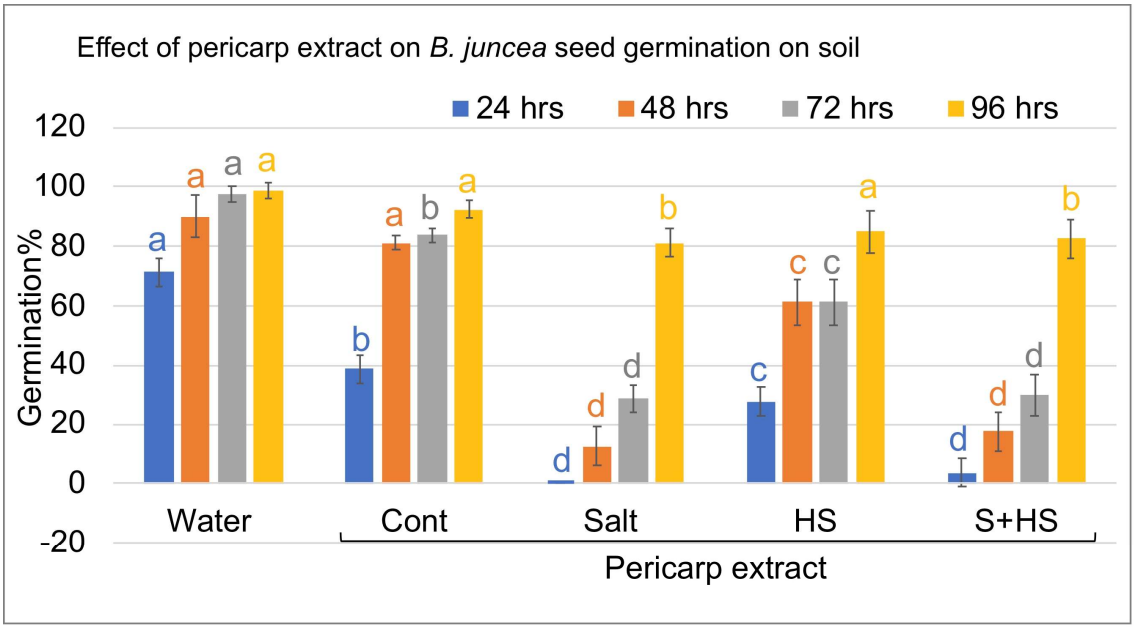

Fig. 2. Pericarp extracts delay seed germination of *B. juncea*. Pericarps derived from plants exposed to salt and heat were extracted and analyzed for their effect on seed germination compared to water. Germination was inspected at different time after sowing and recorded as percentage of germination. Cont, control plants irrigated with water. HS, heat shock applied in three intervals (3h at 37°C) in the course of one week. Vertical bars represent the standard deviation. Different color letters indicate statistically significant differences between treatments at a given time after sowing ($P < 0.05$). Statistical analysis was performed by One-Way ANOVA Calculator plus Tukey HSD (Social Science Statistics).

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3.3. Effect of maternal environment on protein accumulation in dead pericarps: proteome analysis

Proteins derived from pericarps of control or salt-treated plants were subjected to proteome analysis using LC-MS/MS on LTQ-Orbitrap followed by identification and quantification by MaxQuant, using *Brassica rapa* proteins from UniProt as a reference. After filtering out potential contaminant, reverse, only identified by site as well as filtering for proteins expressed in at least two replicates of at least one treatment group, 1167 proteins were documented (Table S1). A principle component analysis (PCA) showed (Fig. 3A) that PC 1 explains about 50% of the variance as it separates the control from salt-treated plants, demonstrating the prominent effect of stress conditions on proteins accumulated within the dead pericarps of *B. juncea*. Functional categorization for biological process (Fig. 3B) showed that among the proteins recognized in this category, 216 proteins were related to metabolic process and 56 proteins were related to response to stimulus. Categorization for protein class (Fig. 3C) identified 181 and 39 metabolite and proteins modifying enzymes, respectively, many of which are hydrolases and oxireductases (Table S2). Also 17 proteins have belonged to the chaperone class and include heat shock proteins HSP17.4, HSP22 and HSP90 as well as multiple chaperonins of the HSP60 family (Table S2). Categorization for pathway revealed many proteins involved in amino acid biosynthesis pathways, TCA cycle intermediates and in pyruvate metabolism (supplementary Fig. S4, Table S3).

Implementing stringent cutoffs and statistical testing with Limma revealed 525 differentially present (DP) proteins between control and salt-treated pericarps; 372 and 153 proteins were up- and down-accumulated in salt pericarps, respectively (Table S4). Functional categorization for biological process revealed 105 proteins are related to metabolic processes and include proteins involved in primary metabolic processes (77 proteins) and oxidation

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reduction processes (20 proteins). In addition, 25 proteins are involved in response to stimulus, including annexin D2 and pathogenesis-related thaumatin superfamily protein (Table S4).

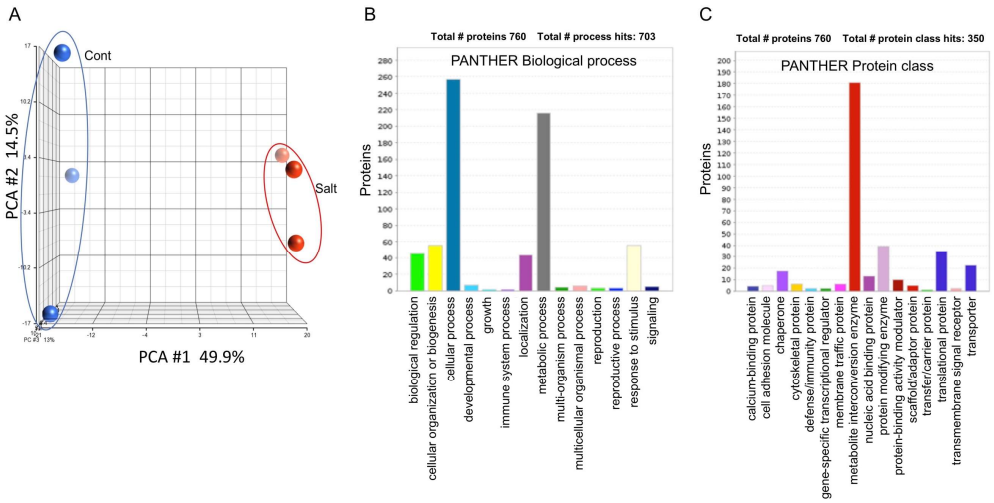

Fig. 3. Proteome analysis of pericarps. (A) Principal Component Analysis (PCA) score plots comparing the proteome profiles between pericarps derived from control (Cont, blue dots) and salt (red dots)-treated plants. Categorization of all proteins present in *B. juncea* pericarps for biological process (B) and protein class (C).

3.4. Maternal environment altered hydrolase activities recovered from dead pericarps

The proteome data highlighted certain protein groups released from dead pericarps including nucleases and proteases (Table S2). We performed in gel assays to compare hydrolytic activities released from pericarps derived from control and stress-treated plants. In-gel nuclease assays showed that pericarps of control and heat shock-treated plants displayed a notable nuclease activity toward denatured salmon sperm DNA, which was completely abolished in pericarps derived from salt-treated plants (Fig. 4A). Similarly, while pericarps of control and HS-treated plants showed strong protease activities, no activity could be recovered from salt-treated plants (Fig. 4B). Conversion assays further confirmed the presence of endonucleases in control and HS pericarps but not in pericarps from salt-treated plants (salt,

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S+HS) as demonstrated by the capacity of pericarp extracts from control and HS plants to convert supercoil plasmid DNA into relaxed and linear forms (Fig. 4C).

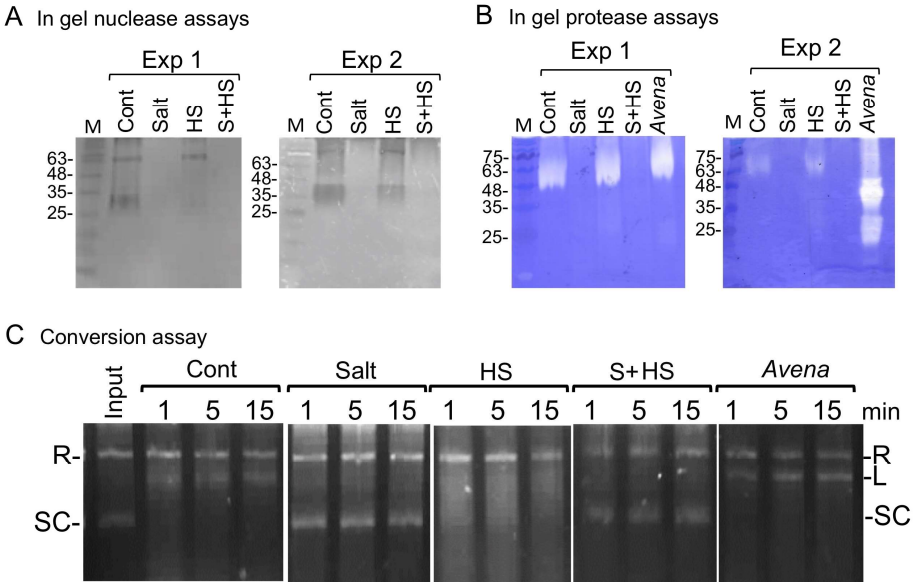

Fig. 4. Maternal growth effect on nuclease and protease activities in dead pericarps of *B. juncea*. Proteins extracted from dead pericarps collected from control, salt (S), heat shock (HS) and S+HS-treated plants were subjected to in gel assays (Exp 1 and Exp 2) for nucleases (A) and proteases (B). M- molecular weight protein marker in kDa. (C) Conversion assay for endonuclease activity. Supercoiled plasmid DNA (1 µg) was incubated for the indicated time points at room temperature with pericarp protein extracts derived from control and stress-treated plants. Input indicates the plasmid DNA used in this assay. SC, supercoil plasmid DNA; R, relaxed form, L, linear form. Extract of *Avena* lemmas and paleas was used as a reference for endonuclease activity.

3.5. Maternal environment alters primary metabolites accumulated in dead pericarps

We performed metabolite profiling of pericarps derived from control and stress-treated plants of *B. juncea* using GCMS and identified 70 primary metabolites (Table S5). A principal component analysis (PCA) of all identified primary metabolites showed that stress has significant effect on the metabolites accumulated in dead pericarps (Fig. 5A). Accordingly, the

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first principal component (PC1) demonstrates 84.5% of the variance separating between salt and other samples (control and HS samples), while PC2 accounting for 12.3% of the variance separated HS from control and salt. The relative content of many amino acids was increased under salt treatment with most notable increase in proline (Fig. 5B). Among the metabolites most affecting the separation of samples on PC1 were (in a decreasing order) proline, fructose, sucrose, glucose, pyroglutamic acid, phosphoric acid and sarcosine (based on eigenvector values), while the metabolites most contributing to the variance on PC2 were proline, valine, cellobiose, sarcosine, isomaltose and serine. Heatmap of all primary metabolites (supplementary Fig. S5) separated the metabolites according to their abundance in the different treatments. It highlighted the dramatic effect of HS treatments on the level of metabolites accumulated in pericarps inasmuch as all primary metabolites were reduced except for isomaltose and cellobiose that were up-accumulated in the pericarps. The TCA intermediates, malic acid, citric acid, fumaric acid and succinic acid were down-accumulated in pericarps derived from stress-treated plants, particularly in pericarps of HS-treated plants (supplementary Fig. S6A). Multiple sugars were up-accumulated in pericarps of salt-treated plants including glucose, fructose, maltose and mannitol but significantly reduced in HS and S+HS treated pericarps (Fig. 6 and supplementary Fig. S6B).

3.6. Maternal environment altered phytohormones accumulation in dead pericarps

Pericarps derived from control and stress-treated plants were subjected to phytohormones analysis by LC-MS. Results showed (Fig. 6) that multiple phytohormones including indole acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA), jasmonic acid (JA) and salicylic acid (SA) are accumulated in the dead pericarps and their level is significantly altered under stress conditions. Thus, while the levels of IAA and ABA are reduced under salt stress, other phytohormones, that is, GA1, GA4, JA and SA were increased

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significantly under salt stress. Interestingly, a relatively high level of SA was accumulated in pericarps regardless of treatment with most notable increase under salt and HS treatment; under the combined S+HS treatment, SA level was indistinguishable from that of the control. GA1 is not accumulated in pericarp derived from control and HS plants, but following exposure to salt stress.

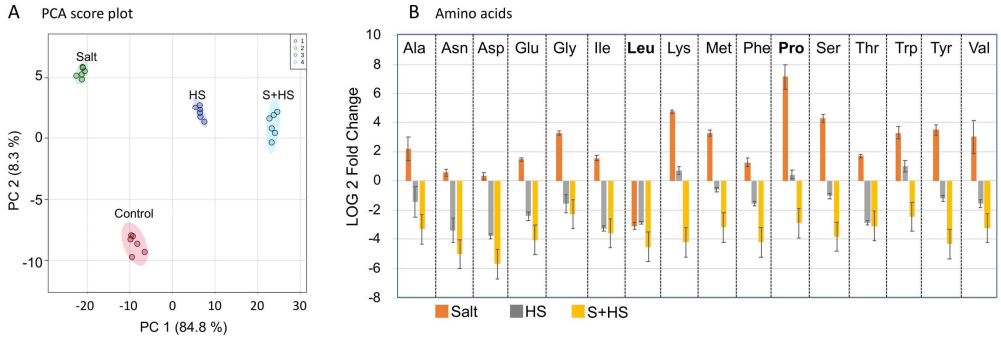

Fig. 5. Primary metabolites in dead pericarps. (A) A PCA score plot of metabolites identified in dead pericarps showing clear separation between treatments. (B) Relative abundance of amino acids shown as LOG2 fold-change of pericarps obtained from stress-treated plants *vs.* control. Amino acids are identified by 3-letter abbreviation. Vertical bars represent the standard deviation.

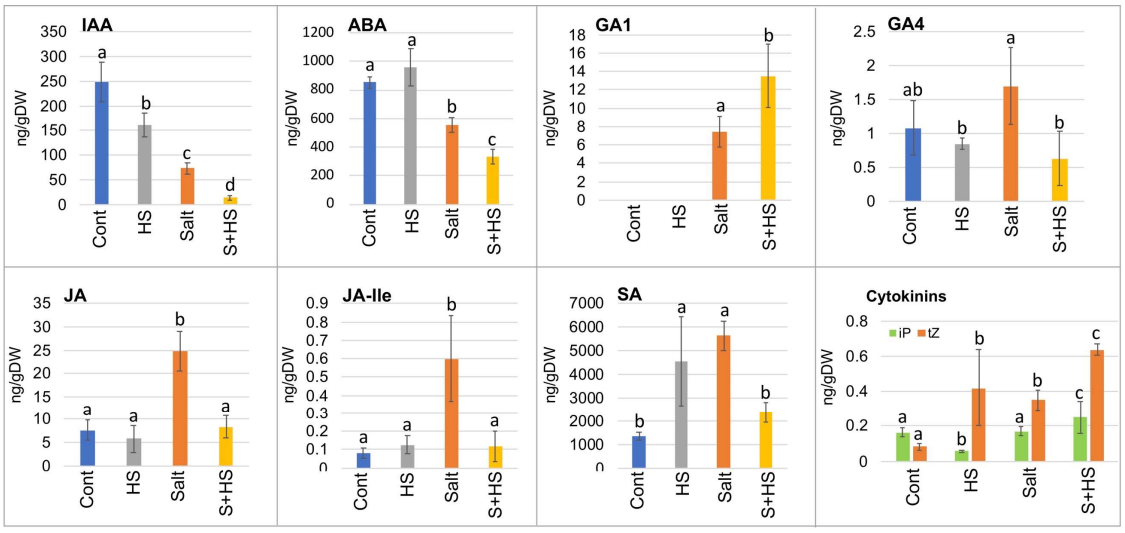

Fig. 6. Dead pericarps accumulate multiple phytohormones whose levels are modified under stress conditions. Content of phytohormones in pericarps derived from control and the indicated stress conditions is given in nanogram per gram dry weight (ng/gDW). IAA, indole

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acetic acid; ABA, Abscisic acid; GA1 and GA4, Gibberellic acid 1 and 4; JA, jasmonic acid; JA-Ile, JA-isoleucine; SA, salicylic acid; iP, isopentenyladenine; tZ, trans-Zeatin. Vertical bars represent the standard deviation. Different letters indicate statistically significant differences between treatments ($P < 0.05$). Statistical analysis was performed by One-Way ANOVA Calculator plus Tukey HSD (Social Science Statistics).

3.7. Correlation between metabolites and proteins

Among the DP protein up-accumulated in salt pericarps is the enzyme Delta-1-pyrroline-5-carboxylate synthase (P5CSA) that plays a key role in proline biosynthesis and its accumulation in pericarps is well correlated with the accumulation of proline to a very high level. Indeed, careful analysis of the proteome and metabolome data revealed that many of the metabolites whose abundance was altered in salt pericarps were highly correlated with the alteration of proteins/enzymes in their respective biosynthetic pathway. For example, reduction in the TCA cycle intermediates succinic acid (succinate) and fumaric acid (fumarate) is well correlated with up-accumulation of succinate dehydrogenase that catalyzes the conversion of succinate to fumarate and with up-accumulation of fumarase that catalyzes the hydration of fumarate to malate (Fig. 7). Likewise, reduction in malic acid (malate) in salt pericarps is well correlated with up-accumulation of malate dehydrogenase that catalyzes the oxidation of malate to oxaloacetate.

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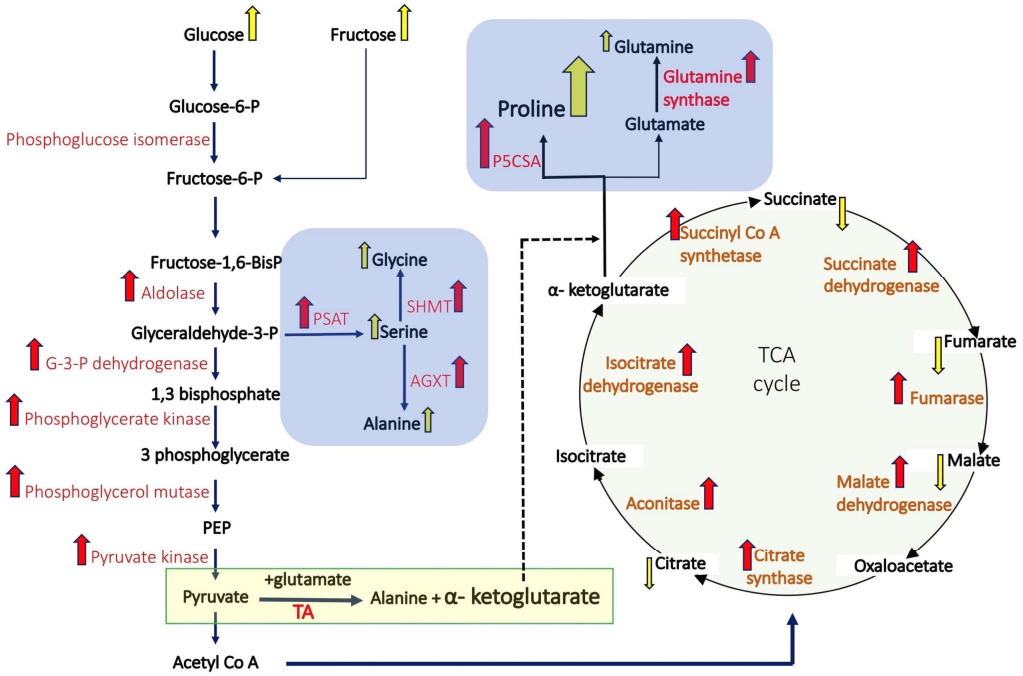

Fig. 7. Proteome-metabolome correlation analysis. The metabolites and the enzymes present in pericarp extracts of control and salt-treated *B. juncea* plants involved in the glycolysis pathway, TCA cycle and amino acids synthesis are shown. Red arrows pointing upward indicate the up-accumulation of enzymes in salt-treated pericarps. Yellow arrows pointing upward and downward indicate up-accumulation and down-accumulation of metabolites, respectively. P5CSA, Delta-1-pyrroline-5-carboxylate synthase A; PSAT, phosphoserine aminotransferase; SHMT, serine hydroxymethyl transferase; AGXT, Alanine glyoxylate aminotransferase; TA, transaminase.

3.8. Maternal environment altered nutrient levels extracted from pericarps or secreted from seeds

We used the Inductively Coupled Plasma (ICP) for analysis of nutrients released from seeds or extracted from pericarps derived from control or stress-treated plants. Results showed (Fig. 8) that the pericarps of *B. juncea* store and release upon hydration multiple nutritional elements including Ca, Mg, P, S and K whose levels were increased significantly following exposure to salt stress (S), heat shock (HS) or S+HS. Interestingly, salt treatment resulted in progeny seeds accumulating and releasing upon hydration high levels of nutritional elements

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including Mg, P, S and K. These elements were accumulated to a significantly lesser extent in progeny seeds derived from S+HS-treated plants. As expected, pericarps derived from salt-treated plants have accumulated high levels of sodium (Na) and chlorine (Cl).

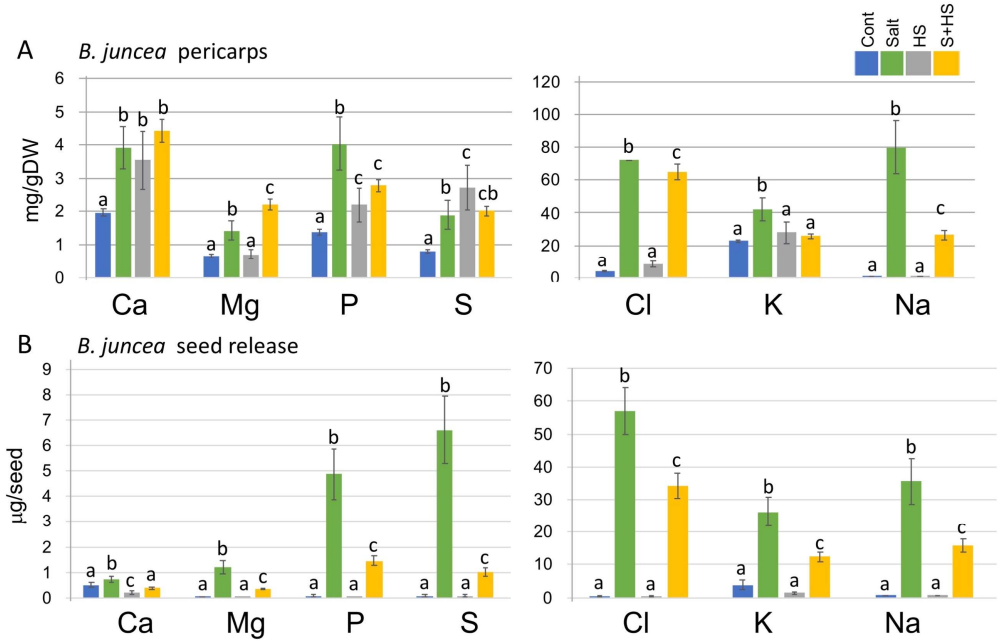

Fig. 8. Effect of maternal environment of nutrient levels. Nutrient levels in *B. juncea* pericarp extract (A) or released from seeds (B) are altered following exposure of mother plants to stress. Pericarps and seeds derived from control (cont), salt (S), heat shock (HS) and S+HS-treated plants were subjected to nutrient detection by ICP-OES. The concentration of each element was calculated either as milligram (mg) per gram dry weight (gDW) or as microgram (µg) per seed. Vertical bars represent the standard deviation. Note that salt-treated plants display (as expected) high levels of chlorine (Cl) and sodium (Na) in pericarps and seeds.

3.9. Pericarps possess bacterial growth promoting substances

We examined the potential of pericarp extracts from treated plants to control microbial growth. To this end, *Escherichia coli* and *Staphylococcus aureus* were grown in a flat-bottom 96-well microtiter plate in LB medium supplemented with PBS or with pericarp extracts of control and stress-treated plants. Ampicillin and kanamycin were used as antibiotic references

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for *E. coli* and *S. aureus,* respectively. Plates were incubated in the dark using a Synergy 4 spectrophotometer (Biotek, USA) and reads (OD_{595}) were taken at 30 min intervals in a course of 12 h. Results showed (Fig. 9) that growth of both *S. aureus* (Fig. 9A) and *E. coli* (Fig. 9B) was accelerated significantly in the presence of pericarp extracts irrespective of their source. As expected both ampicillin and kanamycin completely inhibited growth of the bacteria.

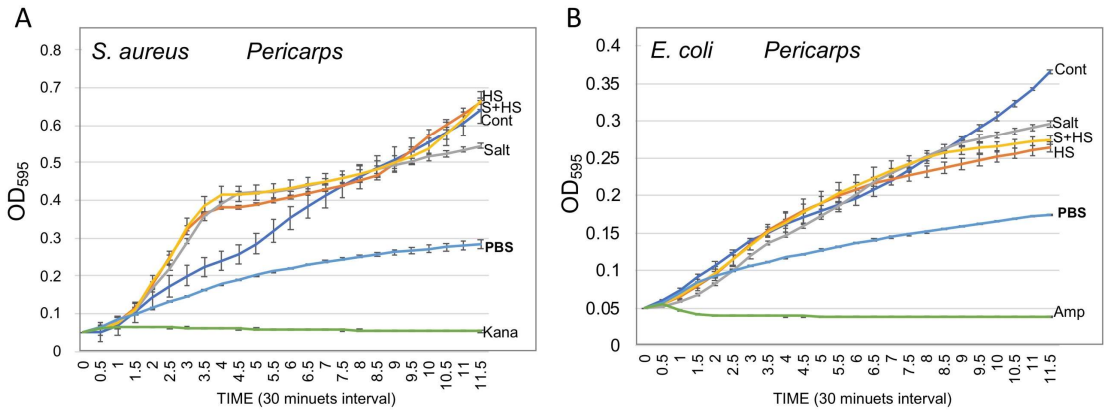

Fig. 9. Pericarps of *B. juncea* contain substances that promote bacterial growth. *Staphylococcus aureus* (A) and *Escherichia coli* (B) were grown in a flat-bottom 96-well microtiter plate in the presence of PBS (control), or in the presence of substances extracted from pericarps derived from control and stress-treated plants. Kanamycin (Kana) and ampicillin (Amp) were used as antibiotic references. Bacterial growth was monitored by measuring the OD_{595} of the culture at 30 min intervals in the course of 12 h. Each treatment was performed in triplicates and vertical bars represent the standard deviation.

4. Discussion

We describe the considerable impact of salinity and short-term exposure to heat stress during flowering and fruit setting of the crop plant *B. juncea* on progeny seed and dead pericarp properties. Our data showed that changes in maternal environment and particularly short episodes of high temperature have dramatic impact on embryo development and composition of metabolites and proteins accumulated in the dead pericarp. The study also highlighted that

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temperature fluctuations are most important in determining crop production inasmuch as short exposure (3h) of plants to high temperature (37°C) was harmful resulting in very high seed abortion. Indeed, exposure of *Brassica napus* plants to short-term heat shock treatments (4h at 35°C daily, in a course of 1 or 2 weeks) during the reproductive stage resulted in substantial loss of yield [29]. Although an increase in global mean temperature by one degree Celsius is predicted to reduce significantly the yields of major crops such as wheat, rice and maize [30] (Zhao et al., 2017), most detrimental effect on yield is exposure of crops to short episodes of high temperature (heat wave/warm spell) during the reproductive stage [31-35]. Thus, heat waves and warm spells that are predicted to increase in frequency in many regions of the world [6,36,37] pose a serious threat on global food production.

The effect of maternal environment on seed and dead pericarp properties of *B. juncea* are consistent with recent published data demonstrating the impact of abiotic stresses on the dispersal unit properties, particularly the properties of the DOEE of wild plants such as *Anastatica hierochuntica* and *Avena sterilis* [14-16]. We showed significant effect of salinity and heat on the composition and level of proteins and metabolites accumulated in dead pericarps. Proteome analysis demonstrated that hundreds of proteins are accumulated in dead pericarps whose abundance is altered under salinity including metabolite modifying enzymes involved in glycolysis, TCA cycle and amino acid biosynthesis, as well as hydrolases (e.g., nucleases, chitinases and proteases), ROS metabolizing and cell wall modifying enzymes as well as multiple chaperones and chaperonins.

Most intriguing is the dramatic effect of short exposure to heat stress on seed viability inducing a complete seed abortion. Heat stress also considerably affected the abundance of primary metabolites in dead pericarps, where the accumulation of all metabolites was significantly reduced except for isomaltose and cellobiose that were up-accumulated under heat stress; both

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are disaccharides that act as reducing sugars. Although not much is known about the role played by these sugars in plant growth and development, several reports have demonstrated increased abundance in plant tissues under stress conditions. Accordingly, an increase in isomaltose was reported under water stress conditions [38-40] or following exposure to ABA, salt and cold in the moss *Physcomitrella patens* [41]. Cellobiose is the major product of cellulose hydrolysis by microorganisms and may function as a chemoattractant for motile cellulolytic bacteria such as *Cellulomonas gelida* [42]. Also, in a recent study cellobiose was found to play a role as a "danger" signal that promote plant defenses, which are commonly triggered by microbe-derived elicitors [43].

Exposure of mother plants to salinity conditions during flowering and seed development had harmful effect on progeny seed and dead pericarp properties. Focusing on dead pericarps we showed significant alteration in storage of proteins and metabolites. Unlike exposure to short episodes of high temperature that reduced considerably the abundance of almost all metabolites in the dead pericarps, under salinity conditions the abundance of many metabolites was increased including increase in abundance of 14 amino acids and multiple sugars. Most notable is the increase in the relative content of proline (over 140-fold), which is commonly induced following exposure of plants to stressful conditions and confers stress tolerance [44-47]. The accumulation of multiple beneficial metabolites in DOEEs has been evolved to ensure the success of offspring in their changing environment by supplying defense molecules against potential soil pathogens, as well as supplying growth factors and nutritional elements for germination and seedling establishment on the one hand, and for mitigation of stress conditions on the other hand [16]. Amino acids accumulated in DOEEs, particularly under stress conditions can serve as an immediate source of nitrogen for the germinating seed [48]. Nutritional elements such as calcium (Ca), potassium (K), phosphorus (P) and sulfur (S) whose accumulation in the seed and the pericarp is induced under salinity condition might be

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available together with soil nutrients for proper growth and development of the germinating seeds and for alleviating abiotic stresses [49].

Interestingly, the analysis of the proteins and metabolites accumulated in pericarps of control *vs* salt-treated plants revealed a very high correlation between the relative content of metabolites and the levels of enzymes involved in their biosynthetic pathways. This correlation indicates that the proteins and metabolites stored in the dead pericarps might well reflect their abundance in live pericarps.

The TCA cycle, which occurs in mitochondria is important for the generation of energy in the form of ATP and reducing molecules as well as for generating multiple precursor metabolites required for the synthesis of other molecules such as nucleotides, lipids and amino acids. TCA intermediates may also function as signaling molecules that control nuclear function [50]. Under salinity conditions the relative contents of the TCA intermediates, namely, citrate, succinate, fumarate and malate were reduced significantly. This is consistent with a recent report addressing the effect of maternal environment on pericarp properties demonstrating considerable reduction in TCA cycle intermediates in pericarps of the desert plant *Anastatica hierochuntica* following exposure to salt [14]. In most cases, exposure to salinity resulted in reduced photosynthesis concomitantly with transient increase in respiration activity until carbon supply is severely depleted [51]. Although there is no a clear trend for the response of the TCA enzymes to salinity, summary of published data revealed that about 50% of the TCA enzymes were upregulated in salt-sensitive plants and about 50% were down-regulated in salt-tolerant plants [51]. Our proteome data of the dead pericarp showed that all TCA enzymes were up-accumulated under salinity stress in parallel with a notable reduction in TCA intermediates. This outcome can be explained if the TCA cycle is not well fueled by pyruvate as a result of reduction in photosynthesis or alternatively by channeling pyruvate for

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production of alanine and α-ketoglutarate, or that α-ketoglutarate produced in the TCA cycle exits the cycle and routed for production of proline.

Dead pericarps of *B. juncea* accumulate various phytohormones whose levels are significantly affected by maternal environment. We observed significant reduction in IAA and ABA content in pericarps derived from plants exposed to salt stress, which was intensified in combination with HS. This is consistent with a previous report demonstrating the accumulation of phytohormones in pericarps of the desert plant *Anastatica hierochuntica* exposed to salt stress [14]. On the other hand, other phytohormones such as GA1, JA, JA-Ile and SA were up-accumulated in pericarps derived from salt-treated plants. Notably, SA was accumulated to the highest levels compared to other phytohormones, particularly under stress conditions. SA is a well-known phytohormone that play key roles in plant immunity [52] and together with other phytohormones including ABA, IAA and ethylene are commonly used in seed priming to enhance seed performance and fate particularly under stress conditions [53] (. Thus, it is possible that phytohormones accumulated in dead pericarps might be released upon hydration to the immediate surroundings of the seeds and prime them to ensure their success in the ecological niche [53].

5. Conclusions

The data presented here highlighted the enormous impact that maternal environment during the reproductive phase, particularly exposure to short episodes of high temperature, might have on seed quantity and quality as well as on the properties of dead pericarps. Dead pericarps of *B. juncea,* commonly considered as agricultural waste, appear to function as a rich storage for multiple beneficial substances including proteins, metabolites, growth factors and nutritional elements and whose levels, composition as well as biological function are changed following mother plants exposure to stress. These data further highlighted the importance of

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dead organs enclosing the embryo in seed biology and ecology and consequently in plant population dynamics and diversity [16]. Considering yearly world production of Brassicas seeds for human consumption of about 60 million tons, roughly the same amounts of dead pericarps are thrown away and their potential use for other purposes such as food additives has not been explored to date. It might be possible to improve pericarp quality by genetic engineering that specifically direct production of valuable proteins and metabolites to the pericarp enabling the production of multiple commodities under the 'canopy' of a single crop plant.

Supplementary materials

Supplementary Figures

Fig. S1. Flow chart describing the experimental procedure.

Fig. S2. Effect of maternal environment on *B. juncea* fruit and seed properties

Fig. S3. Pericarp extracts inhibit seed germination of tomato and *Arabidopsis thaliana*

Fig. S4. Categorization of all proteins present in *B. juncea* pericarps for pathway

Fig. S5. A heatmap of primary metabolites identified in dead pericarps

Fig. S6. Relative abundance of TCA cycle intermediates and sugars

Supplementary Tables S1-S5

Table S1 : List of All proteins (Raw data) identified in the pericarp extracts of *Brassica juncea*

Table S2: List of protein groups involved in response to stress and seed germination

Table S3 : List of proteins involved in the TCA cycle, Amino acid and sugars production

Table S4: Differentially present (DP) proteins

Table S6 : Relative abundance of primary metabolites in dead pericarps

Conflict of interest

The authors declare no conflicts of interest.

 doi:10.20944/preprints202102.0461.v1

Data availability statement

The data that support the findings of this study are available in the main text and in supplementary materials.

Acknowledgments

We acknowledge Dr. I. C. Mori, Mr. T. Matsuura, and Ms. Y. Hojo for technical help with analysis of phytohormone samples. We also thank Liron Summerfield for her technical support. The instrumentation for phytohormone analyses was supported by the Japan Advanced Plant Science Network. This research was funded by the NSFC-ISF grant number 2456/18 to GG.

Author contributions

GG and NN conceived the research and designed the experiments. SB, JRS, RG and NN performed the experiments and analyzed the data with the help from JK, JS, NSP. IG performed the phytohormones analyses. GG supervised the research and wrote the paper. All authors read and commented on the manuscript and approved the final version.

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