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Physiological, Biochemical, and Molecular Response of Cucumber (*Cucumis sativus* L.) during Seed Formation Stage under Temperature Stress Conditions

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Eman El-Remaly [1*]; M. O. Kaseb[1] and A. F. Yousef [2]

[1] Cross-Pollinated Vegetables Research Department, Horticultural Research Institute, Agricultural Research Center, Giza 12619, Egypt
[2] Department of Horticulture, College of Agriculture, Al-Azhar University (branch Assiut), Assiut 71524, Egypt

ABSTRACT

Climate change is a great challenge and a prime factor limiting seed production, which is considered an integral part of food security for providing consumed yields. The experimental layout was split plot in completely randomized block design with four replicates. In 2021 and 2022, the experiments were performed under high and low-temperature stress conditions during the four growing seasons of winter and summer. The three treatments, i.e., calcium boron (Ca + B), salicylic acid (SA), and humic acid (HA) were used to mitigate the adverse effects of low and high-temperature stress on the cucumber seed set. All treatments showed an improvement in seed development. The treatments increased the activity of antioxidant enzymes peroxidase (POD, EC 1.11.1.7), superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11. 1.6), plant hormones, brassinosteroid (BRs) and ethylene in cucumber plants during seed formation stage. The study showed that the increase in the activity of antioxidant enzymes and plant hormones concentrations had an efficient role in mitigating adverse effects of temperature on seed quantity, and nutritional elements. Furthermore, add Ca + B the most improved fruit length, fruit weight, fruit diameter, seed quantity, seed quality, germination percentage, and rate, under high-temperature condition. All treatments increased the expression levels of 9 temperature stress-related genes compared with control. Ca + B application the most induced the expression of *CsGy2G003240* gene (serine/threonine-protein kinase Nek2-like isoform X1). Overall, Ca + B variant best of all improved the physiological, biochemical, and molecular response of cucumber plants during the seed formation.

Keywords: Antioxidant enzymes, Calcium boron, Ethylene, Gene expression. Cucumber seed set.

INTRODUCTION

Global food security is facing a remarkable challenge, represented by climate change (Hampton, Conner et al. 2016). It is well known that seed production is the guarantee for the durability of food crop production. Presently, the global seed trade is valued at about US$54 billion. The expansion of global seed companies has caused the global seed market increase (Le Buanec 2007, Hampton 2009). Cucumber seed yield per unit area is directly influenced by many factors, including the number of fruits, the number of seeds per fruit, and the average seed weight (Zobel and Davis 1949). Unfortunately, the seed yield of cucumber is heavily impacted by environmental factors such as temperature deviations (Golabadi, Ercisli et al. 2019). Temperature stress declines the yield and quality of the seeds due to its direct effects on the seed development stage (Nerson 2005). Rising global temperatures will impact plant growth, development, and productivity, depending on the duration of the temperature stress on various growth stages (Liu, Dong et al. 2021). Temperature stress during flowering and seed set stages can greatly diminish seed yields and decrease the international expansion of crops (Meng, Qin et al. 2004, Kläring 2017).

Although, cucumber is a warm-climate vegetable crop, it is highly affected by thermal deviations. As a thermophilic crop, the optimal developing temperature for cucumbers is 25–30 °C (Meng, Qin et al. 2004). Cucumber plants can often be damaged by temperatures above 35 °C, and lower than 18 °C, causing very high deterioration for a short period at temperatures exceeding upper limit of 45 °C and lower of 15 °C (Meng, Qin et al. 2004, Kläring 2017, Liu, Dong et al. 2021). Temperature stress-accelerated flowering can result in initiation of reproductive stage before accumulating adequate biomass resources required to produce the developing seeds (Zinn, Tunc-Ozdemir et al. 2010). Heat stress can decline the number and size of floral organs and accelerate the development of stigmas and ovules, reducing the period of their receptivity to pollen and pollen tubes (Hedhly, Hormaza et al. 2009, Zinn, Tunc-Ozdemir et al. 2010). Numerous methods are utilized to mitigate the adverse impacts of temperature, including the employment of plant stimulates that promote plant growth and yield (Knight and Knight 2001, Hedhly, Hormaza et al. 2009, Guo, Gull et al. 2022, Gupta 2022). Utilizing growth stimulants products is the most practical option for producing adapted seeds for climate change. The mechanisms of stimulation depended on plant hormones, amino acids, and nutrients, and their physiological, biochemical, and molecular roles under temperature stress (Dos Santos, Ribas et al. 2022, Gupta 2022). The vital components that have been shown to possess a significant impact on environmental stress resistance are the use of calcium and boron jointly, as they work to mitigate the destructive effects of temperature fluctuations (Bonilla, El-Hamdaoui et al. 2004). Calcium takes a key position role in the structure and functioning

[*] Corresponding author.
E-mail address: emanelrmaly@arc.sci.eg
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of cell membranes and therefore the strength of cell walls (Bonilla, El-Hamdaoui et al. 2004, Hocking, Tyerman et al. 2016, Akhtar, Ilyas et al. 2022). Boron is a very significant element in developing cells, pollination, seed set, fruit development structure, and performance of the cell wall (Brown, Bellaloui et al. 2002, Ali, Anwar et al. 2021).

The utilization of salicylic acid (SA) is extremely successful in relieving environmental stress. SA has been identified as a growth regulator that plays diverse physiological roles in early flowering, uptake of nutrients, photosynthesis process and enzyme activities and overcoming injury symptoms under stress condition including temperature tolerance (Senaratna, Touchell et al. 2000, Hayat, Ali et al. 2007, Clarke, Cristescu et al. 2009). Humic acid has a major role in mitigating thermal deviation damages by contributing to the chlorophyll synthesis and activate several enzymes that involved in photosynthesis and respiration. Among the various functional actions of HA, they are able to enhance plant growth, fruit, and seed set under adverse conditions (Kamel, El-Shoraky et al. 2018). During the temperature stress, oxidative injury occurs via generation of reactive oxygen species (ROS). Use the stimulants components have developed enzymatic detoxification systems include peroxidase (POD), catalase (CAT), superoxide dismutase (SOD) to counteract ROS, thereby protecting cells from oxidative damage (Nardi, Pizzeghello et al. 2002, Karakurt, Ozdamar-Unlu et al. 2015, Campobenedetto, Grange et al. 2020). The insufficient quality and quantity of produced seeds under temperatures stress will directly drive poor seed germination. So, the utilization of mitigating stress components increased the plant hormone secretion which enhanced necessary mechanisms for the expansion and development of the embryo, guiding to a new superior seedling (Santner, Calderon-Villalobos et al. 2009, Singh, Prasad et al. 2013). On that point, combinations of factors are controlling seed germination, including plant hormones. Interactions between plant hormones and genes affect seed formation at various conditions. Hence, adjusting the endogenous levels of plants hormones (i.e., abscisic acid, ethylene, and brassinosteroids) and altering the expressions of genes could affect the molecular biology of seed formation. Plant hormones including ethylene, and brassinsteroids are biochemical substances regulating many physiological and biochemical operations inside the plant (Miransari and Smith 2014, Li, Euring et al. 2021). As a response of hormonal balance in the seed, environmental parameters, including temperature, can influence seed germination through increase in ethylene content under stress conditions (Miransari and Smith 2014, Li, Euring et al. 2021). Brassinosteroids (BRs) are very important plant hormones which have wide activities in plant growth and development including cell growth, vascular formation, reproductive growth, seed germination, and production of flowers, fruit, and enhance seed germination (Ali, Anwar et al. 2022). BRs stimulate the synthesis of ethylene to motivate together seed germination by rupturing testa and endosperm, while antagonistically interacting with the inhibitory effects of abscisic acid on seed germination (Xia, Wang et al. 2009). It was recently discovered that calcium can activate The Serine/Threonine Kinases which play a prolific role in the regulation of cell proliferation, programmed death, cell differentiation, and embryonic development (Hsueh 2009, Janczarek, Vinardell et al. 2018). At molecular level, temperature stress regulates the expressions of several genes encoding embryogenesis proteins, mitogen-activated protein kinases (MAPKs), DNA-binding One Zinc Finger (DOF) transcription factor family, Putative ethylene-responsive transcription factor-like, The Serine/Threonine Kinases and stress related proteins during fruits and seeds formation (Cuevas, Abell et al. 2007, Noguero, Atif et al. 2013, Janczarek, Vinardell et al. 2018).

The current study was conducted to produce superior seeds in quality and quantity by alleviating the temperature stress deviations and adverse effects during the cucumber seed sets within the summer and winter seasons. It was hypothesized that exogenously applied Ca + B, SA and HA would help the plants to mitigate the adverse effects of temperature stress. The stress amelioration was studied through physiological, biochemical, and molecular attributes of cucumber.

MATERIALS AND METHODS

1. Experimental location and climate conditions

The plant experiments complied with local and national regulations and followed the rules of Cross-Pollinated Vegetables Research Department, Horticultural Research Institute, Agricultural Research Center (Giza, Egypt). All experiments were performed under greenhouse conditions at the Horticultural Research Departments of Horticultural Research Institute, Agricultural Research Center, Dokki, Egypt. It was carried out during four consecutive growing seasons of summers and winters of 2021 and 2022. The maximum and minimum air temperatures (°C) were recorded by BST-DL13 (B091BRMT7C) thermometer (Table 1). The stress hours for cucumber were calculated as the number of hours higher than 35 °C and lower than 18 °C for summer and winter, respectively. The experimental layout was split plot in completely randomized block design with four replicates . The seeds of the cucumber, Beit alpha cultivar, were obtained from Cross-Pollinated Vegetables Research Department Germplasm Gene Bank. The seedlings were transplanted into the experimental greenhouses with drip irrigation and polythene mulch, on April 22 and April 25 for summer and on September 15, and September 23 for winter in 2021 and 2022, respectively. The seedlings were transplanted on both sides of seedbed (7 $\times$ 1 m^2) with 50 cm space between plants. The experimental unit consisted of 28 plants. Three treatments, calcium boron (200 mg L^{-1} CaO and 0.3 mg L^{-1} B), salicylic acid (100 mg L^{-1} SA), humic acid (3 g L^{-1} HA) with four replicates were applied three times, 20, 40, and 60 days after transplanting. The plants without any treatment were maintained as control. Pollination of the flowers was done manually for 6 fruits on each plant, and the other female flowers that appeared formed were removed to confirm optimal seed filling of the pollinated fruit. Seed harvesting was done at the physiological mature stage where fruit turned yellow and soft. Seeds were extracted, washed, and naturally dried. The seeds from individual fruit were counted and weighed.

2. Morphological characteristics assessments

Phenotypic data were recorded on 20 plants to assess fruit traits i.e., average fruit weight (g) at mature stages, fruit length (cm), fruit diameter (cm), number of mature fruits on the plant, number of days to open first female flower, number of days to seed mature, number of seeds per fruit, weight of seeds per fruit, total seeds weight per plant. For evaluation of quality of produced seeds 100 seeds were randomly selected to estimate seed germination percentage, rate, and seedling length.

GRI % d^{-1}=(G1/1) + (G2/2) + (G3/3) +⋯+(GX/X)

Where, GRI=Germination Rate Index % day^{-1}; G1=Germination percentage × 100 at the first day after sowing, G2=Germination percentage × 100 at the second day after sowing.

Table 1. Actual monthly mean day and night air temperatures (°C)

Season	Month	2020			2021		
Season	**Month**	**Max. Temperature**	**Mini. Temperature**	**No. stress hours>38**	**Max. Temperature**	**Mini. Temperature**	**No. stress hours>38**
Summer	April	40 °C	18°C	120	40.6°C	17°C	126
	May	45°C	23°C	150	46°C	20.5°C	162
	June	45.6°C	25°C	180	46.3°C	24°C	177
	July	47.3°C	25.4°C	210	47°C	25°C	216
Season	Month	Max. Temperature	Mini. Temperature	No. stress hours<15	Max. Temperature	Mini. Temperature	No. stress hours<15
Winter	September	38°C	19°C	45.3 h	36.3°C	18°C	46
	October	35°C	17°C	63 h	33.8°C	16.8°C	65.5
	November	28°C	14°C	75 h	26°C	13.5°C	78
	December	23°C	12°C	90.5 h	21°C	12°C	92.8

The GRI reflects the percentage of germination on each day of the germination period. Higher GRI values indicate higher and faster germination after modification (Esechie 1994).

Final germination percentage (FGP)= [(number of germinated seeds) / (number of cultivated seeds)] ×100

The higher FGP value indicate the greater the germination of seed population according to Scott, Jones et al. (1984).

3. Analysis of mineral elements and protein

Seed samples were ground for every treatment using, The Ultra-Turrax machine (Ika-Werke, Staufen, Germany) and the grinder tube was washed with ethanol after each sample preparation to avoid contamination. The content of macro and micro mineral elements, calcium (Ca), Boron (B), magnesium (Mn), manganese (Mg), nitrogen (N), potassium (K), phosphorus (P), sodium (Na), and zinc (Zn) were estimated in seed samples using an atomic absorption spectrophotometer (Varian AA240; Varian, Palo Alto, CA, USA). The nitrogen content was measured using the Kjeldahl apparatus. Dried, powdered seed samples were analyzed for protein content using official methods of analysis as described by the Association of Official Analytical Chemists (AOAC 2000).

4. Plant hormones estimation

Concerning, brassinosteroid analysis, ripe fruit samples were ground with 10 mL of 80% methanol extraction solution containing 1 mM butylated hydroxytoluene. The mixture was incubated for 4 h at 4 °C. The samples were centrifuged for 10 min at, 3500 g. The supernatants were filtered through a C18-Sep-Pak cartridge (Waters, Milford, MA, United States), and the efflux was collected and dried. The mixture dissolved in 2 mL of PBS containing 0.1 % (v/v) Tween 20 and 0.1% (w/v) gelatin (pH 7.5). The samples were analyzed via indirect enzyme-linked immunosorbent assay. The calibrating samples (epibrassinolide, CAS: 72962-43-7) or test samples (150 μL per well) were put in wells of the plate with the immobilized antibodies. Plates were incubated at 37°C for 30 min. The horseradish peroxidase (HRP)-conjugate (150 μL) was placed within the wells and plates were incubated at 37°C for 20 min. Then, removed the liquid from the wells and washed plates quadruple with washing buffer. Added TMB solution (containing H_2O_2) to the wells and placed the plates at 37 °C for 20 min. Quenched the reaction by adding 2 mol L^{-1} H_2SO_4 (50 μL) into each well. Measured optical absorbance at 450 nm. Calculated the concentration in keeping with the calibration curve (Swaczynová, Novák et al. 2007, Pradko, Litvinovskaya et al. 2015). For ethylene assay, the cucumber mature fruits were determined. Each sample was placed in a 65 mL container with a piece of wet cotton at the bottom and filled with a rubber stopper. After incubation at 23 °C for 1 h, the containers were opened to volatilize the ethylene-induced by cutting. Samples were incubated in a sealed container at 23 °C for another 10 h in the dark and 10 μL of gas was released with a syringe and injected into a gas chromatograph (Agilent 7890B-5977A) equipped with a flame-ionization detector and a capillary column for ethylene measurements. All determinations were made in triplicate (Wang, Zhu et al. 2011).

5. Antioxidant Enzymes Extraction and Activity Assay

For the enzymes assay, 0.3 g of mature fruits were used with 25 mM ice-cold sodium phosphate buffer (pH 7.0). For the homogeneity, the samples were centrifuged at 4 °C for 20 min at 12,000 *g*, and supernatants were used in the finding of enzymatic activity. Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activities were assayed. The POD (EC 1.11.1.7) determination was done spectrophotometrically at 470 nm (Onsa, bin Saari et al. 2004). Catalase activity (CAT, EC 1.11.1.6) was resolute by the enzymatic decomposition of H_2O_2 at 240 nm (Aebi 1984). Superoxide dismutase (SOD) activity was determined by measuring enzyme absorbance at 560 nm using a spectrophotometer (model UV-2401 PC,Shimadzu,Milano,Italy).

6. Identification of genes involved in stress mitigation

The protein sequences of genes involved in, BR, ethylene and antioxidant (AOX) metabolism and transport in *Cucumis sativus*, *Cucumis melo*, *Cucurbita moschata*, *Cucurbita pepo*, *Cucurbita pepo subsp. pepo, Arabidopsis thaliana*, *Cucurbita maxima*, *Luffa aegyptiaca*, *Corchorus capsularis,* and *Momordica charantia* were downloaded from http://cucurbitgenomics.org/organism/16, https:// www. uniprot. org/ uniprotkb/ Q07474/ entry, https:// www. arabidopsis.org/ servlets/ Tair Object? type=locus&name=AT5G20240, and https:// www. ncbi.nlm.- nih.gov/protein/778694283. These sequences were used as queries for protein blast analysis against the cucumber reference genome database (Cucumber (Gy14) v2 Genome, Cucurbit Genomics Database (CuGenDB). The prediction of the candidate genes was based on the gene annotation in the reference genome of cucumber "Gy 14 V2.0" http://cucurbitgenomics.org/organism/16. Genes associated with seed formation such as ethylene, BR, and AOX were selected. Based on the resequencing data of plants polymorphisms of the selected genes were examined. Characteristics and structural analysis of genes associated with temperatures stress mechanisms in cucumber seeds were done. Names of 9 genes along with their accession numbers, their location, and their function annotation (Table S1) were retrieved from two online tools: i) http://cucurbitgenomics. org/organism/16 and ii) ExPASy http://web. expasy. org/computepi/databases. MEGA X software was used to draw phylogenetic trees. Clustal W tool was used to align protein sequences and neighbor-joining method with 1000 bootstrap replicates to construct trees (Hall 2013).

7. Total RNA Isolation, cDNA Synthesis and gene Expression Analysis by qRT -PCR

Genes linked to the ethylene, BR, and AOX metabolism and transport were selected from the cucumber genome database (Cucumber, Gy14) v2 Genome, Cucurbit Genomics Database (CuGenDB) (Table S1). To check the expression patterns of selected genes in cucumber, fruits were collected 20 days after pollination, and RT-qPCR was performed consistent with the manufacturer's instructions, total RNA was extracted using RNA Kit (TianGen, China). 1% agarose gel was accustomed to check RNA degradation and contamination. RNA quality and integrity were checked using Nano Drop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Extracted RNA was used for the cDNA synthesis for RT-qPCR, with M-MLV polymerase (Promega, USA) a Bio-Rad iQ1 real-time PCR system (Bio-Rad, Hercules, CA, USA). Specific primers for each gene are listed in Table S2. *Actin1* was used as a reference gene for normalizing gene expression values (Xie, Liu et al. 2018). Three independent biological replicates were used for gene expression analysis, the complete data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Jarošová and Kundu 2010).

8. Statistical analysis

Data were statistically analyzed, as split plot design with five replicates using analyses of variance (ANOVA) in both seasons with the Stat soft statistical package (MSTATC) software program (Michigan State University, East Lansing, MI, U.S.A.). Probabilities of significance differences between treatments were compared by Duncan's multiple range tests at 5% probability level (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

Results

1. Fruit characteristics

The treatments were investigated on cucumber plants under high and low-temperature stress conditions, the average of mature fruit weight, the typical fruit length, and the average fruit diameter were measured. As shown in Figure 1 according, the seasonal effect of average fruit weight at maturity stage and fruit diameter significantly increased within summer season, while no significant differences were observed in fruit length for both seasons. To study the role of treatments on fruit characteristics, Ca +B treatment gave the heaviest fruit weight, largest diameter, and highest length compared with other treatments and control. With relation to interaction effects between the treatments and the cultivation season, significantly the highest fruit parameters were recorded for treated plants compared to untreated control ($p \leq 0.05$) under summer season. However, there was significant increase within the fruit weight, length, and diameter with relevance Ca + B treated in summer season stress followed by the same treatment under winter conditions stress compared with other treatments and control. On the other hand, the seasonal effect clearly leading to a reduction in fruit numbers in winter compared to summer season without any significant differences. Specifically, the quantity of matured fruits was increased from 2 to 4 fruits/ plant due to the treatment effect where calcium boron-treated plants had the best fruit number compared with other treatments and control (Figure 2). Interestingly, a positive trend was observed, with salicylic and humic substance-treated plants altogether fruit characteristics in summer than winter.

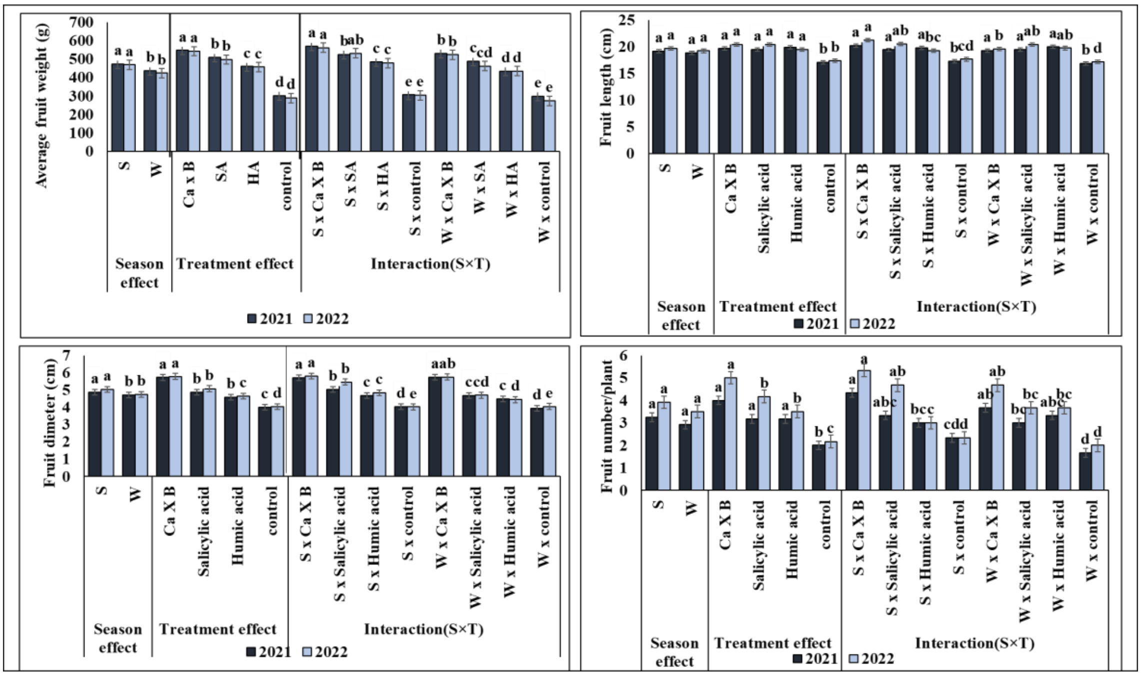

Figure 1. Effects of seasonal variation, different temperatures stress [Summer (S) and Winter (W)] mitigation treatments, and their interaction on the average fruit weight (g), fruit length (cm), fruit diameters (cm), and fruit numbers/ plant. Different small letters represent a significant difference at 0.05 level of probability by Duncan's test.

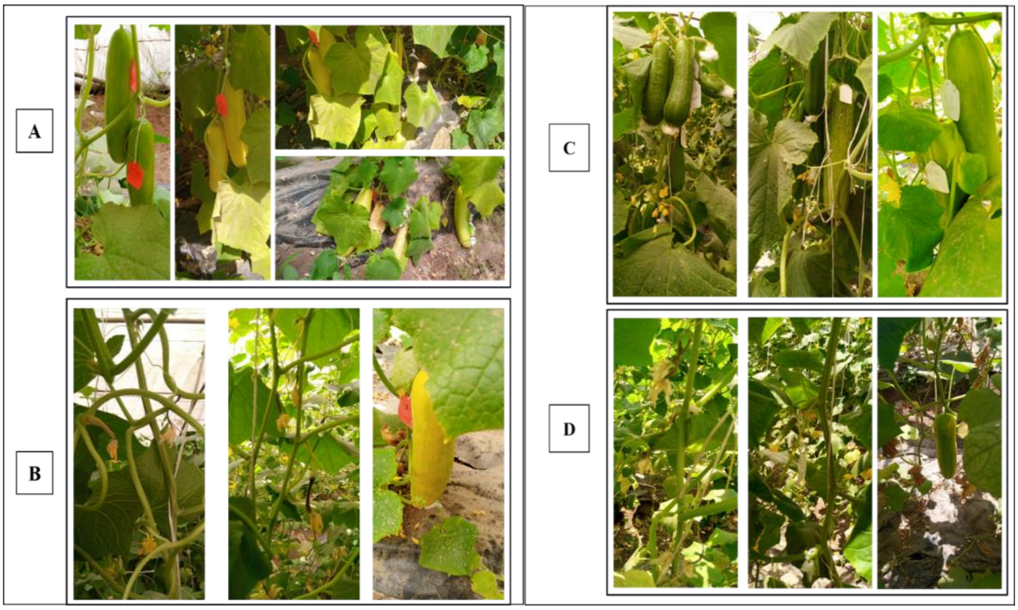

Figure 2. The calcium boron treatment effects on the fruit set (A and C) compared with the control fruit set (B and D) under high and low temperatures stress conditions, respectively.

Effects of seasonal temperature stress on the number of days to first female flower opening and days to seed formation are presented in Figure 3. The number of days to first female flower and number of days to seed formation within the Ca + B, salicylic, and humic substance treated plants were significantly under within the control plants, with the positive trend towards the summer season which is the normally expected early seed yield. Furthermore, Ca+ B treated plants significantly had the smallest amount of period to flower opening and seed formation under heat stress conditions.

2. Seed characteristics

There were significant differences between the number of seeds fruit^{-1} and the seed weight (g) fruit^{-1} between all treatments, where the Ca + B had the best effects on the number and weight of seeds (Figure 3). Furthermore, the season affects significantly seed number and weight, with a significant increase in summer season. Regarding, temperatures stress, some treatments were accustomed to alleviate the stress injure on seed quality and quantity. The treatment Ca+ B encouraged seed number and weight under heat stress season (Figure 4 A, B, C, and D). The whole number of seeds was 522 seed fruit^{-1} as average two summer seasons in treated plants with Ca + B under heat conditions compared with 179 seed fruit^{-1} for control plants under low temperature which weighted 14.14 g fruit^{-1} and 3.85 g fruit^{-1}, respectively (Figure 3).

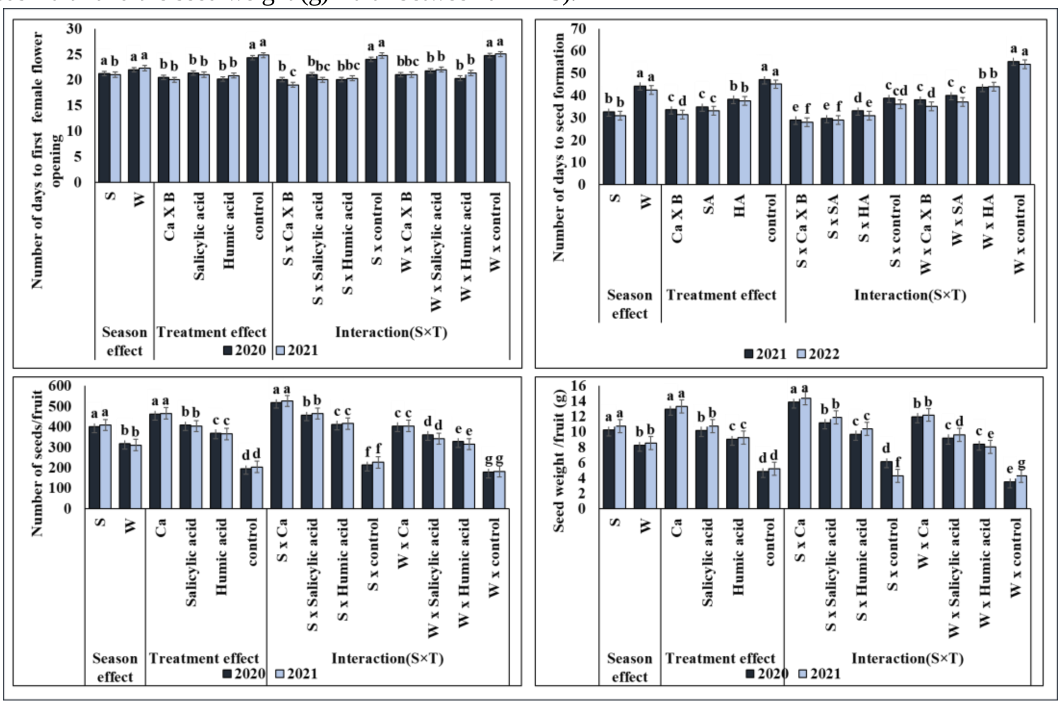

Figure 3. Effects of different temperatures stress [Summer (S) and Winter (W)] mitigation treatments on number of days to first female flower opining, number of days to seed formation, number of seed /fruit, and seed weight fruit^{-1}. Different small letters represent a significant difference at 0.05 level of probability by Duncan's test.

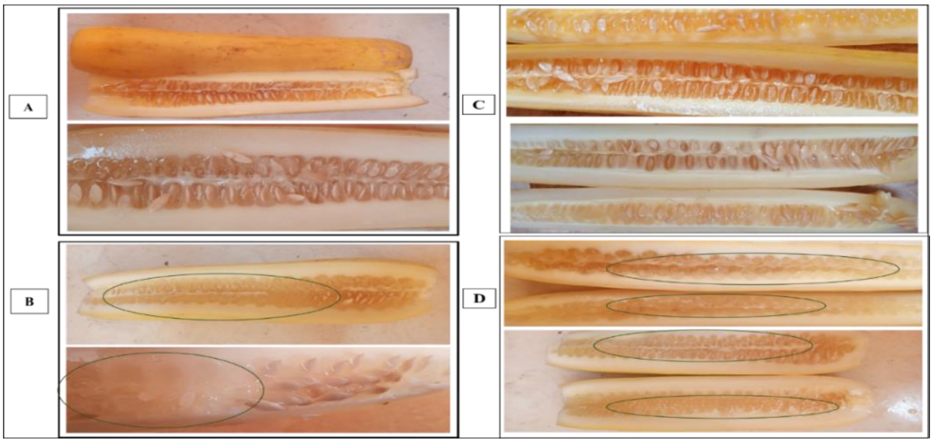

Figure 4. the calcium boron treatment effects on the seed set (A and C) compared with the control seeds set (B and D), all circled areas showed no seeds formed under high and low temperatures stress conditions, respectively.

The Ca + B, SA, and HA significantly influenced positively - total seed yield of plant^{-1} was 59.87, 37.205, and 29.15 g plant^{-1}, respectively, under high- temperature stress and 44.24, 27.85, and 28.05 g plant^{-1}, under low temperature stresses conditions, respectively (Figure 5). The total seed yield of plant^{-1} dramatically decreased to 14.29 and 5.6 g plant^{-1} in high and low temperatures for control plants, respectively. Some characteristics were evaluated to determine the standard of produced seeds. The stress conditions for mother seeds decreased the produced seedling length, however, the treatments with Ca +B, SA, and HA significantly increased the seedling length (Figure 5). The seedling length of mother seeds produced from the Ca + B gave the tallest seedling. The germination percentage differences were observed consistent with the treatment (Figure 5). Overall, the effects of Ca +B were greater than other treatments on germination percentage, that led to the germination of some seeds inside the fruit under heat- stress conditions (Figure 6), particularly at high temperatures. The germination rate of seeds with Ca +B, SA, and HA pretreated mother plants increased, especially under Ca +B, reaching 91.3 % (Figure 5). However, there was no obvious difference in the rate of seed germination between SA and HA treatments.

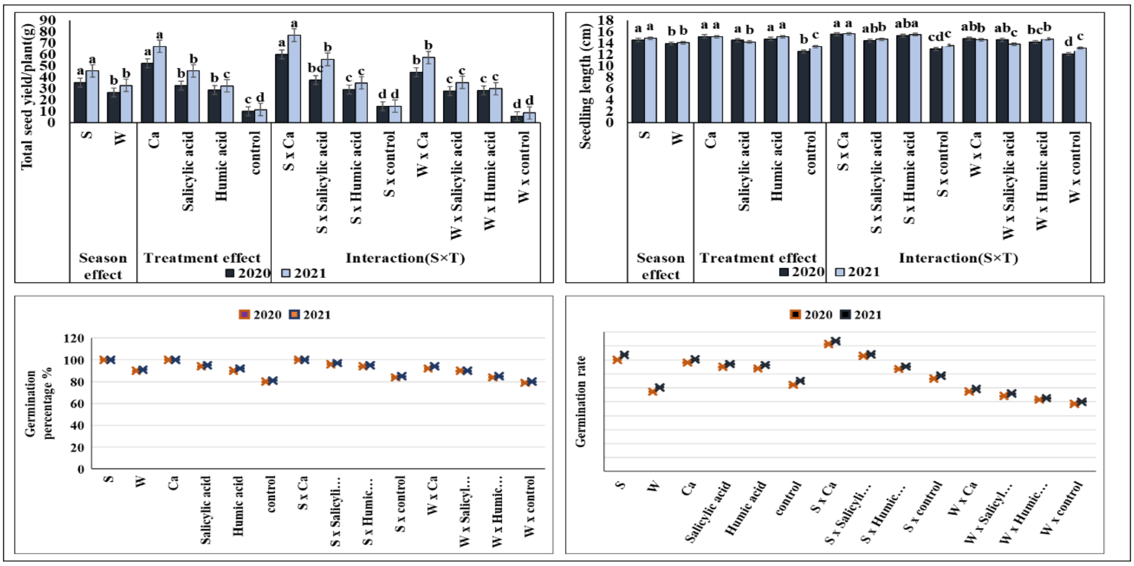

Figure 5. Effects of seasonal variation, different temperatures stress [Summer (S) and Winter (W)] mitigation treatments, and their interaction on total seed yield/ plant, seedlings length, germination percentage, and germination rate, in produced seeds. Different small letters represent a significant difference at 0.05 level of probability by Duncan's test.

Figure 6. The calcium boron treatment effects on seed inside the fruit led to seed germination under high temperature stress.

3. Antioxidant enzymes and endogenous plant hormones

The treatments markedly enhanced the enzyme activities of POD, CAT, and SOD in cucumber fruits (Figure 7). Regarding temperature degree stress, the summer season with heat stress conditions induces the AOX significant increase in cucumber fruits compared with low-temperature stress. At treatment, Ca + B, SA, and HA, motivated AOX at high-temperature more than in low-temperature seasons compared with control. POD, CAT, and SOD activities in control plants were significantly lower than the enzyme activities in treated plants. According to the interaction effect, Ca+ B proved the highest activity of AOX with high-temperature stress conditions followed by low-temperature stress conditions. In addition, SA induces a higher significant increase in AOX compared with HA application and control under two-season conditions.

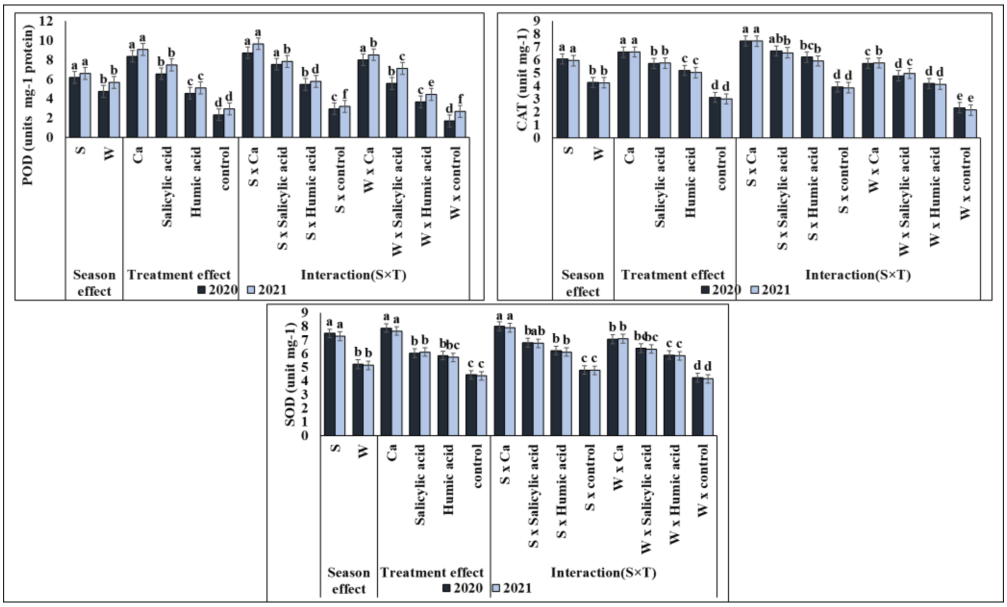

Figure 7. Effects of seasonal variation, different temperatures stress [Summer (S) and Winter (W)] mitigation treatments, and their interaction on POD, CAT, and SOD enzymes activities in mother plants. Different small letters represent a significant difference at 0.05 level of probability by Duncan's test.

The BR and ethylene contents are presented in Figure 8. Regarding, the seasonal temperatures effect there is a significant difference between high and low-temperature conditions with the obvious increase and vast superiority for high-temperature stress for BR (Figure 8) and ethylene (Figure 8). As for the treatments effect, the Ca + B led to a vast increase in BR and ethylene contents compared with other treatments and control. The results proved that the SA application gave a significant increase in BR and ethylene compared with HA and control. According to the intervention effect, the results showed significant variation between all treatments compared with the control. The Ca + B treatment induced the highest BR and ethylene concentration under high-temperature stress followed by SA and HA treatments under summer season conditions with a remarked increase with high temperature compared with all treatment's effects in winter season with low-temperature stress conditions.

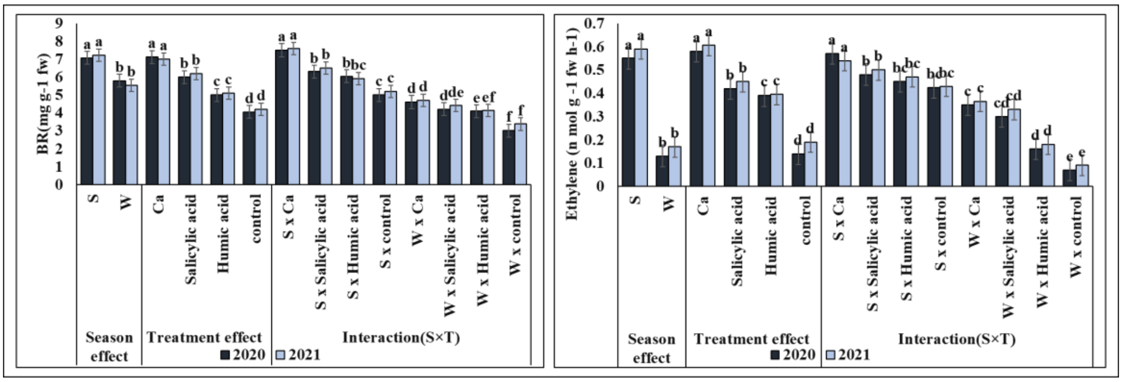

Figure 8. Effects of seasonal variation, different temperatures stress [Summer (S) and Winter (W)] mitigation treatments, and their interaction on BR and ethylene in mother plants. Different small letters represent a significant difference at 0.05 level of probability by Duncan's test.

4. Mineral elements and protein contents in produced seeds

Mineral elements and protein concentration in produced seeds are presented in Figure 9,10, and 11. Generally, the seed produced from Ca + B treated plants revealed the highest concentrations of all major (N, P, K) and minor (Mg, Mn, Zn, Ca, B, Na) elements. The nitrogen concentration was under high-temperature conditions higher than under low-temperature conditions (Figure 9). The data revealed that N concentration reached 12 mg g^{-1} for Ca + B treatment. The protein content in seeds is positively related to N content. The protein amount of cucumber seeds reached 75 mg g^{-1} with Ca + B application under heat-stress conditions (Figure 9). All results proved that all treatments significantly increased the protein concentration in seeds with the highest content towards the Ca + B treated plants. Significant differences were observed between treatments, therefore the produced seeds from pr-treated mother plants had the highest mineral elements concentration compared with seeds of non-treated plants. All results proved that the Ca + B showed the highest elements concentrations in seed under high temperature, followed by the SA and HA treatment compared with the control. Cucumber seeds which produced from Ca + B treated plants contained a higher amount of Nitrogen (12 mg g^{-1}),(Figure 9) phosphorous (9.5 mg g^{-1}), potassium (22.6 mg g^{-1}), calcium (15.65 mg g^{-1}), sodium (9.3 mg g^{-1}) (Figure 10) magnesium (6.7 mg g^{-1}), manganese (0.23 mg g^{-1}), zinc (0.8 mg g^{-1}), and boron (0.95 mg g^{-1}) (Figure 11) when compared with mineral composition of control seeds. The high-temperature stress encourages a significant increase in mineral concentrations than under low-temperature conditions with various treatments. It is worth noting that SA treatment revealed higher effects than HA, except with the boron concentrations.

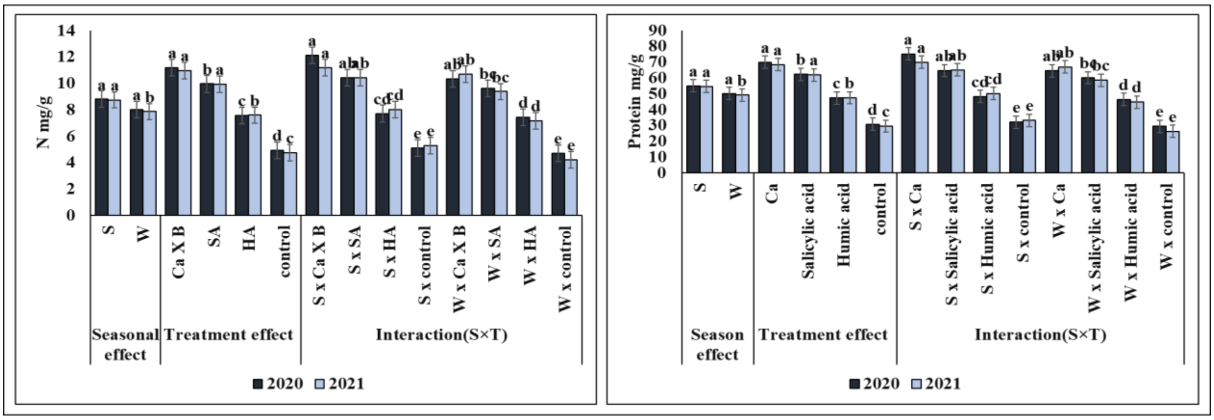

Figure 9. Effects of seasonal variation, different temperatures stress [Summer (S) and Winter (W)] mitigation treatments, and their interaction on nitrogen and protein contents in the produced seeds. Different small letters represent a significant difference at 0.05 level of probability by Duncan's test.

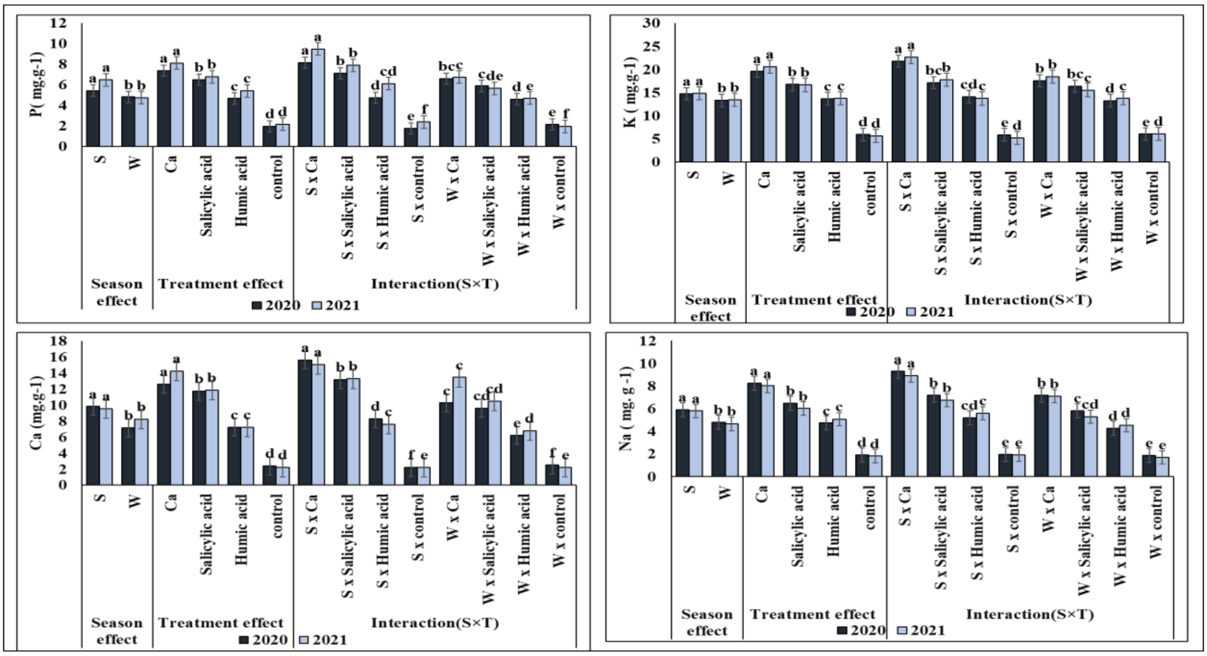

Figure 10. Effects of seasonal variation, different temperatures stress [Summer (S) and Winter (W)] mitigation treatments, and their interaction on phosphor, potassium, calcium, sodium, in the produced seeds. Different small letters represent a significant difference at 0.05 level of probability by Duncan's test.

5. Gene expressions

To further understand the plant response to high and low-temperature stress, the candidate genes and the gene expression analysis were carried out and presented in Table 2 and Figure 12 and 13. In general, the treatment with Ca + B exert a strong effect on related proteins, antioxidant, BR, and ethylene gene expression levels, while the genes were downregulated with respect to the control. An expression profile was obtained, in which the treatment stimulated a higher accumulation of three scavenger gene transcripts: CAT-*CsGy4G025230*, POD- *CsGy4G005180*, and SOD-*CsGy4G010750*. Interestingly, cucumber plants were treated with Ca +B upregulation of proteins related to the seed formation genes, *CsGy2G003240* with function, serine/threonine-protein kinase Nek2-like isoform X1. Also, expression of the gene *CsGy2G025870* responsible for mitogen activated protein kinase kinase 2-like and gene *CsGy2G025820* with the Cellulose synthase function was a higher expression with Ca+ B treatments, especially under high-temperature stress. The down-regulation of the genes coding for antioxidant enzymes, protein kinase, cellulose, BR, and ethylene might be correlated to slow down the consequence under low-temperature conditions. On the other hand, the high temperature stress exerted a stronger effect at the expression level. Indeed, the Ca + B treatment led to upregulation of the expression of all the studied temperature stress related genes related to protein (*CsGy2G003240*, *CsGy2G025870*), Cellulose (*CsGy2G025820*), POD (*CsGy4G005180*), SOD (*CsGy4G010750*), CAT (*CsGy4G025230*), BR (*CsGy6G029150*), and ethylene (*CsGy4G022650*, *CsGy6G022520*).

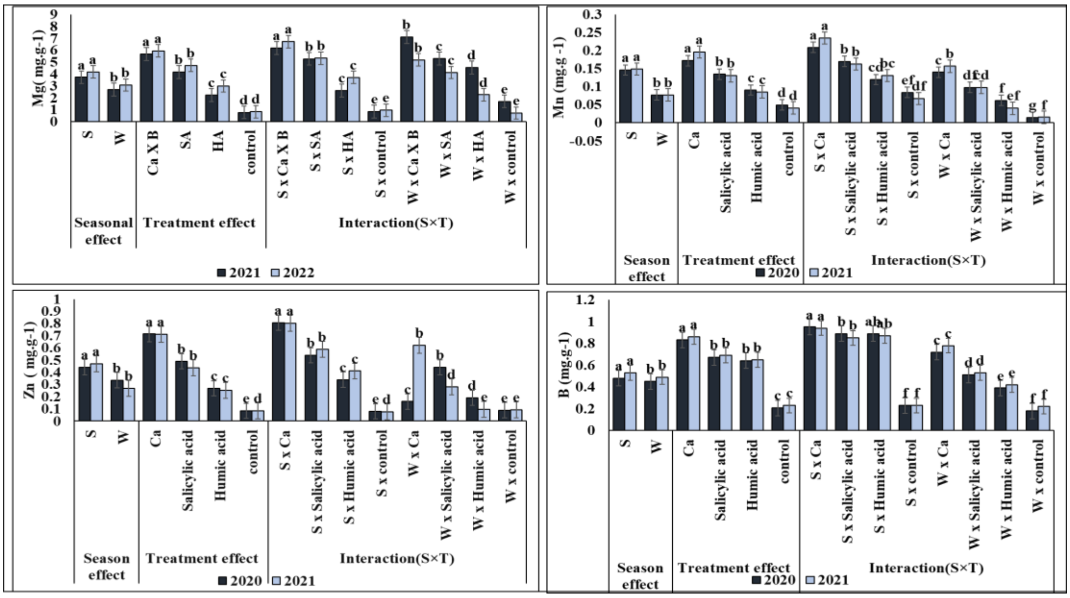

Figure 11. Effects of seasonal variation, different temperatures stress [Summer (S) and Winter (W)] mitigation treatments, and their interaction on magnesium, manganese, zinc, and Boron in the produced seeds. Different small letters represent a significant difference at 0.05 level of probability by Duncan's test.

Table 2. Candidate genes related to cucumber temperature stress tolerance.

		Gene ID	Location	Gene Function Annotation
1	protein	*CsGy2G003240*	Chr2: 2112890.. 2120663 (-)	serine/threonine-protein kinase Nek2-like isoform X1
2	protein	*CsGy2G025870*	Chr2: 33383658 .. 33385190 (+)	mitogen-activated protein kinase kinase 2-like
3	Cellulose	*CsGy2G025820*	Chr2: 33282069 .. 33316987 (-)	Cellulose synthase
4	POD	*CsGy4G005180*	Chr4: 3732444 .. 3735094 (-)	Peroxidase
5	CAT	*CsGy4G025230*	Chr4: 30688897 .. 30692995 (+)	Catalase
6	SOD	*CsGy4G010750*	Chr4: 9907339 .. 9916663 (+)	Superoxide dismutase [Cu-Zn]
7	BR	*CsGy6G029150*	Chr6: 27853373 ...27855339 (1.97 Kb)	dof zinc finger protein DOF5.7
8	ethylene	*CsGy4G022650*	Chr4: 29053290 .. 29053739 (+)	Putative ethylene-responsive transcription factor-like
9	ethylene	*CsGy6G022520*	Chr6: 22960811 .. 22963402 (+)	Pollen Ole e 1 allergen/extensin

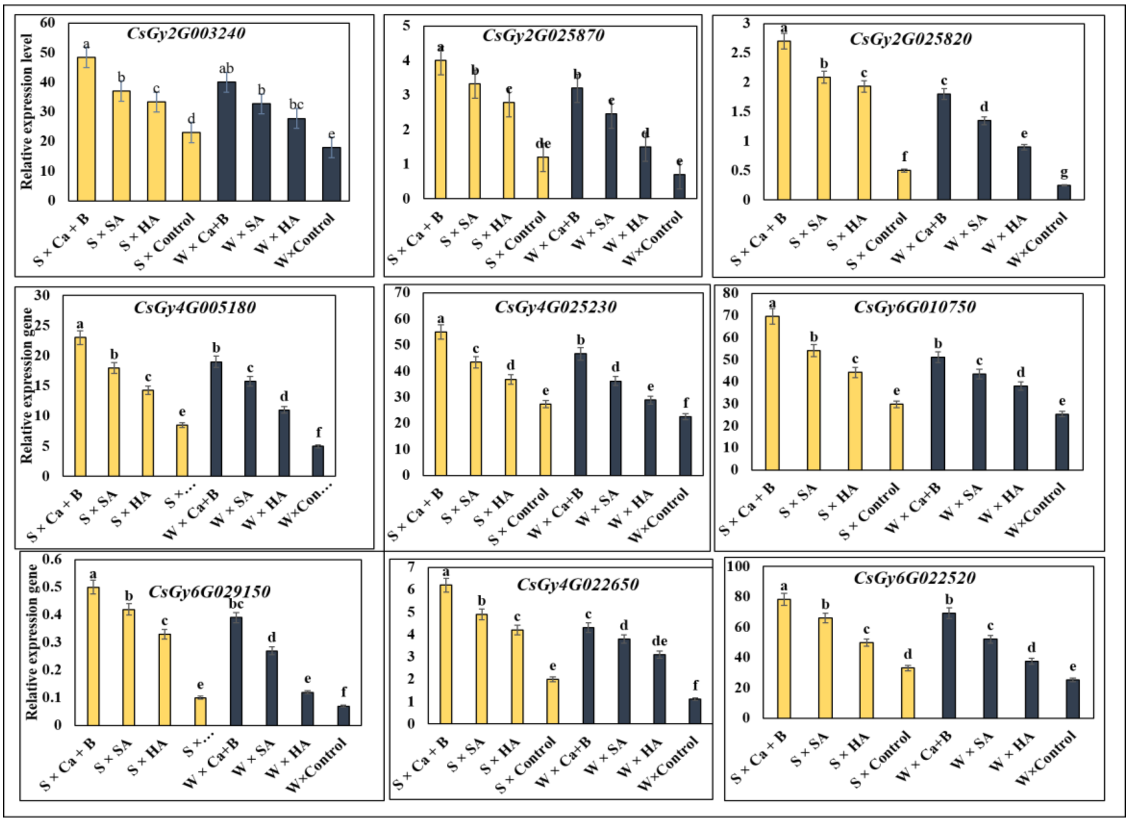

Figure 12. Relative expression genes under temperatures stress [Summer (S) and Winter (W)]. The expression levels of genes under different temperatures stress mitigation treatments were measured in qRT-PCR assays. Different small letters represent a significant difference at 0.05 level of probability by Duncan's test.

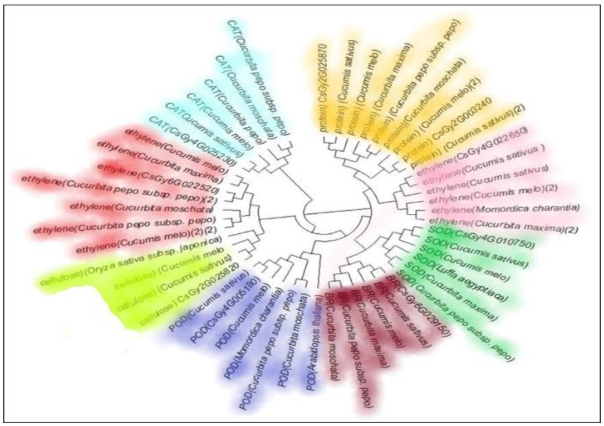

Figure 13. Maximum likelihood trees genes Note: involved in CAT, POD, SOD, BR, ethylene, cellulose, and protein metabolism and transport with those from *Cucurbita moschata, Cucurbita pepo, Cucumis sativus, Cucumis melo, Cucurbita pepo subsp. pepo, Arabidopsis thaliana, Cucumis melo, Cucurbita maxima, Luffa aegyptiaca,* and *Momordica charantia*. The protein sequences were used to make a phylogenetic tree by the neighbor-joining method.

Discussion

Under low and high temperatures stress conditions, calcium boron, salicylic and humic acid, were employed to enrich cucumber seed production that directly caused physiological, biochemical, and molecular responses in treated plants (Brown, Bellaloui et al. 2002, Bonilla, El-Hamdaoui et al. 2004, Hocking, Tyerman et al. 2016, Akhtar, Ilyas et al. 2022). Calcium boron is a product possess calcium that is widely acknowledged for its essential function in building strong cell walls for sturdier plants and increasing vitality (Hocking, Tyerman et al. 2016). Increasing calcium levels in the tissue enhance leaf cuticle and fruit skin thickness, leading to larger, firmer, better quality fruit that travels better and stores longer (Hocking, Tyerman et al. 2016). Boron is also fundamental in the pollination process and seed maturation, so peak demand timing for boron starts ahead of flowering and continues through the fruit set. So, the treatments were used three times after the flowering stages start. To mitigate temperature stress, boron synergies with calcium to improve cell wall integrity during the cell division stages (Brown, Bellaloui et al. 2002, De Silva, Kämper et al. 2022). The environmental conditions including temperature degree play a significant role in variation in seed size, weight, and numbers. Also, Fruit weight, length, and diameter were acknowledged joined of the foremost important seed quality parameters (Zobel and Davis 1949, Globerson. D, Dagan. A et al. 1975 , Nerson 2005). All treatments Ca+B, SA, and HA, positively impacted fruit parameters, whereas the variability in fruit size and shape was mirrored in seed yield, seedling emergence, and growth (Figure 1). The varying level of fruit sizes sometimes may well be an indicator of seed number and weight. Also, poor seed quality has contributed to the poor germination rate and seed germination percentage which was detected with the control plants (Zobel and Davis 1949, Golabadi, Ercisli et al. 2019). One of the essential ways to improve seed quality how to mitigate adverse abiotic stress including temperature stress. In current study, the applied treatments were highly effective for all experimental parameters. The fruit weight, length, diameter, number of seeds/ fruits, the weight of seeds/fruit, and total seeds yield/plant were boosted significantly with all treatments. The advancement of studied parameters may be due to the alleviating adverse conditions on mother plants during temperature stress and motivating the physiological, biochemical, and molecular responses to overwork without any deterioration, and the nutritional role of these compounds on the mother plants (Geetharani, Vijayakumar et al. 2007).

In this study, seed production was largely improved by Ca + B treatment where treated plants showed a significant increase in fruit traits, seed quantity, and quality under high and low-temperature stress conditions in two successive seasons. The second significant temperature stress mitigation treatment is salicylic acid (SA) which plays a fundamental role in stress tolerance and its ability to induce a protective impact on plants under stress (Senaratna, Touchell et al. 2000, Hayat, Ali et al. 2007). Several signaling molecules such as calcium, ethylene, and salicylic acid have been identified in plants. It has been documented that exogenous calcium and SA application boosted the cytoplasmic Ca^{2+} level, which is well demonstrated as a messenger in various plants. (Knight and Knight 2001). It has been reported that exogenous treatments application raised the cytoplasmic Ca_2^+ level, which is well documented as a messenger in various plants (Nerson 2005). Calcium boron is an essential component for pollination, these two nutrients are immobile in the plant and, once absorbed, cannot be redistributed. Among these adverse environmental factors, low and high temperature is a common threat to plants establishment, flowering and seed/fruit set in many crop species (Ganie, Akhter et al. 2013).

To improve stress mitigation, humic acid (HA) stimulates antioxidant enzymes, accelerates plant metabolism, regulates stomatal openings, enhances crop stress resistance, improves plant photosynthesis, and promotes plant balanced growth. According to several reports, HA significantly increased fruit weight, diameters, length, and total yield, additionally, decreasing the harmful effects of stressors on plants compared with control plants (Nardi, Pizzeghello et al. 2002, Farnia and Moradi 2015, Campobenedetto, Grange et al. 2020). HA can also influence the cell division and enhance protein synthesis, which result in enhancing total protein content in plants. HA stimulates production of plant enzymes and hormones. It also increases enzyme catalysis, peroxidase, and superoxide dismutase and enhances respiration and photosynthesis processes. These signaling molecules activate a variety of signal transduction pathways, a number of which relieve the plant to beat the stresses (Nardi, Pizzeghello et al. 2002, Farnia and Moradi 2015, Campobenedetto, Grange et al. 2020). The protein amount of cucumber seeds reached to 75 mg/g with Ca + B application under heat stress conditions, which was higher than the values of cucumber seeds in other studies (Nerson 2002, Nerson 2005, Kläring 2017, Liu, Dong et al. 2021, Gupta 2022). The mineral composition of cucumber seeds produced from Ca + B treated plants contained three times higher than the amount in control seeds. Treated cucumber plants overcame physiological deterioration during the seed set process. The nutrient content is responsible for seed quality and ensures successful crop production which increases the yield up to 15-20% (Ambika, Manonmani et al. 2014). The physiological quality assessed through germination rate and percentage, and seedling length steadily coincided with Ca + B treatment. The Ca + B treated mother plants commonly support the production of good seedlings compared to other treatments and control due to sufficient energy content in seeds. Fruit and seed weight are important physical indicators of seed quality that affect produced seedlings (Navitha, Sujatha et al. 2019). The nutrient content of the seeds relies on seed weight and varies from species to species, therefore, heavily weighted seeds help in producing adequate performance on seed germination survival and initial seedling growth compared to lightweight seeds (Geetharani, Vijayakumar et al. 2007, Navitha, Sujatha et al. 2019)**.** The AOX, BR, and ethylene biosynthesis accumulated in the treated plants was higher than in the control plants. The results proved that Ca + B could be the most promising treatment for BRs, ethylene content, and AOX capacity. The results confirmed that AOX, BRs, and ethylene accumulation contribute to plant abiotic stress tolerance (Corbineau, Xia et al. 2014, Li, Euring et al. 2021). However, Ca + B treatments induced the highest seed yield and quality and at the same time caused seed germination inside the fruit, which, of course, badly affected the produced seed yield. This problem caused a decrease in seed yield, which could have doubled. It may be caused by ethylene increase where the ethylene has an effective role in seed germination, particularly under heat stress conditions similar to the study conditions (Corbineau, Xia et al. 2014).

Ethylene can motivate the seeds to be ready to germinate completely (Corbineau, Xia et al. 2014). At different stages of seed germination, the expression of various genes ends up in the assembly of proteins, necessary for seed germination and dormancy release. Proteins necessary for seed germination are accumulated after ripening, under seed drying conditions, leading to the release of dormancy (Talanova, Topchieva et al. 2006, Miransari and Smith 2014). All applied treatments induced the enhanced -expression of temperature stress-related genes. In the case of the calcium boron treatment, the gene expression was at a more elevated level compared to other treatments. *CsGy2G003240* gene was in the higher expression. This gene is responsible for serine/threonine-protein kinase, and it recreates a role in the regulation of cell differentiation and embryonic development for seeds (Janczarek, Vinardell et al. 2018). *CsGy2G025870* [Mitogen-activated protein kinases (MAPKs)] is the member of a dynamic protein kinase network through which diverse stimuli regulate the spatiotemporal activities of complex biological systems. MAPKs regulate critical cellular procedures needed for homeostasis such as the expression of cytokines and proteases, cell cycle progression, cell adherence, motility, and metabolism (Janczarek, Vinardell et al. 2018). *CsGy6G029150* is related to BR formation, which is responsible for pollen formation and its fertility. The DOF (DNA-binding One Zinc Finger) family of transcription factors is involved in many fundamental functions in higher plants, including responses to light and phytohormones as well as roles in seed development and germination (Noguero, Atif et al. 2013). *CsGy4G022650* is putative ethylene-responsive transcription factor-like, which is related to stress response and development through a different mechanism (Phukan, Jeena et al. 2017). *CsGy6G022520* is responsible for Pollen Ole e 1 allergen/extension. It has a big role in pollen germination and pollen tube growth (Alché, M'rani-Alaoui et al. 2004). From the previous description, we can conclude that all these genes are directly related to plant growth and development, especially the seed set stage, and all of them are heavily described under temperatures and stress conditions.

CONCLUSION

There is no escaping the conclusion that the capability of the world's seed industry to supply the amounts of quality seed required for agricultural production will be impaired unless the temperature stress is overwhelmed. The temperature stress at critical times during seed maturation could deteriorate the seed yield and quality. This research was a real attempt to maximize cucumber seed production with the climate changes challenges including temperature deviations which faces the cucumber cultivation time in Egypt. The results revealed that the use of the calcium boron product contribute to increasing seed production in cucumber as seed quantity and quality. On the other hand, using calcium boron causes a new unexpected problem that seeds germinated inside the fruit. So, the use of calcium boron components should use in a proper dose and at the right time to evade the bad impact on seed production. All recommendations in this study were done on seed production manually, regardless of the cross-pollination methods. In addition, the nutritional balance and its effects on seed quality required further study to evaluate the physiological role of nutrition on biochemical and molecular responses in plants and their effects on seed quality under stress conditions. Although summer was the most proper season for seed production from the point of view of the quality and quantity under greenhouse conditions, further studies should be carried out to study the effects of pollination techniques under diverse environmental conditions in open fields and on the large scale.

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الاستجابة الفسيولوجية والبيوكيميائية والجزيئية لمحصول الخيار أثناء مرحلة تكوين البذور تحت ظروف الاجهاد الحراري

ايمان بسيوني عبد السلام الرميلي[1] ، محمد عمر محمد كاسب[1] و أحمد فتحي يوسف [2]

[1] قسم بحوث الخضر خلطية التلقيح- معهد بحوث البساتين –مركز البحوث الزراعية
[2] قسم البساتين جامعة الأزهر

الملخص

يمثل تغير المناخ تحديًا كبيرًا وعاملًا رئيسيًا يحد من إنتاجية المحاصيل . يعد إنتاج البذور جزءًا لا يتجزأ من الأمن الغذائي لتوفير البذور اللازمة لزراعة المحاصيل المستهلكة . تم تنفيذ التجربة في نظام القطع المنشقة في تصميم قطاعات كاملة العشوائية في 4 مكررات . في عامي 2021 و 2022 نفذت التجارب في ظل ظروف الاجهاد الحراري العالي والمنخفض خلال مواسم النمو الأربعة في فصلي الشتاء والصيف .تم استخدام ثلاث معاملات ،كالسيوم بورون (Ca + B)، وحمض الساليسيليك (SA) ، وحمض الهيوميك (HA) للتخفيف من آثار الاجهاد الحراري لدرجات الحرارة المنخفضة والعالية على انتاج بذور الخيار .أظهرت النتائج أن جميع المعاملات أظهرت تحسنًا في تكوين البذور .علاوة على ذلك ، زادت جميع المعاملات من نشاط مستوى إنزيمات مضادات الأكسدة البيروكسيديز والكتاليز والسوبروكسيد ديستيميز وكذلك الهرمونات النباتية ، البرسينوستيرودات والإيثيلين في النباتات الأم أثناء مرحلة تكوين البذور .أظهرت الدراسة أن نشاط إنزيمات مضادات الأكسدة وتركيزات الهرمونات النباتية كان له دور فعال في التخفيف من الآثار السلبية لدرجة الحرارة على كمية البذور ، ونسبة البروتين في البذور ، والعناصر الغذائية .علاوة على ذلك ، فإن Ca + B كان الأكثر تأثيرا في تحسين طول الثمرة ، ووزن الثمرة ، وقطر الثمرة ، وكمية البذور ، وجودة البذور ، ونسبة الإنبات ، ومعدل الإنبات ، وطول الشتلات تحت ظروف اجهاد الحرارة العالية كمادت جميع المعاملات الي زيادة مستويات التعبير عن 9 جينات مرتبطة بالاجهاد الحراري مقارنة بالكنترول . اظهرت النتائج ان Ca + B هو أكثر مستحث لتعبير الجين .CsGy2G003240 (serine / threonine-protein kinase Nek2-like isoform X1) . بشكل عام ، أثبت Ca + Bانه الأفضل في تحسين الاستجابة الفسيولوجية والكيميائية الحيوية والجزيئية لنبات الخيار أثناء مرحلة تكوين البذور.