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Physiological Responses of Cucumber Seedlings to Combined High-Temperature and High-Humidity Stress at Different Leaf Stages

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Abstract: The growth and development of plants are closely tied to growth stages, such as germination, flower bud differentiation, photosynthesis, water and fertilizer use efficiency, stress resistance, etc. Previous studies on the stress resistance of plants with different leaf stages have primarily focused on single-factor environmental conditions. However, there has been a lack of systematic research on the physiology of plant seedlings under combined high-temperature and high-humidity (HH) stress, and the relationship between cucumber growth stages and HH tolerance remains unclear. In this study, we analyzed the phenotype, photosynthetic characteristics, reactive oxygen species content, and antioxidant enzyme activity of cucumber seedlings at 1-, 2-, 3-, and 4-leaf stages under control (25 °C + 80%RH, CK) and HH (42 °C + 95%RH) stress, aiming to clarify the relationship between growth stage and cucumber HH tolerance. The results indicated that the HH tolerance of cucumber seedlings increases with leaf stage. Seedlings at 1-leaf and 2-leaf stages were most sensitive to HH, whereas 4-leaf seedlings showed the greatest tolerance. Under HH stress, the biomass, chlorophyll content, net photosynthetic rate, and photosynthetic electron transfer rate were significantly reduced compared to CK. Simultaneously, there was an increase in reactive oxygen species content and antioxidant enzyme activity. The relative values for dry weight, total chlorophyll content, net photosynthetic rate, Fv/Fm, qP, ETR, and Y (II) in 1-leaf and 2-leaf seedlings were significantly lower, while ROS accumulation and changes in antioxidant enzyme activity were significantly higher compared to 4-leaf seedlings. This lays a foundation for future studies on the growth and physiological response of cucumber plants at different growth stages under varying temperature and humidity combined stresses.

Keywords: cucumber; growth stage; high-temperature and high-humidity stress; dual stress; physiology

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1. Introduction

Vegetables are one of the essential foods in people's daily diets, with high nutritional and economic value. To meet the market demand for year-round production and balanced supply, most vegetables are grown in facilities. However, during the growth and development of vegetables, they are often subjected to various abiotic stresses within the facility, such as salt stress [1], nitrate stress [2], drought stress [3], heat stress [4], and heavy metal stress [5], which greatly affect the yield and quality of vegetables. Meanwhile, HH stress is also one of the main stresses faced by facility crops, which not only limits their vegetative growth but also significantly reduces their photosynthesis and production. When seedlings were subjected to HH stress, the growth of their leaves and roots were inhibited. Similar results were reported by Weng et al. [6], as well as a significant decrease in plant dry weight and leaf dry weight [7,8]. Pollen abortion occurred, fruit setting rate decreased, and ovary

development was restricted [9]. In addition, it was reported that HH stress would make it easy to cause diseases which seriously threaten the yield and quality of vegetables [10,11].

At present, only a few studies on the HH tolerance of vegetables have been reported. It was determined that Fv/Fm, Pn, chlorophyll content, leaf length, leaf width, plant height, MDA, and stem diameter could be used as identification indexes of early tolerance to HH stress [6]. With the occurrence times and accumulative days of HH stress events, the growth of tomato was inhibited, the corresponding losses of physiological indexes of tomato increased, and flower bud differentiation was also affected [12,13]. Moreover, Zheng et al. [14] found that the activities of sucrose-metabolizing enzymes in young tomato fruits were changed under HH stress, which reduced fruit soluble sugar content. In cucumber, researchers found that HH stress has a serious impact on photosynthesis and yield formation in cucumber [15,16]. In our previous research, HH stress had a significant inhibitory effect on the vegetative growth, reproductive growth, and physiological status of cucumber [7]. The strength of cucumber's HH tolerance may be related to the leaf stage of seedlings, which should be further studied.

It was concerning that there were significant differences in the HH resistance of different varieties [17]. The leaf stage of seedlings may also be one of the main factors affecting the stress tolerance of plants [18], which has been reported in wheatgrass [19], plantain trees [20], *Elymus sibiricus* [21], maize [22], etc. Shao et al. discovered that the most significant effects of waterlogging and high-temperature combined stresses on maize yield occurred at the third-leaf stage, followed by the sixth-leaf stage and tasselling stage [22]. The aging process of *E. sibiricus* was associated with an increase in oxidative stress, indicating that there was an increase in ABA, particularly in the roots [21]. In our previous study, it was shown that cucumber plants at the 6-leaf stage were more tolerant than those at the 2- and 4-leaf stages under low-temperature and high-humidity stress [23]. Similarly, we wondered if there would be similar patterns of cucumber seedlings under HH stress.

In this study, we analyzed the phenotype, photosynthetic characteristics, reactive oxygen species content, and antioxidant enzyme activities of cucumber seedlings at different leaf stages under HH stress, aiming to clarify the relationship between growth stage and HH tolerance. This research will lay a foundation for the physiological and molecular response of plants under HH stress and provide a reference for further studies of other combined stresses.

2. Materials and Methods

2.1. Plant Materials and Experimental Treatment

The cultivar 'Jinchun No. 4' used in this experiment was considered a variety sensitive to HH, which was purchased from Tianjin Kerun Cucumber Research Institute. The cucumber seeds were firstly soaked in hot water at 55 °C for 15 min, then soaked in 25 °C water for 4 h, and germinated in a constant-temperature incubator at 28 °C for 24 h. When the cucumber seeds germinated and the buds were 1–2 mm exposed, we selected the germinated seeds that grew consistently and sowed them in 50-well trays. They were cultivated in growth chambers (Jiangnan, Ningbo) at 25/18 °C (14 h/10 h), a relative humidity of 80% (80%RH), and daytime photosynthetically active radiation of 200 $\mu mols^{-1}m^{-2}$. When the seedlings grew to the 1-, 2-, 3-, and 4-leaf stages, the seedlings were subjected to high-temperature and high-humidity stress (42 °C + 95%RH, HH) and the control treatment (25 °C + 80%RH, CK). The treatments lasted for 8 h and temperature and humidity recorders (Jinan, China, Jianda Renke) were used to monitor the internal environment of the growth chambers in real time during the whole treatment. Three replicates were set for each leaf stage, with 10 seedlings per replicate. Before this experiment, cucumber seedlings at the same leaf stage with consistent growth conditions were selected. The physiology indexes of the seedlings were sampled at 6 h after treatment, and the phenotypes and growth indexes of the seedlings at different leaf stages were analyzed when the treatments finished.

2.2. Biomass

After 8 h of treatment, seedlings at different leaf stages were quickly washed with clean water, wiped to remove the free water on the leaf surface, and then used for biomass measurement, including leaf fresh weight, top part fresh weight, and root fresh weight. Then, all these samples were transferred into envelopes, heated at 105 °C for 30 min, and then dried at 65 °C until reaching a constant weight, which lasted about 3 days. After that, the dry weights of leaves, top parts, and roots were measured and analyzed. Each index of cucumber seedlings at different leaf stages was measured with three biological replicates.

2.3. Photosynthesis and Chlorophyll Fluorescence

After 6 h of treatment, the first fully spread leaf of each treatment was selected for photosynthesis and chlorophyll fluorescence determination. The photosynthetic parameters were measured using a LI-COR6800 plant photosynthesis analyzer (LI-COR, Lincoln, NE, USA), which included the net photosynthetic rate (A), intercellular CO_2 concentration (Ci), transpiration rate (E), and stomatal conductance (gsw). Before powering on, the CO_2 steel cylinder should be firstly installed, and the desiccant H_2O scrub, H_2O humidifier, and soda lime CO_2 scrub should also be tested and correctly equipped. When the blade chamber is closed, the machine performs warmup tests by checking itself for 10 to 15 min. After that, the parameters of the environment were set as follows: 500 μmol s^{-1} flow rate, 0.1 kPa press valve, 10,000 rpm fan speed, and 'sun + sky' ambient. We created a new record file, clamped on the blade, and matched the IRGAs, then logged our remarks in the files.

Leaves were fully adapted to dark conditions which were placed with leaf clamps in a dark environment for no less than 30 min before this experiment. Chlorophyll fluorescence parameters were measured using a Dual PAM-100 dual-channel chlorophyll fluorescence analyzer (Walz, Effeltrich, Germany) under the 'Fluo+P700' measure mode, and then began to test the device by setting the measure light, saturated pulsed light, and actinic light. When the height of ΔF(Fm'-F) showed between 1/3 and 2/3 of Fv(Fm-Fo) in slow kinetics, we began to measure all these chlorophyll fluorescence parameters including PSII maximum photochemical efficiency (Fv/Fm), photochemical quenching coefficient (qP), electron transfer rate (ETR), non-photochemical quenching coefficient (NPQ), actual photochemical efficiency of PSII (Y(II)), quantum yield of unregulated energy dissipation (Y(NO)), and quantum yield of non-photochemical quenching (Y(NPQ)). All the data above can be downloaded from the report.

2.4. Chlorophyll Content

Samples were taken from the first fully spread leaf at each leaf stage 6 h after treatment. Three replicates were taken for each treatment and then were stored in a −80 °C freezer for future use. The chlorophyll content indexes, including chlorophyll a, chlorophyll b, carotenoids, total chlorophyll content, and total pigment content A, were determined by dissolving a 0.1 g leaf sample in 9 mL of 95% ethanol, and extracting for 24 h in a dark environment, inverting and mixing every 6 h. Then, we measured the absorbance at 665 nm, 649 nm, and 470 nm using a UV-4802 spectrophotometer (Unico, Shanghai, China) with 95% ethanol as the control. Total chlorophyll content was calculated by the mathematical expression given below.

$$\text{Chlorphyll a} = 13.95A_{665} - 6.88A_{649} \quad (1)$$

$$\text{Chlorphyll b} = 24.96A_{649} - 7.32A_{665} \quad (2)$$

$$\text{Carotenoids} = \frac{1000A_{470} - 2.05\text{Chlorphyll a} - 114\text{Chlorphyll b}}{245} \quad (3)$$

$$\text{Total chlorphyll} = \text{Chlorphyll a} + \text{Chlorphyll b} \quad (4)$$

$$\text{Total pigment} = \text{Chlorphyll a} + \text{Chlorphyll b} + \text{Carotenoids} \quad (5)$$

2.5. Reactive Oxygen Species and Antioxidant Enzyme Activity

Samples were taken as per the method in Section 2.4, and used for reactive oxygen species determination, including hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\bullet -}$). The activities of antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), were also measured. The method for determining the indexes above was also performed with reference to Amin et al. [23] with a little revision.

For H_2O_2 determination, we added 0.2 g leaf samples to 0.1% trichloroacetic acid, and then fully ground them. After that, they were subjected to a 4 °C centrifuge at 12,000 r/min for 15 min. Next, 0.5 mL of supernatant was mixed with 0.5 mL 10 mM PBS7.0 and 1 mL 1 M KI, and the data were analyzed at 390 nm after 1 h of dark treatment.

$O_2^{\bullet -}$ was extracted with 65 mM PBS7.8, and we added 1 mL of supernatant to 0.75 mL PBS7.8 and 0.25 mL 10 mM hydroxylamine hydrochloride after being centrifuged at 10,000 r/min for 10 min. Then, we let the reaction solution stand at room temperature for half an hour, and extracted 2 mL of the reaction solution into 2 mL 7 mM α-naphthylamine and 2 mL p-aminobenzene sulfonic acid. After that, the data were measured and recorded at 530 nm after a 30 min water bath at 30 °C.

Leaf samples of about 0.5 g were taken and added to 6 mL 0.05 M PBS 7.8 for the reaction solution preparation. After that, the reaction solution was centrifuged at 4 °C at 11,000 r/min for 20 min, and the supernatant could be extracted for the determination of SOD, POD, and CAT.

A few kinds of solution were prepared for the SOD preparation, including 0.13 M methionine (Met), 0.75 mM nitro-blue tetrazolium (NBT), 0.02 mM riboflavin (Rib), and 0.1 mM EDTA-Na_2. We took 0.05 mL of the reaction solution and added it to a mixed solution including 1.5 mL PBS 7.8, 0.3 mL Met, 0.3 mL NBT, 0.3 mL EDTA-Na_2, 0.25 mL dH_2O, and 0.3 mL Rib. We placed the reaction solution in a fully illuminated environment for about 10 min to 20 min until the reaction solution became dark blue, and covered the reaction solution with a black cloth to terminate it immediately. The data were measured at 560 nm and two tubes without enzyme solution were used as the control, one of which was in the dark during the whole experiment, and the other was in light.

The reaction solution for POD measurement was prepared with 0.7 mL 0.05 M PBS 7.0, 1 mL 0.2% 2-methoxyphenol, and 1 mL 0.3% H_2O_2. The enzyme solution was added into the reaction solution and the absorbance was measured at 470 nm at 30 s intervals for 3 min.

Catalase activity was assayed by measuring a 30% H_2O_2 reduction; 1.9 mL 0.05 M PBS 7.0 and 1 mL 0.3% H_2O_2 were mixed and prepared for the reaction, and 0.1 mL was added to the reaction solution and the 30% concentration of H_2O_2 at 240 nm was measured at 30 s intervals for 3 min.

2.6. Data Processing

The relative values of each leaf stage were calculated and the calculation formula was as follows:

$$R = \frac{X_T}{X_{CK}} \quad (6)$$

X_T is the treatment value and X_{CK} is the control value in Formula (1), and multiple comparisons were conducted between different leaf stages using Tukey's test, $p < 0.05$. All the indexes for cucumber seedlings were measured at the same leaf age stages with 3 replicates under treatment and control separately. The relative values were compared in pairs. In addition, all the data were statistically analyzed using IBM SPSS Statistics 26.0 and presented using GraphPad Prism 6 software.

3. Results

3.1. Growth of Cucumber Seedlings with Different Leaf Stages Under HH Stress

The sensitivity of cucumber seedlings to HH treatment varied among different leaf stages. In brief, the tolerance to HH gradually increased with the increase in leaf stages. At 8 h after HH treatment, all leaves and growth tips of the 1-leaf seedlings were wilted and the whole plant was dead. For seedlings of the 2-leaf stage, the leaves showed severe chlorosis, with a curled growth tip, but the seedlings were still upright and not dead. In comparison, although the growth tips of the 3-leaf seedlings were curled, and parts of the leaves were damaged, the injured area was significantly smaller than that of the 2-leaf seedlings. However, there was only a little injury on the leaf edges of the 4-leaf seedlings, while other parts of those leaves remained dark green, with normal growth pip and normal plant growth (Figure 1).

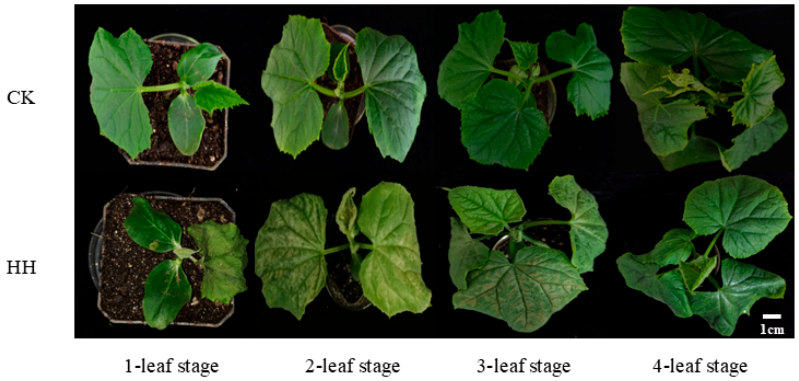

Figure 1. Phenotypes of different leaf-stage cucumber seedlings at 8 h after HH stress.

For biomass, there were significant differences among the seedlings of different leaf stages under HH stress. Overall, the relative biomass of seedlings increased with the increase in the leaf stages. However, there was no significant difference in the relative values of each part of the seedlings between the 3-leaf and the 4-leaf seedlings (Figure 2), indicating that the 3-leaf and the 4-leaf seedlings had similar HH tolerance. The relative values of the leaf fresh weight, top part fresh weight, root fresh weight, and whole-plant fresh weight of the 1-leaf and the 2-leaf seedlings were significantly lower than those of the 4-leaf seedlings (Table S1), and there was no significant difference between the 1-leaf and the 2-leaf seedlings (Figure 2A–D). The relative values of leaf dry weight, top part dry weight, root dry weight, and whole-plant dry weight of the 1-leaf and the 2-leaf seedlings were significantly lower than those of the 4-leaf seedlings (Table S1, Figure 2E–H). The relative values of leaf dry weight, top part dry weight, and whole-plant dry weight of the 1-leaf seedlings were also significantly lower than those of the 2-leaf seedlings (Table S1, Figure 2E,F,H). All of the above indicate that HH stress significantly affected the dry matter accumulation of cucumber seedlings, and the effect decreased with the increase in the leaf stages. The relative values of root fresh weight and root dry weight of the 1-leaf seedlings decreased by 0.15 and 0.13, respectively, compared to the 4-leaf seedlings (Table S1). There was no significant difference in the relative values of root fresh weight and root dry weight between adjacent leaf stages (Figure 2C,G).

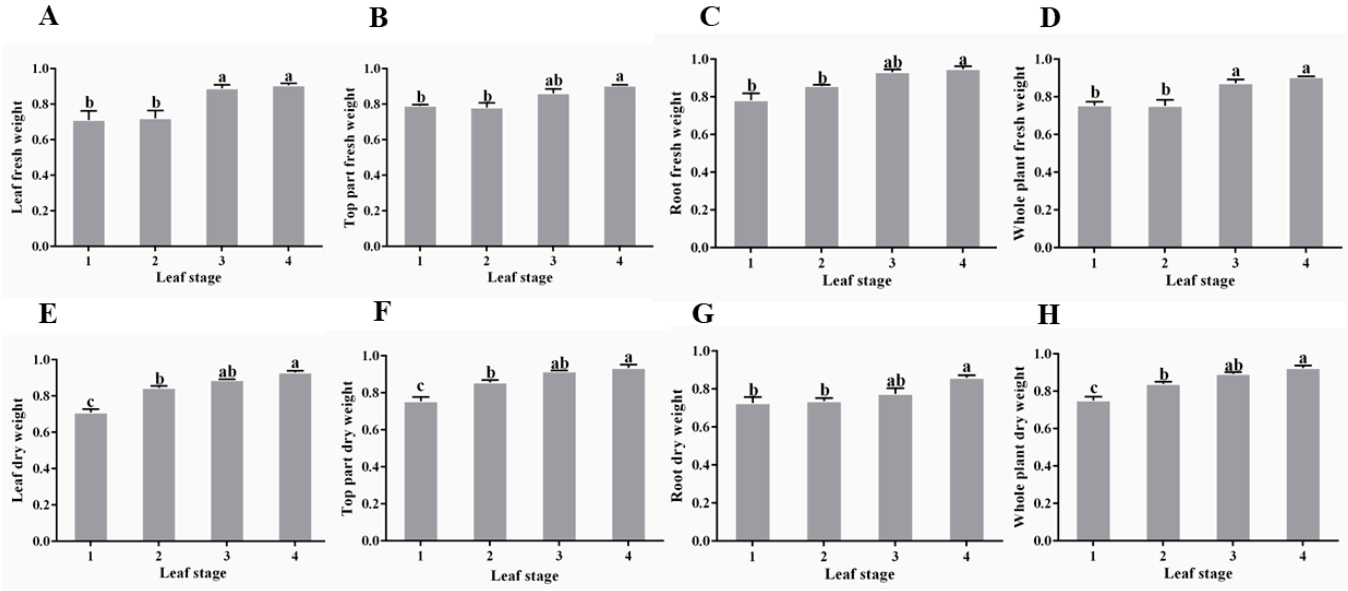

Figure 2. Relative biomass of different leaf-stage cucumber seedlings 8 h after treatments. (**A**) Leaf fresh weight; (**B**) top part fresh weight; (**C**) root fresh weight; (**D**) whole-plant fresh weight; (**E**) leaf dry weight; (**F**) top part dry weight; (**G**) root dry weight; (**H**) whole-plant dry weight. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, $p < 0.05$. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment.

3.2. Photosynthesis of Cucumber Seedlings with Different Leaf Stages Under HH Stress

Chlorophyll and carotenoids are the main photosynthetic pigments of plants, and the relative value of chlorophyll content can partly reflect the HH tolerance of cucumbers at different leaf stages. Overall, the relative values of chlorophyll a, chlorophyll b, carotenoids, total chlorophyll content, and total pigment content increased continuously with the increase in cucumber leaf stages. The relative values of different pigments of the 4-leaf seedlings were significantly higher than those of the 1-leaf seedlings, while there was no significant difference between the 1-leaf and the 2-leaf seedlings (Table S2, Figure 3). There was a significant difference in chlorophyll b and total pigment content between the 2-leaf seedlings and the 3-leaf seedlings, with decreases of 0.08 and 0.09, respectively (Table S2, Figure 3B,E). Only total pigment content showed a significant difference between the 3-leaf and the 4-leaf seedlings, while there was no significant difference among other types of pigments (Table S2, Figure 3E).

There were significant differences in the relative values of photosynthetic parameters among cucumbers at different leaf stages (Table S3). After HH stress, the relative values of the net photosynthetic rate of seedlings at all leaf stages were less than 1, indicating the inhibitive effects, but the decrease in the 4-leaf seedlings was the smallest, only 0.38, and the net photosynthetic rate of the 4-leaf seedlings was significantly higher than those of the 1-leaf, the 2-leaf, and the 3-leaf seedlings (Table S3, Figure 4A), indicating that the 4-leaf seedlings had the strongest resistance to HH stress among all leaf stages. For the 1-leaf and the 2-leaf seedlings, the relative values of stomatal conductance were significantly higher than those of the 3-leaf and the 4-leaf seedlings (Table S3). It was indicated that the increase in stomatal conductance of the 1-leaf and the 2-leaf seedlings under HH stress was significantly higher than that of the 3-leaf and the 4-leaf seedlings compared to the control condition (Table S3, Figure 4B). The relative values of the intercellular carbon dioxide concentration for seedlings at all leaf stages were greater than 1 (Table S3). The relative value of the 4-leaf seedlings was the lowest (1.05) and significantly lower than that of the 1-leaf and the 2-leaf seedlings (Table S3, Figure 4C), indicating that HH stress could lead to an increase in the intercellular carbon dioxide concentration in cucumber

seedlings compared with the control condition, and the increase in the 4-leaf seedlings was the smallest. In addition, the transpiration rate of seedlings at different leaf stages significantly increased to varying extents under HH stress, indicating that the transpiration rate of the 1-leaf seedlings increased the most (0.78), and was significantly higher than that of the 3-leaf and the 4-leaf seedlings (Table S3, Figure 4D).

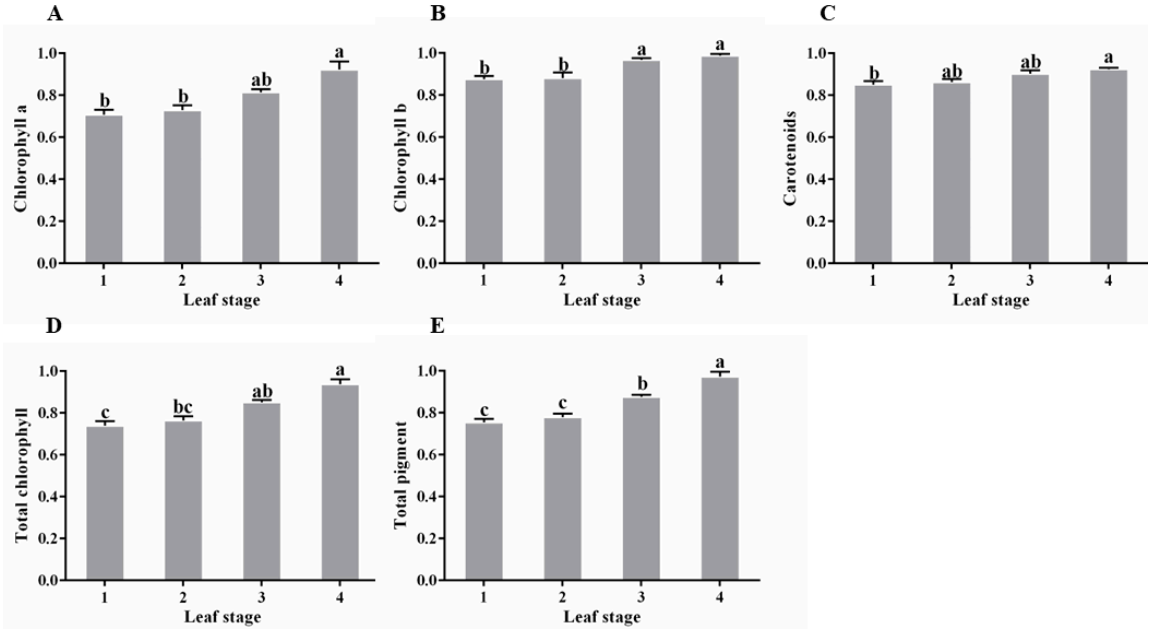

Figure 3. Relative values of photosynthetic pigment content of different leaf-stage cucumber seedlings 6 h after HH stress: (**A**) chlorophyll a; (**B**) chlorophyll b; (**C**) carotenoids; (**D**) total chlorophyll; (**E**) total pigment. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, $p < 0.05$. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment.

HH stress had a serious and different-degree impact on the photosynthetic electron transfer process in the PSII of seedlings at different leaf stages (Figure 5). Although the relative values of Fv/Fm for different leaf-stage seedlings were all less than 1, the 4-leaf seedlings had the smallest decreased value (0.07), while the 1-leaf seedlings had the largest decrease (0.47) (Table S4, Figure 5A). qP represented the opening degree of PSII reaction centers. Although the relative value of qP of the 4-leaf seedlings was less than 1 (0.93) under HH stress, it was significantly higher than those of the 1-leaf (0.72) and the 2-leaf (0.82) seedlings (Table S4, Figure 5B). In contrast, the relative value of NPQ of the 1-leaf seedlings was significantly higher than those of the 2-leaf, the 3-leaf, and the 4-leaf seedlings (Table S4, Figure 5C), showing that the 1-leaf seedlings consumed the most energy for heat dissipation under HH stress. The relative values of ETR and Y(II) for seedlings at different leaf stages showed the same trends of change. Although the ETR and Y(II) of 3-leaf and 4-leaf seedlings decreased after HH stress, the degree of reduction was significantly lower than those of the 1-leaf and the 2-leaf seedlings (Table S4, Figure 5D,E). In addition, the relative values of Y(NPQ) and Y(NO) for the 3-leaf and the 4-leaf seedlings were significantly lower than those of the 1-leaf and the 2-leaf seedlings, which were close to 1, indicating that the HH tolerance of the 3-leaf and the 4-leaf seedlings was significantly higher than that of the 1-leaf and the 2-leaf seedlings (Table S4, Figure 5F,G).

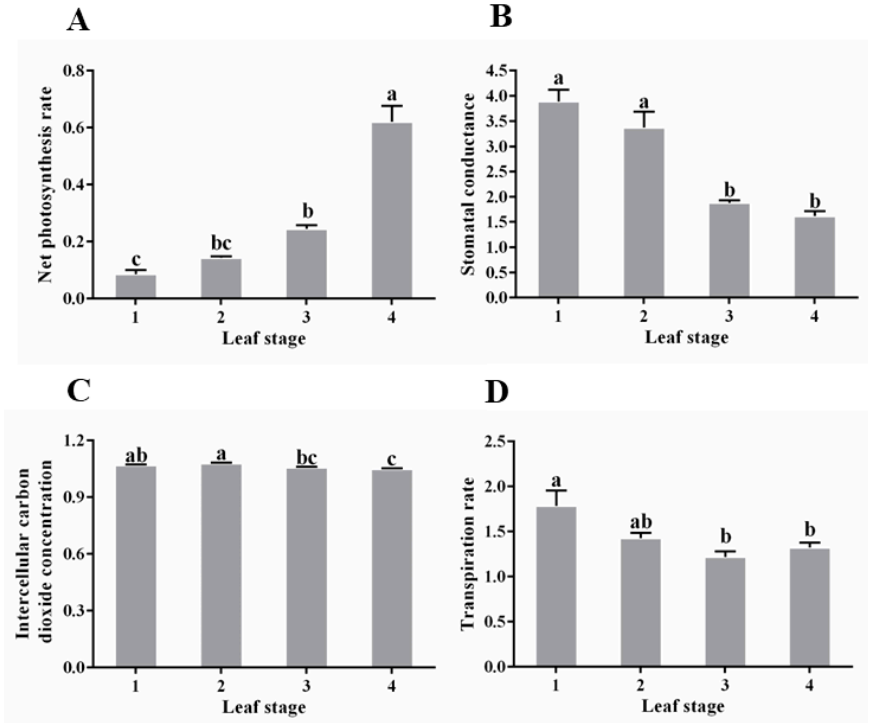

Figure 4. Relative values of photosynthesis parameters of different leaf-stage cucumber seedlings 6 h after HH stress. (**A**) Net photosynthesis rate; (**B**) stomatal conductance; (**C**) intercellular carbon dioxide concentration; (**D**) transpiration rate. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, $p < 0.05$. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment.

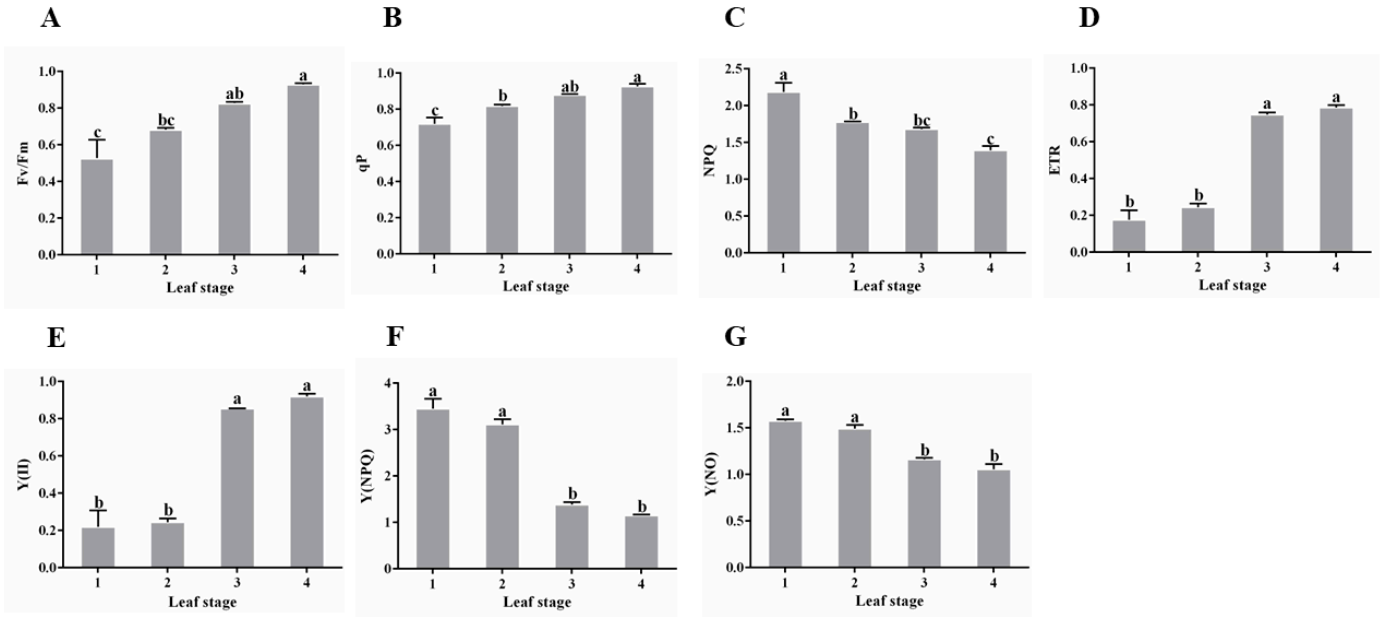

Figure 5. Relative values of chlorophyll fluorescence parameters in different leaf-stage cucumber seedlings 6 h after HH stress. (**A**) Fv/Fm; (**B**) qP; (**C**) NPQ; (**D**) ETR; (**E**) Y(II); (**F**) Y(NPQ); (**G**) Y(NO). Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, $p < 0.05$. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment.

3.3. Antioxidant Properties of Cucumber Seedlings with Different Leaf Stages Under HH Stress

There were significant differences in the accumulation of reactive oxygen species (ROS) in seedlings of different leaf stages under HH stress. As shown in Table S5 and Figure 6, the relative values of $O_2^{\bullet -}$ and H_2O_2 in different leaf-stage seedlings were both greater than 1 and gradually decreased with an increase in leaf stages. This indicated that HH stress significantly increased the accumulation of $O_2^{\bullet -}$ and H_2O_2 in young seedlings.

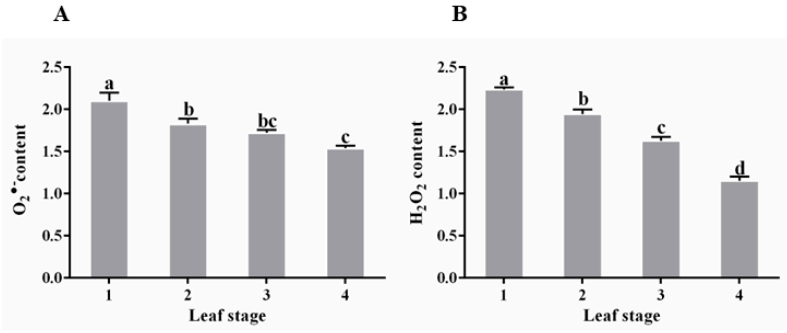

Figure 6. Relative value of reactive oxygen species content in different leaf-stage cucumber seedlings 6 h after HH stress. (**A**) $O_2^{\bullet -}$ content; (**B**) H_2O_2 content. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, $p < 0.05$. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment.

There were significant differences in antioxidant enzyme activities among cucumber seedlings at different leaf stages. After 6 h of HH stress, the relative values of SOD activity in seedlings were all greater than 1, while the relative values of POD activity and CAT activity were both less than 1 (Table S5, Figure 7), indicating that HH stress increased the activity of SOD, while it lowered the activity of POD and CAT in plants. The relative values of SOD activity in the 1-leaf and the 2-leaf seedlings were significantly higher than those in the 3-leaf and the 4-leaf seedlings, while the relative values of POD activity were significantly lower than those in the 3-leaf and the 4-leaf seedlings (Table S5, Figure 7A,B). For CAT activity, the relative values in the 4-leaf seedlings were significantly higher than those in the 1-leaf, the 2-leaf, and the 3-leaf seedlings (Table S5, Figure 7C). All the above results indicated that the antioxidant enzyme activity changes in the 1-leaf and the 2-leaf seedlings were significantly higher than those in the 4-leaf seedlings under HH stress.

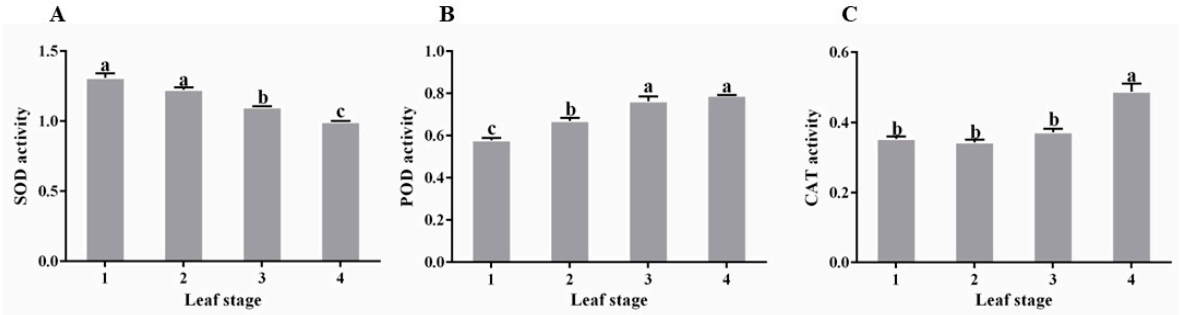

Figure 7. Relative values of antioxidant enzyme activities in different leaf-stage cucumber seedlings 6 h after HH stress. (**A**) SOD activity. (**B**) POD activity. (**C**) CAT activity. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, $p < 0.05$. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment.

4. Discussion

The tolerance of plants to abiotic stress depends not only on the type and duration of stress, but also on the plant growth stage [24]. The growth stage is a major driving force for changes in the traits and performance of plants. Wu et al. found that younger garlic plants

were more sensitive to low temperatures and showed significant inhibition of vegetative growth [25]. Elder plants always exhibited stronger self-regulation abilities, and were less sensitive to environmental changes in many traits [26]. Our previous research on cucumber seedling tolerance to low-temperature and high-humidity conditions found that the fresh and dry weights of plants at different leaf stages were significantly reduced compared to the control, while the decrease in the 2-leaf seedlings was significantly higher than that of the 4-leaf and the 6-leaf plants [23]. Until now, there have only been a few reports on the growth response of plants to HH stress at a single growth stage [7,27], and studies on trait responses at different growth stages are scarce. This study found significant differences in the phenotypes of seedlings at different leaf stages after 8 h HH stress (Figure 1). One-leaf seedlings wilted and died, while the two-leaf, the three-leaf, and the four-leaf seedlings still grew upright. After HH stress, the damaged area of leaves decreased with the increase in leaf stages. In terms of biomass, the relative values of fresh and dry weight, whether on the top part or root part, were all less than 1, indicating that HH stress inhibited the accumulation of biomass (Table S1, Figure 2). The relative values of fresh weight and dry weight increased with the increase in leaf stages, indicating that the higher the leaf stage of cucumber seedlings, the stronger their water retention capacity and dry matter accumulation ability, and the stronger their resistance to HH stress.

The chloroplast is one of the main target organs of plants under oxidative stress, and chlorophyll content is related to the resistance of plants to stress [28]. Pshibytko et al. found that the total chlorophyll content in leaves of 4-day-old barley plants significantly decreased after being subjected to high-temperature stress [29]. Amin et al. found that the chlorophyll content of cucumber seedlings at different leaf stages significantly decreased after low-temperature and high-humidity stress, and the degree of decrease continued to weaken with increasing leaf stage [23]. It has been reported that HH stress could significantly reduce the chlorophyll content of tomato seedlings [27], but the chlorophyll content of different leaf-stage plants under HH stress was currently unclear. In this study, the degree of leaf chlorosis varied among seedlings of different leaf stages under HH stress (Figure 1), which might be related to the decrease in chlorophyll content (Table S2, Figure 3E). This research also showed that relative values of various chlorophyll indicators in the 4-leaf seedlings were significantly higher than those in the 1-leaf and the 2-leaf seedlings. Except for the relative value of pigment content in the 4-leaf seedlings, which was significantly higher than that in the 3-leaf seedlings, there was no significant difference with other indicators compared to the 3-leaf seedlings (Table S2, Figure 3). This indicated that the chlorophyll content of the 1-leaf and the 2-leaf seedlings was more sensitive to HH stress compared to the 4-leaf seedlings.

The leaves are the main organ of photosynthesis. Their photosynthetic capacity is greatly influenced by the external environment, and vary with different leaf stages. There were significant differences in the relative values of net photosynthetic rate, stomatal conductance, intercellular carbon dioxide concentration, and transpiration rate among seedlings of different leaf stages (Table S3, Figure 4). Although the net photosynthetic rate of different leaf-stage seedlings decreased after HH stress, the relative value of the 4-leaf seedlings was significantly higher than that of other leaf-stage seedlings (Table S3, Figure 4A). Due to the influence of chlorophyll content on light absorption and conversion, it was believed that this was also the main factor leading to different degrees of net photosynthetic rate decline among different leaf-stage seedlings (Figures 3 and 4A). Tong et al. found that the transpiration rate of cucumber seedlings significantly increased after HH stress [7]. In this study, we further found that the increase in transpiration rate was related to leaf stages, and the increased degree of transpiration rate of the 3-leaf and the 4-leaf seedlings was significantly lower than that of the 1-leaf seedlings (Table S3, Figure 4D). The stomatal conductance and transpiration rate showed the same trend of change, and the increase in stomatal conductance of the 3-leaf and the 4-leaf seedlings was significantly smaller than that of the 1-leaf and the 2-leaf seedlings (Table S3, Figure 4B,D). In addition, although the relative intercellular CO_2 concentration of the 4-leaf seedlings

was significantly lower than that of the 1-leaf and the 2-leaf seedlings, the relative net photosynthetic rate of the 4-leaf seedlings was significantly higher than that of the 1-leaf and the 2-leaf seedlings (Table S3, Figure 4A,C). Shang et al. believed that this was because elder plants stored more energy and carbon sources during growth than younger plants [30], and therefore they had stronger regulatory ability and were less affected by changes in environmental factors.

The PSII reaction center is sensitive to HH stress in terms of photosynthetic electron transfer. Yang et al. found that the Fv/Fm, qP, and ETR of tomato seedlings under HH stress were significantly reduced compared to the control [31]. Tong et al. showed that there was no significant difference in Fv/Fm and NPQ between cucumber seedlings under HH stress and control, while qP and ETR decreased significantly [7], which was not completely consistent with the results obtained in this study. In our study, we found that the Fv/Fm, qP, ETR, and Y (II) of seedlings at different leaf stages showed varying degrees of decrease after HH stress, while NPQ, Y (NO), and Y (NPQ) showed an upward trend (Figure 5). Furthermore, our analysis also revealed that the decrease in Fv/Fm, qP, ETR, and Y (II) of the 4-leaf seedlings was significantly lower than that of the 1-leaf and the 2-leaf seedlings after HH stress, and the increase in NPQ, Y (NO), and Y (NPQ) was also significantly lower than that of the 1-leaf and the 2-leaf seedlings (Table S4, Figure 5). Therefore, it was believed that the 4-leaf seedlings had significantly stronger HH tolerance than the 1-leaf and the 2-leaf seedlings due to their higher PSII reaction center opening ratio, lower energy dissipation, higher light energy utilization efficiency, and stronger self-regulation ability.

The self-repair ability of different leaf-stage seedlings under abiotic stress gradually increases with the increase in leaf stages. The activities of SOD, POD, and CAT in cucumber seedlings were significantly upregulated under low-temperature and high-humidity stress compared to the control, and the increase was greater with leaf stage [23]. As far as we know, there is no report on the antioxidant enzyme activities of cucumber seedlings at different leaf stages under HH stress. Only a few studies found that HH stress could significantly affect antioxidant enzyme activities. However, the reports on changes in SOD, POD, and CAT activities were not consistent [7,32]. This study found that the activities of SOD, POD, and CAT showed different trends under HH stress, with SOD activity significantly increasing and POD and CAT activities significantly decreasing (Table S5, Figure 7). This might be closely related to the time point of sampling. During the ROS clearing process, different antioxidant enzymes had a sequential effect, and these antioxidant enzymes generally showed a dynamic trend of first increasing and then decreasing after stress. Therefore, to understand the changes in antioxidant enzyme activities in cucumber seedlings under HH stress, it is necessary to take samples and measurements at various time points during the whole process to clarify the biochemical mechanism of cucumber seedlings responding to HH stress. In addition, there were significant differences in the changes in antioxidant enzyme activities among seedlings at different leaf stages. The changes in SOD, POD, and CAT activities in the 4-leaf seedlings were significantly lower than those of the 1-leaf, the 2-leaf, and the 3-leaf seedlings (Table S5, Figure 7), indicating that the 4-leaf seedlings with strong HH tolerance had stronger self-regulation abilities, which was consistent with patterns observed in biomass and photosynthetic indicators.

Overall, this study showed that cucumber seedlings with elder leaf stages had stronger HH tolerance, while seedlings at younger leaf stages suffered more severe damage (Figure 1), presented much more decline in biomass (Table S1, Figure 2) and photosynthesis effect (Tables S2–S4, Figures 4 and 5), showed a larger accumulation of reactive oxygen species (Table S5, Figure 6), and a greater change in antioxidant enzyme activities (Table S5, Figure 7).

In addition, Kuk and Shin reported that the youngest leaf on the same cucumber plant at the four-leaf stage was more tolerant than elder leaves to 5 °C low-temperature stress [33], because of less of an increase in lipid peroxidation and H_2O_2 content, lower photosynthetic activity and chlorophyll a fluorescence (Fv/Fm), higher induction of antioxidant activity, and the expression of APX isozymes in response to low temperature. However, Munné-

Bosch and Lalueza measured oxidative stress markers in leaves and organelles, together with ABA levels in leaves of 2- and 7-year-old *Cistus clusii* dunal plants and concluded that meristematic tissues maintained the capacity to make new leaves with no symptoms of oxidative stress for several years, the leaves of elder plants show higher oxidative stress than those of young plants when exposed to adverse climatic conditions, age-induced oxidative stress occur both in chloroplasts and mitochondria, and newly emerged leaves accumulate higher amounts of ABA even without environmental stress as plants age [34]. Therefore, further research could be conducted to clarify the effects and mechanism of HH stress on different-age leaves of the same plant and on different-age plants at the adult stages of cucumber.

5. Conclusions

High-temperature and high-humidity stress did serious harm to cucumber seedlings. This study explored the physical effect on seedlings with various leaf stages under HH stress. It was shown that seedlings at the 1-leaf and the 2-leaf stages were the most sensitive to HH, and the 4-leaf seedlings were the most tolerant to HH. Compared with the control, biomass, chlorophyll content, net photosynthetic rate, and photosynthetic electron transfer rate of cucumber seedlings under HH stress were significantly decreased. At the same time, reactive oxygen species content and antioxidant enzyme activity were increased. The relative values of various indicators such as dry weight, total chlorophyll content, net photosynthetic rate, Fv/Fm, qP, ETR, and Y (II) of the 1-leaf and the 2-leaf seedlings were significantly lower than those of the 4-leaf seedlings, while the relative values of ROS accumulation and changes in antioxidant enzyme activity were significantly higher than those of the 4-leaf seedlings. Our findings contribute to a deeper understanding of seedling damage under HH stress and provide a piece of important information for further basic mechanism research towards the HH tolerance of cucumber plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10121369/s1, Table S1: Biomass of different leaf-stage cucumber seedlings at 8 h after treatments; Table S2: Photosynthetic pigment content of different leaf-stage cucumber seedlings at 6 h after treatments; Table S3: Photosynthesis of different leaf-stage cucumber seedlings at 6 h after treatments; Table S4: Chlorophyll fluorescence parameters of different leaf-stage cucumber seedlings at 6 h after treatments; Table S5: Reactive oxygen species content and antioxidant enzyme activities of different leaf-stage cucumber seedlings at 6 h after treatments.

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