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Article

Salvia miltiorrhiza Derived Carbon Dots and Their Heat Stress Tolerance of Italian Lettuce by Promoting Growth and Enhancing Antioxidant Enzyme Activity

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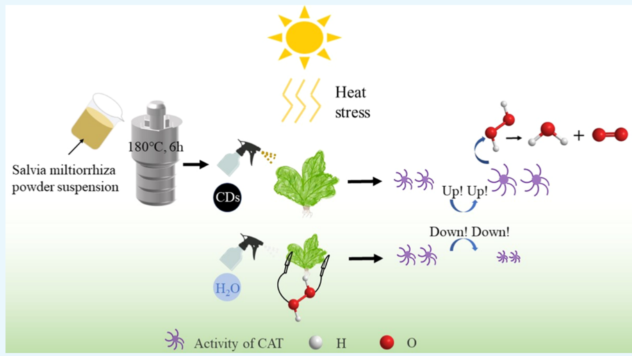
ABSTRACT: With global warming, plants often suffer damage from high temperatures during the growth process, which inhibits their growth. In this work, carbon dots (CDs), synthesized by *Salvia miltiorrhiza* (*S. miltiorrhiza*) with a one-step hydrothermal method, were selected as heat-resistant enhancement agents for plants. Inspired by this background, this work studied Italian lettuce grown at 25, 35, and 45 °C and treated with CD and deionized water control (sprayed on leaves). The results showed that the biomass, chlorophyll content, net photosynthetic rate, activities of SOD (superoxide dismutase), POD (peroxidase), CAT (catalase), soluble sugar, and soluble protein contents of lettuce treated by CDs were increased while the contents of malondialdehyde (MDA) and proline (Pro) were decreased at 35 and 45 °C. The application of CDs at 35 and 45 °C could maintain the growth of plants by reducing oxidative damage and lipid peroxidation especially at the temperature of 35 °C, the growth status of lettuce treated by CDs was no different from that of lettuce grown naturally at the optimal temperature of 25 °C, or even better than the latter. This finding verified that the CDs could significantly improve the high-temperature tolerance of lettuce, thus alleviating the heat stress of plants.



1. INTRODUCTION

Plants may be stressed by various abiotic factors in nature during their growth, such as high temperature, drought, salt and alkali, heavy metals, ultraviolet light, and so on.[1] Heat stress hampers plant growth by affecting a multitude of biological functions, ultimately leading to hindrance in plant growth and yield loss.[2] Due to human activities and environmental pollution, more and more greenhouse gases are emitted, leading to global warming.[3] Abnormal high temperatures, drought, and other severe weather problems are occurring frequently in many places in summer.[4] The response of plants to high-temperature stress affects the stability of various proteins and membranes, causes metabolic imbalance, and changes the efficiency of major cellular enzyme reactions in plants.[5] High-temperature stress leads to oxidative stress and the generation of reactive oxygen species (ROS), including superoxide anions $O_2^{\bullet-}$, hydrogen peroxide (H_2O_2), hydroxyl radicals (⊕OH), etc, which leads to cell injury symptoms under high temperatures.[6] Previous studies have shown that rising temperatures lead to lower yields of rice,[7] corn,[8] and wheat.[9]

Recently, the application of CDs and other nanomaterials to plants has provided new ideas for agricultural production.[10,11] Because of their low toxicity, stability, good biocompatibility, and photoluminescence properties, CDs have become the focus of many research studies.[12] In previous works, CDs complemented Fe^{2+} of *Cucumis melo*,[13] enhanced the nitrogen fixation activity of *Azotobacter chroococcum*,[14] and enhanced the photosynthesis of plant,[15] etc. In recent years, CDs have been shown to mimic the action of antioxidant enzymes and thus protect against oxidative damage caused by abiotic stresses; for example, to alleviate the abiotic stress of rice,[16] to reduce the toxicity of heavy metals to wheat seedlings,[17] etc. Therefore, CDs are a promising alternative to protect plants from abiotic stresses by removing ROS.

Salvia miltiorrhiza is one of the most widely and longest-used herbal medicines for numerous maladies throughout Asia.[18] Its main components are tanshinone and salvianolic

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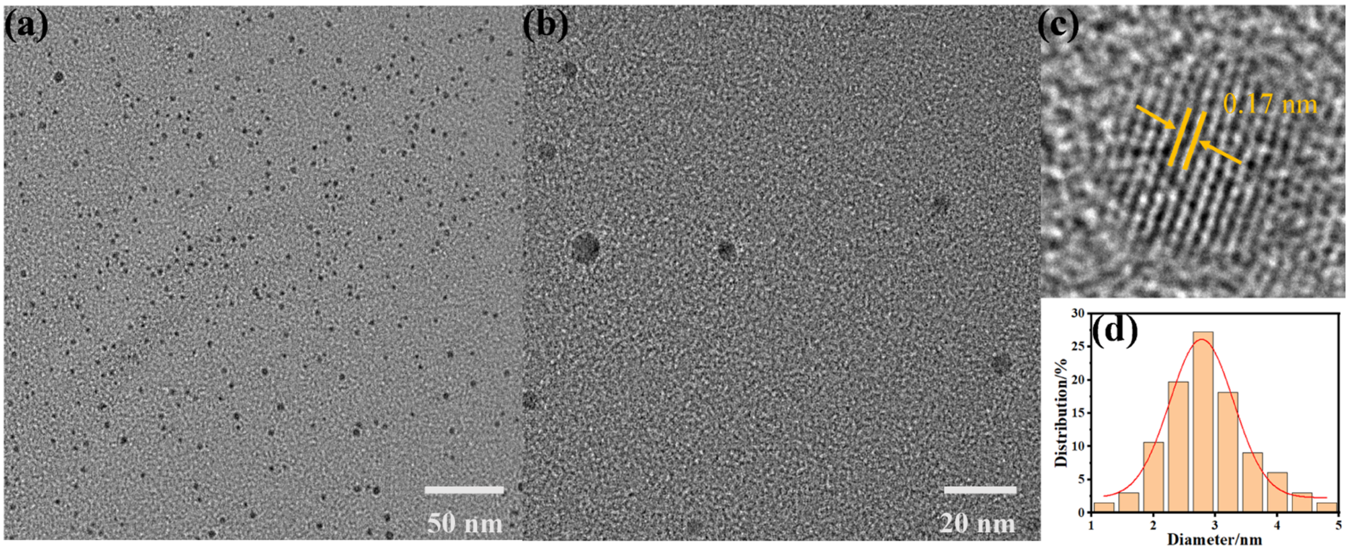

Figure 1. (a, b) HRTEM images, (c) lattice fringe image, and (d) size distribution of the prepared CDs.

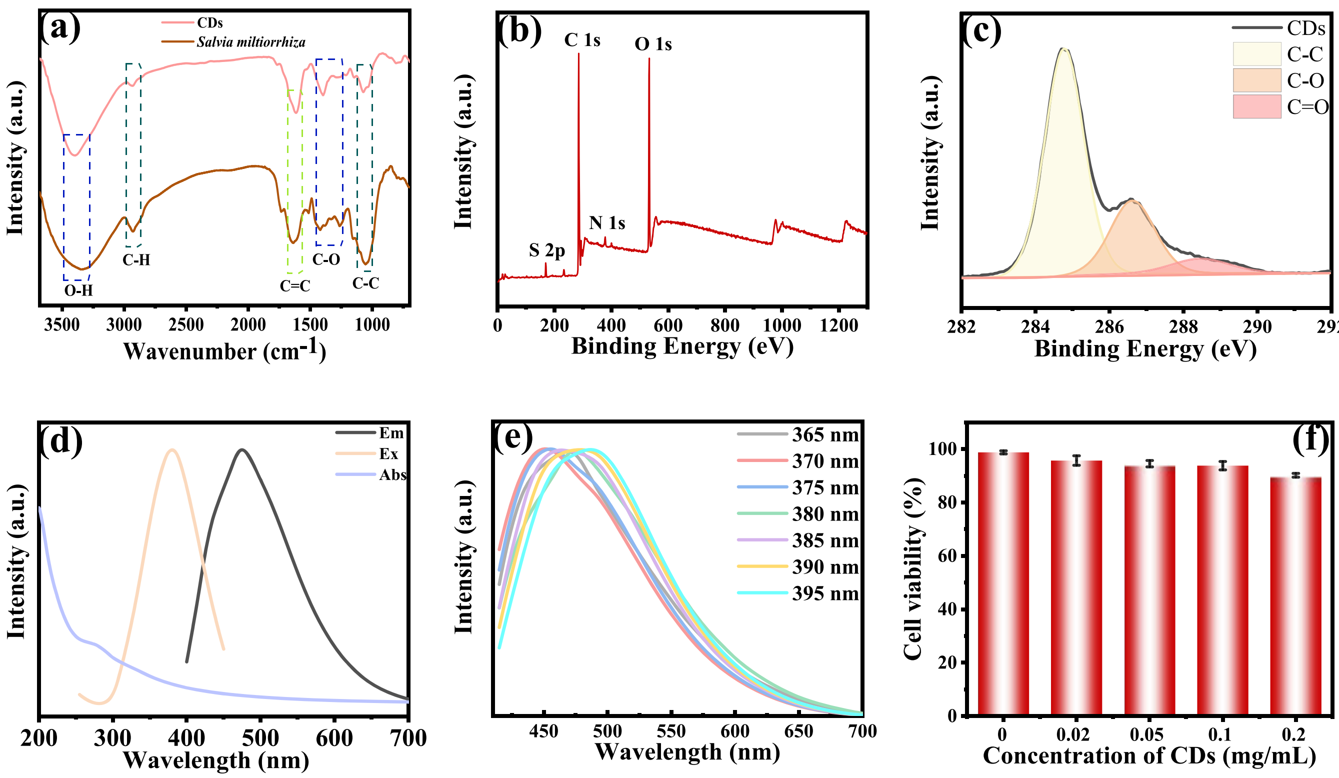

Figure 2. FTIR spectrum (a); full XPS pattern (b) and high-resolution XPS spectra of C 1s (c); UV−vis absorbance, FL excitation, and FL emission spectra (d); FL emission spectra at different excitation wavelengths (e); and cytotoxicity assessment of different concentrations of the prepared CDs (f).

acid compounds, which have a wide range of pharmacological effects, including the treatment of cardiovascular and cerebrovascular diseases,[19] anti-inflammatory,[20] antitumor,[21] antioxidation,[22] etc. *S. miltiorrhiza* and its preparations are used clinically in combination with other drugs to treat a variety of diseases, including cancer, gastric ulcer, and diabetes.[23] Previous studies have indicated that *S. miltiorrhiza* has anti-inflammatory properties. The use of medicinal herbs or their extracts is currently attracting attention as a promising chemopreventive strategy.[24] Inspired by Li et al.'s experiment on reducing oxidative damage of plants,[25] the application of *S. miltiorrhiza* in biology, especially in plants, is greatly limited due to its low solubility in water and the large particle size of its extracts. In this study, we used *S. miltiorrhiza* as raw material to synthesize CDs. Inspired by the above analysis, we combined the properties of *S. miltiorrhiza* with the advantages of CDs. We want to improve the heat resistance of lettuce with the aim to provide a reference for the annual production of cold-season vegetables under high temperatures and harsh climates. In this work, we measured the photosynthetic indexes of plants closely related to heat stress, which can preliminarily reflect the physiological function of plants. In addition, the

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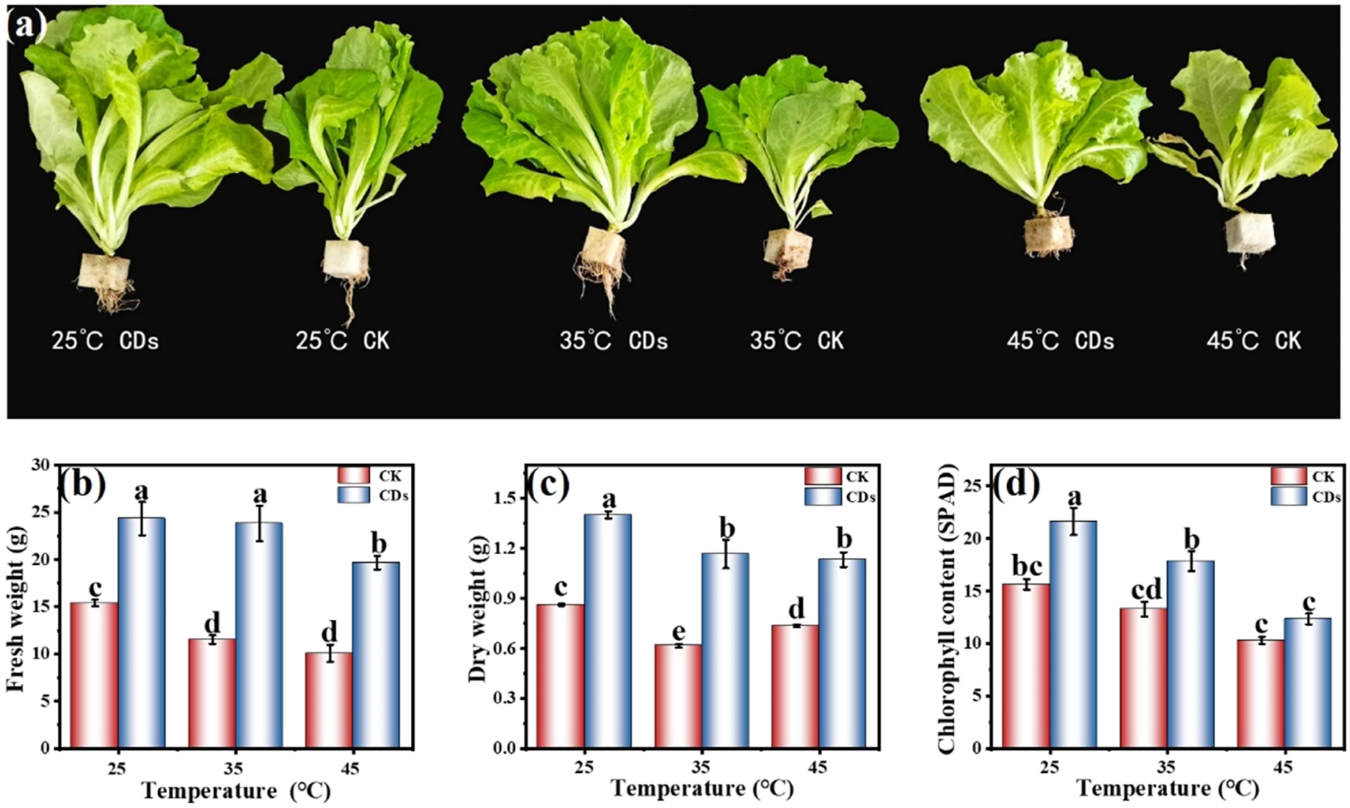

Figure 3. Effect of different treatments on the growth of Italian lettuce (a): fresh weight (b), dry weight (c), and chlorophyll content of Italian lettuce (d). $N = 4$. Average ± SD. Different letters indicate significant differences among different treatments at $p < 0.05$ level.

activities of three important antioxidant enzymes were measured to more intuitively show the antioxidant mechanism of the CDs.

2. RESULTS AND DISCUSSION

2.1. Synthesis and Characterization of CDs. The synthesized CDs exhibit excellent water solubility. As shown in the transmission electron microscopy images in Figure 1a,b, the carbon dots prepared by *S. miltiorrhiza* were spherical nanoparticles with an average diameter of 2.04 nm. The high-resolution transmission image of *S. miltiorrhiza* CDs showed obvious lattice fringes with a crystal plane spacing of 0.17 nm (Figure 1c).[26]

To understand the chemical composition and surface state of the CDs of *S. miltiorrhiza*, Fourier transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS) were tested. The surface of pure *S. miltiorrhiza* contains abundant phenolic hydroxyl or o-phenolic dihydroxyl; therefore, in FTIR, the wide absorption bands observed at 3200−3600 cm^{-1} are attributed to the stretching vibration of O−H, which also proves that the CD surfaces contain *S. miltiorrhiza* polymer. The peaks at 2926, 1611, 1391, and 1070 cm^{-1} correspond to the stretching vibrations of C−H, C=C, C−O, and C−C, respectively (Figure 2a).[27−29]

Further in-depth detection of the surface functional groups of the CDs was carried out through X-ray photoelectron spectroscopy (XPS) analysis. The survey spectrum indicates that the CDs mainly consist of carbon (C, 70.63%), nitrogen (N, 1.16%), oxygen (O, 26.03%), and sulfur (S, 2.18%). The four peaks observed at 168.3, 286, 379.4, and 531.7 eV correspond to S 2p, C 1s, N 1s, and O 1s (Figure 2b). The strong binding energy peak at 284.7 eV (C−C) belongs to the CD-graphitized S 2p carbon structure (Figure 2c).[30] Subsequently, the optical properties of the CDs were fully investigated.

From the fluorescence spectrum and the UV−vis spectrum of CDs in Figure 2d we can see two absorption peaks detected at approximately 280 and 325 nm. The peak at 280 nm is attributed to the $\pi \rightarrow \pi^*$ transitions of aromatic C=C, indicating the formation of a graphite carbon core in the CDs. The acromial at 310−350 nm are derived from the n−π^* transition of C=O.[31,32] Then, the optical properties of CDs were further explored. Under 380 nm excitation, the emission peak of CDs is located at 470 nm. The emission spectra of CDs at different excitation wavelengths are shown in Figure 2e, which indicates that the fluorescence emission has an excitation-dependent property. With the increase in the excitation wavelength, the emission peak gradually shifts to red, which proves the luminescence mechanism of CD surface states.[33,34]

As shown in Figure 2f, compared with plant cells, animal cells do not contain cell walls and absorb nanoparticles more easily. Therefore, animal cells are more sensitive to CDs than plant cells. HeLa cells were used for cytotoxicity assays of CDs. After treatment with different concentrations of *S. miltiorrhiza* CDs, the survival rate of HeLa cells in each group was higher than 89%. These data suggest that *S. miltiorrhiza* CDs have good biocompatibility and can be used in plants.

2.2. Effect of CDs on Italian Lettuce Growth. Heat stress can lead to the depletion of carbohydrate reserves.[35] As shown in Figure 3a−c, in terms of biomass, there were significant differences between the three groups treated with CDs at different temperatures and those treated with deionized water. The treatment with CDs increased the biomass and

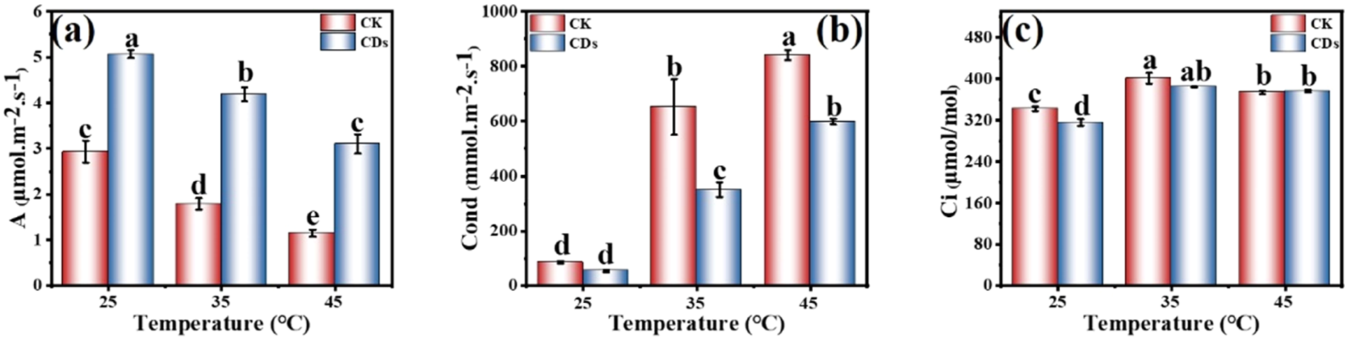

Figure 4. Net photosynthesis of Italian lettuce (a), stomatal conductance of Italian lettuce (b), and intercellular carbon dioxide concentration (c) of the Italian lettuce in different treatments. $N = 4$. Average ± SD. Different letters indicate significant differences among different treatments at $p < 0.05$ level.

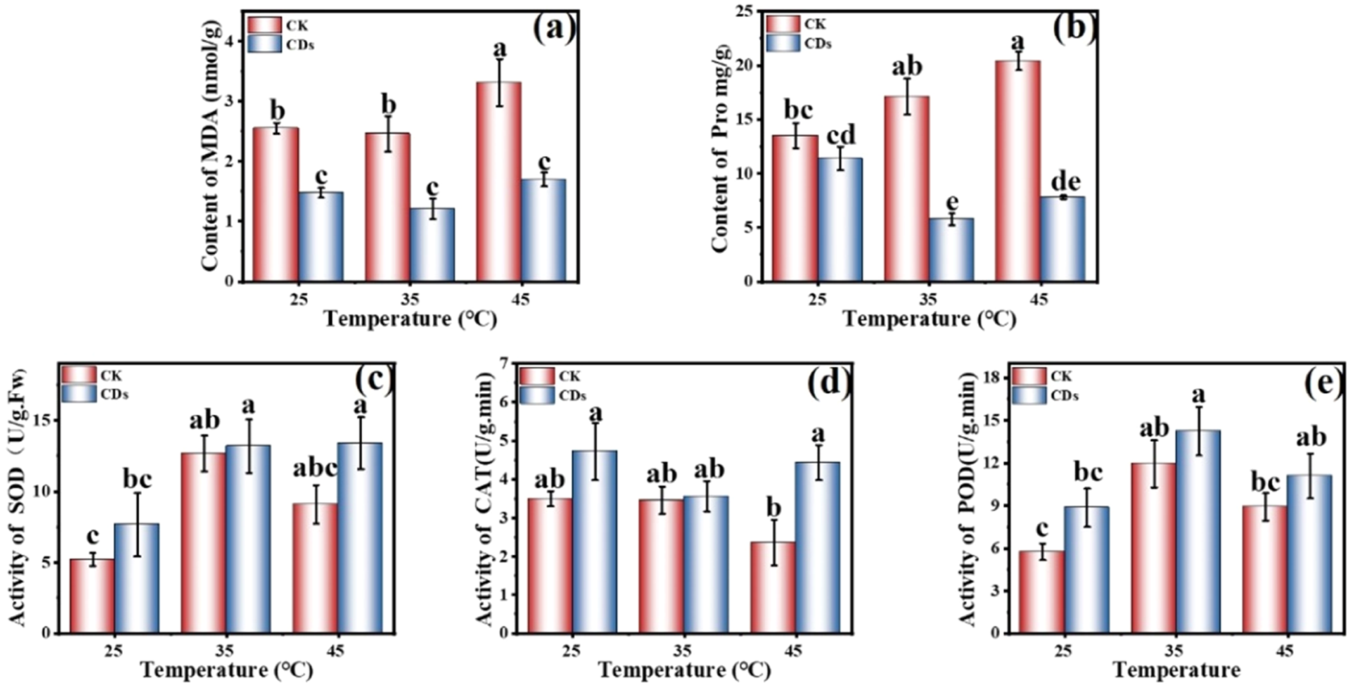

Figure 5. MDA contents (a), proline contents (b), SOD, CAT, and POD activities (c−e) of the Italian lettuce in different treatments. $N = 4$. Average ± SD. Different letters indicate significant differences among different treatments at $p < 0.05$ level.

chlorophyll content of lettuce. With the increase in temperature, the biomass of the treatment group decreased in turn. Interestingly, the fresh weight and dry weight of lettuce seedlings treated with CDs at 35 and 45 °C were significantly increased compared with those treated with deionized water at the optimal temperature (25 °C). The fresh weight and dry weight of the group treated with CDs at 35 °C increased by 54.7 and 35.2%, respectively, compared with that control group treated with deionized water at 25 °C. Thus, even when the lettuce seedlings were treated with high temperatures and sprayed with CDs, they still had more biomass than the plants grown normally without treatment. The changes in chlorophyll content in plant leaves can reflect the physiological activity of leaves. In general, the chlorophyll content decreases with the increase of temperature when heat stress occurs.[36] The results showed that the chlorophyll content of the plants increased at different temperatures after spraying with CDs. Among them, the chlorophyll content of plants grown at 25 and 35 °C was significantly increased after the carbon spot treatment. The chlorophyll content in the group treated with CDs at 35 °C was 14.5% higher than that in the group treated with deionized water at 25 °C (Figure 3d). In conclusion, in a high-temperature environment, the spraying of *S. miltiorrhiza* CDs plays a crucial role in the effective resistance of lettuce seedlings to heat stress.

Photosynthesis is a process in which plants absorb light energy, perform energy conversion and electron transfer, and eventually convert light energy into chemical energy, which is crucial to plant growth. The change in temperature is bound to affect photosynthesis, where the net photosynthetic rate of leaves significantly decreases with the increase in temperature. Heat stress results in loss and efficiency of photosynthetic pigments, abnormalities in cystic tissue, disruption of electron transport chains in mitochondria and chloroplasts, and reduced production of light absorption.[37−39] As shown in Figure 4a, in this experiment, the net photosynthetic rates of leaves in the three groups treated with CDs at different temperatures and that in the group treated with deionized water all decreased as the temperature increased. The group treated with deionized water at 25 °C was used as the control group, and the net

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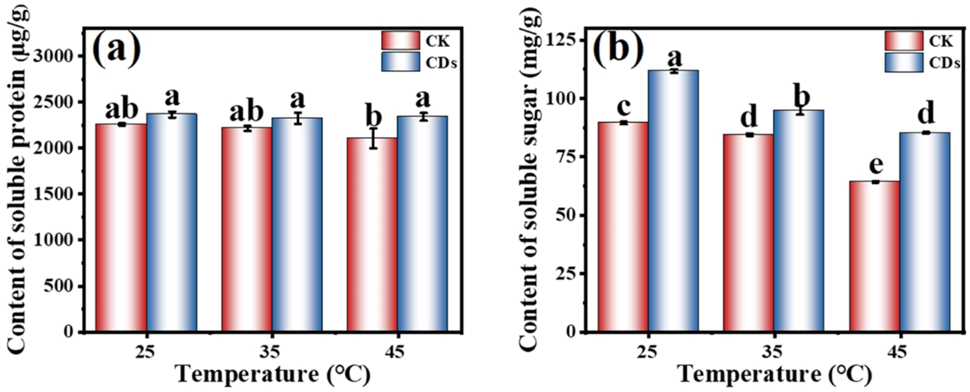

Figure 6. Soluble protein content (a) and soluble sugar content (b) in the Italian lettuce in different treatments. $N = 4$. Average ± SD. Different letters indicate significant differences among different treatments at $p < 0.05$ level.

photosynthesis rates of lettuce leaves in the groups treated with CDs at 35 and 45 °C were significantly increased, and the net photosynthesis rates of lettuce under high-temperature treatments increased by more than 43.8 and 6.5%, respectively.

Stomata control the majority of gaseous exchange between the atmosphere and the leaf interior; in general, conditions of high light intensity, open stomata.[40] From Figure 4b,c, corresponding to the trend of net photosynthesis rate, it can be seen that the stomatal conductance of the three treatment groups at different temperatures and deionized water treatment group all increased with the increase of temperature, and the stomatal conductance of lettuce leaves sprayed with CDs was all lower than that of the control group without spraying. From the above two photosynthetic indexes, *S. miltiorrhiza* CDs play a decisive role in the resistance of lettuce seedlings to high temperatures.

2.3. Antioxidant Defense System of Italian Lettuce. MDA is a product of lipid peroxidation and is commonly used as a biomarker for oxidative damage levels.[41] Pro content in plants reflects the stress resistance of plants to some extent. Plants with strong stress resistance tend to accumulate more proline. Ingredients such as proline and MDA are biochemical parameters that indicate that a plant has been damaged; therefore, the higher the damage, the more severe the damage.[42] Therefore, we studied the MDA content, proline content, and antioxidant defense system of lettuce seedling cells, including the activities of POD, SOD, and CAT. As shown in Figure 5a, the membrane lipids of lettuce seedlings were subjected to different degrees of oxidative damage after high-temperature treatment. The application of CDs significantly reduced the content of MDA by 41.9, 50.6, and 49.2% at three temperatures, respectively.

Interestingly, the MDA level in lettuce seedlings sprayed with CDs under 45 °C temperature stress was significantly lower than that of seedlings not sprayed with CDs at 25 °C optimal temperature. In Figure 5b, in the three temperature treatments, the proline content of the plants sprayed with CDs was lower than that of the nonsprayed group, and the proline content at 35 and 45 °C was significantly reduced by 66.3 and 61.3%, respectively. In conclusion, spraying *S. miltiorrhiza* CDs can well protect the leaves of lettuce seedlings from oxidative damage.

ROS is not only a byproduct of plant aerobic metabolism but an important signaling molecule involved in the regulation of plant growth and development as well as various stress responses. SOD is a major $O_2^{\bullet-}$ scavenger, and its enzymatic action leads to the generation of H_2O_2 and $O_2^{\bullet-}$; POD can reduce hydrogen peroxide.[43] CAT can act directly on H_2O_2, and CAT in peroxisomes has no affinity for H_2O_2, so most H_2O_2 can be removed. As shown in Figure 5d, at high temperatures of 45 °C, the CAT activity of lettuce seedling leaves treated with CDs was significantly higher than that of the deionized water treatment group. Interestingly, compared with that of the lettuce seedlings treated with deionized water under 25 °C, the CAT activity level of lettuce seedling leaves treated with carbon point under 35 °C showed no significant difference. This indicated that CDs reduced the stimulation of antioxidase and enhanced the tolerance of lettuce to high temperatures. As shown in Figure 5c,e, under heat stress, activities of SOD and POD first increased and then decreased, and the highest activity was found at 35 °C. CDs had no significant effect on the SOD and POD activity. Among the three temperature treatment groups, compared with that in the deionized water treatment group, the reactive oxygen species in the carbon point treatment group activated the antioxidant system of the lettuce seedling leaves by significantly increasing the activities of CAT, decreased the contents of MDA and proline, and made the lettuce show lower oxidative damage, indicating that CDs mainly catalyzed the removal of ROS. The antioxidant defense effect of lettuce leaf cells was enhanced to alleviate the high-temperature stress of lettuce.[44]

2.4. Effect of CDs on the Assimilation of Nutrition by Italian Lettuce. The synthesis of plant protein is related to various stress factors, and the content of soluble protein changes under high-temperature stress. After the CD treatments, the contents of these permeable substances also increased obviously under heat stress. As shown in Figure 6a, under 45 °C, soluble protein content in the CD treatment groups was significantly higher than that in the deionized water treatment group. This suggests that CDs can promote plant growth. The defense system of lettuce may be stimulated by temperature stress and produce more antioxidant enzymes to reduce damage, thus leading to an increase in protein content. This is consistent with the results of the antioxidant enzyme activity mentioned earlier.[45]

Endogenous soluble sugar is involved in the regeneration process of plants, so it is of great significance to explore whether CDs affect the content of soluble sugar. As shown in Figure 6b, the soluble sugar contents of the CD treatment groups were significantly higher than that of the deionized water control group at three temperatures, an increase of 24.7, 12.34, and 32.56%, respectively. These results indicated that

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CDs could effectively reduce the activities of the sucrose-degrading enzyme, acid invertase, and sucrose synthase under heat stress, resulting in an increase in the total soluble sugar content.[46] In conclusion, CDs can effectively improve the tolerance of lettuce seedlings to high-temperature stress and alleviate the damage to the plant.

2.5. Growth of Lettuce Seedlings Treated by CDs under Heat Stress. Under heat stress, the biomass, chlorophyll, and photosynthetic system of lettuce are affected, and the growth of the plant is delayed and the stem is fragile. Furthermore, the degree of biofilm lipid peroxidation of lettuce is increased after the plant is stressed, which is specifically manifested as the increase in the malondialdehyde content. Correspondingly, the content of proline as an osmotic protectant is also increased, and the activity of antioxidant enzymes is also decreased. However, the plants treated with carbon point had a good protective effect on the plant, which was embodied in promoting the plant to grow at a normal or better level and increasing the content of soluble sugar and soluble protein. The activity of antioxidant enzymes was increased, and the scavenging effect on reactive oxygen was enhanced. Therefore, the degree of biofilm lipid peroxidation was weakened, and the contents of MDA and proline were decreased. In conclusion, a series of studies have demonstrated that CDs can improve the thermal stress resistance of Italian lettuce by promoting growth and enhancing antioxidant enzyme activity.

3. CONCLUSIONS

This study reveals that under a high-temperature environment, spraying CDs on leaf surface increased the biomass, antioxidant enzyme activity, and osmotic regulation substance content in leaf, alleviated the damage to the plasma membrane of lettuce cell due to high temperature, and enhanced the tolerance of lettuce to a high-temperature environment. These results provide a good solution for CDs in the resistance of cold-like vegetables to high temperatures and provide a possibility for normal and orderly agricultural production under harsh climates.

4. EXPERIMENTAL SECTION

4.1. Synthesis of CDs. CDs were synthesized using a one-step hydrothermal process. First, 15 g of *S. miltiorrhiza* powder and 300 mL of distilled water were added to a beaker, stirred with a glass rod, followed by treatment with ultrasonication for 30 min until the solution was well mixed. After transferring the suspension to a 500 mL Teflon-lined stainless steel autoclave and heating at 180 °C for 6 h, the resulting mixture was centrifuged to remove the sediment after cooling to room temperature. The supernatant liquid was purified with a 0.22 μm filter membrane at room temperature to remove large particles. The resulting solution was used to remove small molecules using a cellulose dialysis bag (300 Da), freeze-dried, and then stored at 4 °C until needed.[25]

4.2. Preparation of the *S. miltiorrhiza* Extract. Fifteen grams of *S. miltiorrhiza* powder was mixed with 300 mL of deionized water, stirred evenly with a glass rod, and then vibrated with ultrasonic vibration for 30 min; the supernatant was the extract of *S. miltiorrhiza*.

4.3. Cytotoxicity of CDs. The cytotoxicity of CDs was assessed via their effects on the cell viability of HeLa cells by CCK assays. First, HeLa cells were cultured (37 °C, 5% CO_2) in a 96-well plate for 24 h. Then, the medium was discarded and the cells were transferred to 100 mL of medium with different concentrations of CDs (0, 0.02, 0.05, 0.1, and 0.2 mg/mL). Next, the medium was discarded again, and the HeLa cells were washed twice with Dulbecco's phosphate-buffered saline. The medium containing 10% CCK8 (100 μL) was added to each well. After 1 h, the cell viability was measured using a microplate reader at 450 nm.

4.4. Plant Growth and Cultivation. The plant growth and cultivation experiments were carried out in the College of Horticulture of South China Agricultural University (SCAU Main Campus Teaching & Research Base). Two-week-old Italian lettuce (*Lactuca sativa* L. var. *ramosa Hort*) seedling was used to study the effects of CDs on plant growth. Seedings of uniform size were selected and placed in the light incubators; after one week of growth at a relative humidity of 60%, the illumination intensity was controlled at 200 mmol/m^{-2}/s^{-1}. Then, the temperature of the incubator was set at 25/20 °C (14/10 h), 25/35/20 °C (10/4/10 h), and 25/45/20 °C (10/4/10 h). The growth substrate was a mixture of peat soil and vermiculite, and the appropriate nutrient solution was irrigated every other day until harvest. Spraying deionized water and CDs (2 mg/mL) small sprayer over the leaf surface. While applying fluid on the surface of the leaf, it was made sure that the blades were moist and the liquid did not drip. Three replicates of six seedlings were set for each treatment. CDs and deionized water were set for each temperature, the treatment group sprayed with CDs solution at the high-temperature time simulates with the high-temperature period of a natural day 25/20 °C (14/10 h) was the positive control group, the treatment group sprayed with deionized water was the negative control group for each temperature set, 10 days later, the growth parameters of biomass, chlorophyll content, photosynthesis, soluble sugar content, soluble protein content, MDA content, proline content, SOD, POD, CAT were measured.

4.5. Methods and Analyses of Measured Parameters. The fresh and dry weights of each plant were obtained from an average of six lettuce plants. The content of chlorophyll was measured by SPAD (spad502). A photosynthesis system (Li-Cor 6400; Li-Cor, Inc., Nebraska) was used to monitor the net photosynthetic rate. The SOD activity in lettuce seedlings was determined by the NBT reduction method.[47] The CAT activity in lettuce seedlings was determined by the previously reported methods.[48] The POD activity of lettuce seedlings was determined by the guaiacol method.[49] The content of MDA and proline in lettuce seedlings of each treatment was determined by the MDA (malondialdehyde, MDA, Content Assay Kit, Colorimetric Method) and Pro (proline, PRO, Content Assay Kit, Colorimetric Method) content kit produced by Shanghai Sangon Biological Engineering Company. The contents of soluble sugar and soluble protein was assessed by the anthrone colorimetry method and the Coomassie Brilliant Blue method.[50]

4.6. Statistical Analyses. All data (mean ± SD, n = biological replicates) were analyzed using SPSS 2017 (SPSS Inc., Chicago, IL). Comparison between treatments was performed by independent samples t-test (two tailed) or one-way ANOVA based on Duncan's multiple range test (two tailed). Different lowercase letters mean the significance at $p <$ 0.05.

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Notes

The authors declare no competing financial interest.

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