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Foliar Application of Carnosine and Chitosan Improving Drought Tolerance in Bermudagrass

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Abstract: Drought stress is one of the crucial factors affecting plant growth and development in turfgrass species, especially during the summer season. Exogenous plant growth regulators are an effective and convenient approach to mitigating the adverse effects of drought stress on plant growth. The objectives of this study were to reveal the effects of exogenous carnosine or chitosan on turf performance and physiological indexes in bermudagrass (*Cynodon transvaalensis* × *C. dactylon*) in response to drought stress. Bermudagrass was foliar sprayed with carnosine or chitosan, and dose-dependent effects on turf quality were observed under drought stress. Under drought stress, foliar application of either carnosine (0.03%) or chitosan (10 mg L^{-1}) significantly increased turf quality, chlorophyll content, leaf relative water content, and decreased electrolyte leakage, malonaldehyde, and hydrogen peroxide content in comparison with untreated control in bermudagrass. Moreover, exogenous carnosine treatment significantly enhanced the activities of both catalase and peroxidase, but chitosan application only increased catalase activity. The results of this experiment were beneficial to the development of new plant growth regulators and would provide helpful insights for turf management under drought-stressed conditions.

Keywords: bermudagrass; drought stress; carnosine; chitosan; antioxidant enzymes

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1. Introduction

Drought stress is considered to be one of the most important factors limiting plant growth [1]. Water deficit induced various physiological problems such as endogenous hormone disorder [2], reduced antioxidant capacity [3], chlorophyll degradation [4], and decreased photosynthetic capacity [5], resulting in inhibition in plant growth and development [6,7]. To deal with the negative effects of drought stress, plants undergo several physiological and metabolic changes in order to adapt to the adverse environments [8]. For example, plants subjected to abiotic stresses, including drought stress, could decrease oxidative damages through an antioxidant defense system of which plants evolved to be able to detoxify reactive oxygen species (ROS), including superoxide (O_2^-), singlet oxygen, hydroxyl radical, and hydrogen peroxide (H_2O_2) [9,10]. Under drought stress, the dynamic balance of free radicals would be broken, and a great deal of ROS accumulated in plants [11–13]. As the frontline enzyme in the antioxidant system, superoxide dismutase (SOD) rapidly converted O_2^- to H_2O_2, thereby reducing the production of hydroxyl radicals [3]. Then, H_2O_2 was further broken into H_2O and O_2 by peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) in different metabolic pathways [3]. However, when stresses reached beyond the scope of self-regulation, the injury appeared in plants [14].

A great number of approaches were conducted to improve drought tolerance, such as cultivating drought-resistant varieties [15], deficit irrigation [16], drought hardening [17], as well as chemical regulation [18]. Among them, the application of exogenous chemicals has been considered a highly efficient and convenient approach to promoting drought tolerance in plant species, especially in perennial turfgrass species [19]. For example, a

harpin protein from *Ralstonia solanacearum*, PopW, application improved drought tolerance in alfalfa (*Medicago sativa*) due to alterations of endogenous hormone content and gene expression related to drought stress and leaf senescence [20]. Chitosan mitigated drought stress in white clover (*Trifolium repens*) by regulating polyamine metabolism, endogenous hormones, and antioxidant defense [21]. However, it is necessary to point out that more new plant growth regulators associated with higher quality and yield, high efficiency, and pollution-free production are also demanded to meet the diverse requirements of the market [22].

As a natural dipeptide, carnosine is predominantly found in animal muscle tissues, of which the main components are *β*-alanine and L-histidine [23,24]. Carnosine was considered to be a natural antioxidant due to its antioxidant properties that could improve enzymatic activities and non-enzymatic antioxidants to reduce oxidative damage by scavenging superoxide radicals and hydroxyl radicals [25–29]. To our knowledge, this is the first report about carnosine ameliorating drought stress in plants.

Chitosan, chemically named (1,4)-linked 2-amino-deoxy-β-D-glucan, is a derivative of the natural polysaccharide chitin [30,31]. Chitin played roles in inducing various defense responses in plants [32]. Chitosan was found to be used as an edible film coating to extend the storage time of fruits and vegetables, such as in plums (*Prunus Salicina*) and loofah (*Luffa cylindrica*) [33,34]. It also could stimulate growth and development under non-stressed conditions in various plant species. Previous studies documented that chitosan improved rice (*Oryza sativa*) yield [35] and increased floret size as well as inflorescence length in dendrobium orchid (*Dendrobium*) [36]. In addition, chitosan promoted drought tolerance by increasing the activity of cytoprotective enzymes to scavenge O_2^- and H_2O_2 in plant cells and decreasing the accumulation of organic substances in wheat grass (*Agropyron repens*) cultivars [37]. Foliar application of chitosan inhibited the reduction of both dry matter and oil yield of *Thymus daenensis* grown under drought stress [38]. However, little research was documented about chitosan-induced positive effects in perennial turfgrass species.

Bermudagrass (*Cynodon transvaalensis* × *C. dactylon*) is an important warm-season turfgrass extensively used in home lawns, sports fields, and parks [39]. A previous study reported that drought stress caused a decrease in turf quality (TQ), leaf relative water content (RWC), and an increase in electrolyte leakage (EL) in all genotypes of bermudagrass [40]. We hypothesized that exogenous carnosine or chitosan could improve the drought resistance of bermudagrass. Therefore, this research aimed to determine whether foliar application with either carnosine or chitosan could enhance the drought tolerance of bermudagrass and explore the regulating mechanism at the physiological level by evaluating TQ, cellular membrane stability, and activities of antioxidant enzymes. This study would provide new growth regulators for turf management under drought-stressed conditions.

2. Materials and Methods

2.1. Experiment 1—Screening the Optimal Concentration of Carnosine and Chitosan Affecting Plant Growth

Sods of bermudagrass (cv. 'Tifway') (10 cm diameter and 3 cm thickness) were collected from a turf farm at Nanjing Agricultural University. The PVC tubes (11 cm diameter and 50 cm long) were set vertically and filled with coarse sand. One side of the PVC tube was closed. We used PVC tubes as pots and planted each sod on the open end of each PVC tube. Materials were maintained in a greenhouse with natural light and an average temperature of 25/19 °C (day/night) for 60 days. Plants were watered three times weekly and mowed every two days to establish root and canopy.

To determine the effects of carnosine or chitosan on turf quality under drought stress, 60-day-old plants were subjected to drought stress and foliar sprayed with 0.01%, 0.03%, 0.05%, 0.07%, and 0.09% chitosan (dissolved in water and 1% acetic acid = 40:1), as well as 2, 4, 6, 8, and 10 mg L^{-1} carnosine (dissolved in water) every 5 days, respectively. Both chemicals were purchased from Aladdin Inc. For all treatments, irrigation was withheld

until leaves were completely wilted. Drought control plants without chemicals were foliar sprayed with distilled water. Each treatment included 3 pots as 3 replicates and was sprayed at 0, 5, 10, and 15 d, respectively.

2.2. Experiment 2—Effects of Exogenous Carnosine or Chitosan on Drought Tolerance in Bermudagrass

Sods of bermudagrass (cv. 'Tifway') (10 cm diameter and 3 cm thickness) were collected from a turf farm of Nanjing Agricultural University and established in a greenhouse with the same environmental conditions as described in Experiment 1. After root and canopy establishment, plants were moved into a growth chamber (Xubang, Jinan, Shandong province, China) with day/night temperature controlled at 30/25 °C, 600 μmol m^{-2} s^{-1} photosynthetically active radiation, 60% relative humidity, and 14 h photoperiod for one week before treatments. Each treatment was performed in 5 PVC tubes as 5 replicates. Plants with carnosine or chitosan application were treated with the optimal concentration of carnosine (0.03%) or chitosan (10 mg L^{-1}) every 5 d, respectively, according to preliminary experiments. Drought treatment was performed as same as in Experiment 1. Distilled water was foliar sprayed for control plants (control) under both well-watered and drought stress. A completely randomized trial was used, and all tubes were randomly relocated once a week to avoid environmental impacts across the growth chamber.

2.3. Determination of Growth and Physiological Indexes

Soil water content (SWC) was monitored using a time domain reflectometry (TDR) (Mini Trase Kit 6050X3, Soil Moisture Equipment Corp., Santa Barbara, CA, USA). At 0, 5, 10, 15, and 20 days of treatments, a 20 cm long probe was vertically inserted into the soil of each tube, and the value of SWC was read directly.

According to the American National Turfgrass Evaluation Program (NTEP), TQ was visually evaluated by turf performance from 1 to 9 [41]. A rating of 9 represented plants with green color and dense turf canopy, 1 was completely dead plants, and 6 was the minimally acceptable quality rating.

RWC was analyzed as previously described by Barrs and Weatherley [42]. Fully expanded leaves were collected from tubes and immediately weighed as fresh weight (FW). Then fresh samples were moved into tubes with deionized water overnight in the dark at 4 °C and blotted dry, and then weighed immediately to obtain turgid weight (TW). Samples were moved into an oven at 80 °C for at least 72 h to obtain dry weight (DW). Leaf RWC was calculated based on the following formula:

$$\text{RWC (\%)} = [(\text{FW} - \text{DW})/(\text{TW} - \text{DW})] \times 100 \quad (1)$$

For leaf chlorophyll content (Chl), 0.35 g of fresh leaves was sampled and immediately soaked in 10 mL dimethylsulfoxide at room temperature in darkness for the extraction. The absorbance at 663 and 645 nm was measured with a spectrophotometer (Ultrospec 2100 pro, Biochrom Ltd., Cambridge, UK) to obtain the Chl content of samples [43].

EL was evaluated following the method of Blum and Ebercon [44]. Fresh leaves (0.2 g) were immersed in 30 mL of deionized water and shaken at 25 °C for 24 h. The initial conductivity of the liquid was measured by a conductivity meter (Orion Star A212, Thermo Scientific Inc., Waltham, MA, USA) as $C_{initial}$. Samples were then autoclaved at 121 °C for 20 min, and the maximal conductivity (C_{max}) was measured after cooling and shaking for another 24 h. EL was calculated based on the following formula:

$$\text{EL (\%)} = (C_{initial}/C_{max}) \times 100 \quad (2)$$

2.4. Measurements of Antioxidant Enzyme Activity, Malondialdehyde, and Hydrogenperoxide Content

The antioxidant enzyme activity of leaves was determined according to the method described by Zhang and Kirkham [45]. The enzymatic solution was extracted from fresh leaves (0.35 g) with a 4 mL cold mixture of 50 mM potassium phosphate and 1 mM ethylenediaminetetraacetic acid with a pH of 7.8. The homogenate was centrifuged at

4 °C and 12,000 rpm for 30 min. The supernatant was collected for further determination of SOD, CAT, POD, APX activity, and malonaldehyde (MDA) content.

For the analysis of SOD activity in leaves at 20 d, the formation rate of p-nitro blue tetrazolium (NBT) was recorded at the absorbance of 560 nm with a spectrophotometer. Activities of CAT, POD and APX in leaf tissues were measured by changes in absorbance at 240, 460, and 290 nm, respectively, at 20 days of the experiment [45].

MDA content was measured using the modified method by Zhang and Kirkham [45]. An antioxidant enzyme extraction solution of 1 mL was mixed with a 2 mL reaction solution of 20% trichloroacetic acid (TCA) and 0.5% thiobarbituric acid (TBA). The solution was incubated at 95 °C for 30 min and then centrifuged at 10,000× *g* for 10 min. The absorbance of the supernatant was recorded at 450, 532, and 600 nm with a spectrophotometer.

For hydrogen peroxide (H_2O_2) content, 0.3 g fresh leaves were homogenized with 5 mL 0.1% trichloroacetic acid. Then 0.5 mL supernatant was mixed with 0.5 mL 10 mM phosphate buffer (pH 7.0) and 1 mL 100 mM potassium iodide solution placed at room temperature in darkness for 1 h. The absorbance at 390 nm was measured by a spectrophotometer. The content of H_2O_2 was calculated according to the standard curve [46].

2.5. Statistical Analysis

The data were analyzed using the SPSS software version 21.0 (SPSS Inc., Chicago, IL, USA). A one-way ANOVA was used, followed by Fisher's protected least significant difference for multiple comparison test. The values were reported as means with standard error for all parameters. Differences were considered to be significant at $p < 0.05$.

3. Results

3.1. Determination of the Optimal Concentration of Carnosine and Chitosan

In order to determine the optimal concentration of carnosine or chitosan for improving drought tolerance, different concentrations of carnosine and chitosan were foliar sprayed in bermudagrass.

In the early period of drought stress (0–10 d), no obvious difference in TQ was found in treatments with or without foliar application of both carnosine and chitosan (Figure 1A,B). At 20 d of drought treatment, the decline in TQ was lesser with the chitosan application than the untreated drought alone, and TQ with 0.03% and 0.05% chitosan was distinctively higher than that with 0.01%, 0.07%, and 0.09% (Figure 1A). As shown in Figure 1B, at 15 d of drought treatment, the TQ of treatments, including both drought control and 2 mg L^{-1} carnosine, was significantly reduced compared with other treatments. At 20 d of drought stress, a higher TQ by 88.6% was found in leaves with the application of 10 mg L^{-1} carnosine than in the untreated drought control. No significant difference was observed in plants with foliar application of 8, 6, and 4 mg L^{-1} in comparison with that of drought control. In addition, TQ in treatment with 2 mg L^{-1} carnosine was significantly lower than drought control but without difference with 4 and 6 mg L^{-1} treatments. Therefore, 0.03% chitosan and 10 mg L^{-1} carnosine were selected as the optimal concentration for the next experiment.

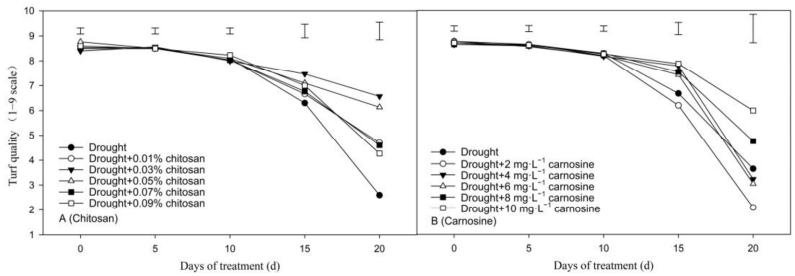

Figure 1. Effects of different concentrations of carnosine or chitosan on turf quality under drought stress in bermudagrass. 'Drought' represents plants without foliar application of any chemicals but only sprayed with distilled water. Vertical bars represent LSD values where a significant difference ($p < 0.05$) was detected among different treatments at the same time point.

3.2. Effects of Carnosine and Chitosan on Soil Water Status

Under well-watered conditions, SWC was about 12.7%, and no significant difference was observed in SWC between control and treatments from 0 d to 20 d (Figure 2A,B). Under drought stress, the SWC of all treatments decreased to about 61.7% compared with 0 d, and there was no obvious difference in SWC between the control and treatments.

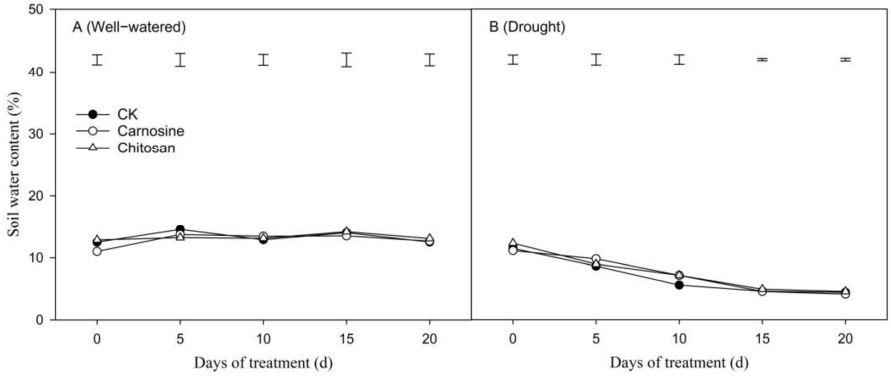

Figure 2. Effects of carnosine and chitosan on the soil water content in bermudagrass. 'CK' represents control plants sprayed with distilled water under both well-watered and drought stress. Vertical bars represent LSD values where a significant difference ($p < 0.05$) was detected among different treatments at the same time point.

3.3. Effects of Carnosine and Chitosan on Turf Performance

TQ of different treatments was maintained at the level of about 8.2–8.7 in bermudagrass under well-watered conditions. At 10 d and 15 d of drought treatments, plants with carnosine showed higher TQ than both control and chitosan treatments (Figure 3B). At 20 d of drought stress, foliar application of both carnosine and chitosan resulted in significantly greater TQ by 92.9% and 67.3% than the control, respectively.

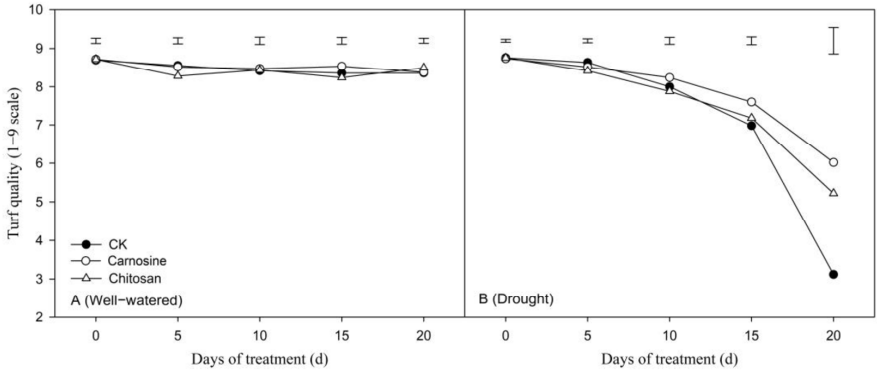

Figure 3. Effects of carnosine and chitosan on turf quality in bermudagrass. 'CK' represents control plants sprayed with distilled water under both well-watered and drought stress. Vertical bars represent LSD values where a significant difference ($p < 0.05$) was detected among different treatments at the same time point.

Under well-watered conditions, RWC was between 92.1% and 97.3% among treatments with or without foliar application of carnosine or chitosan (Figure 4A). At 15 d and 20 d of drought stress, carnosine treatment caused obviously higher RWC than control by 8.8% and 87.2%, respectively. A significant increase of 75.1% was also observed in chitosan treatment compared with drought control at 20 d of the experiment.

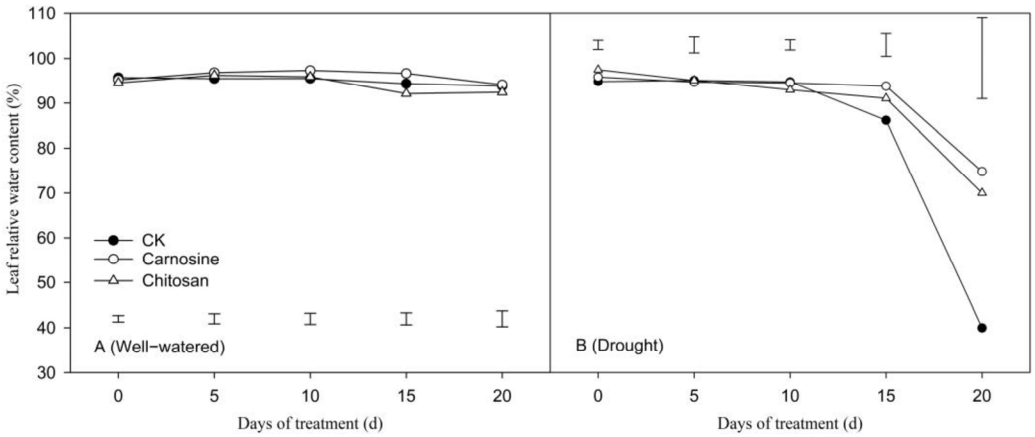

Figure 4. Effects of different concentrations of carnosine and chitosan on leaf relative water under drought stress in bermudagrass. 'CK' represents control plants sprayed with distilled water under both well-watered and drought stress. Vertical bars represent LSD values where a significant difference ($p < 0.05$) was detected among different treatments at the same time point.

Under drought stress, a significant difference induced by carnosine and chitosan treatments was not found in Chl content until 20 d of the experimental period compared with untreated control. Leaf Chl content of carnosine and chitosan treatment was 1.1-fold greater than the control, respectively, at 20 d (Figure 5B).

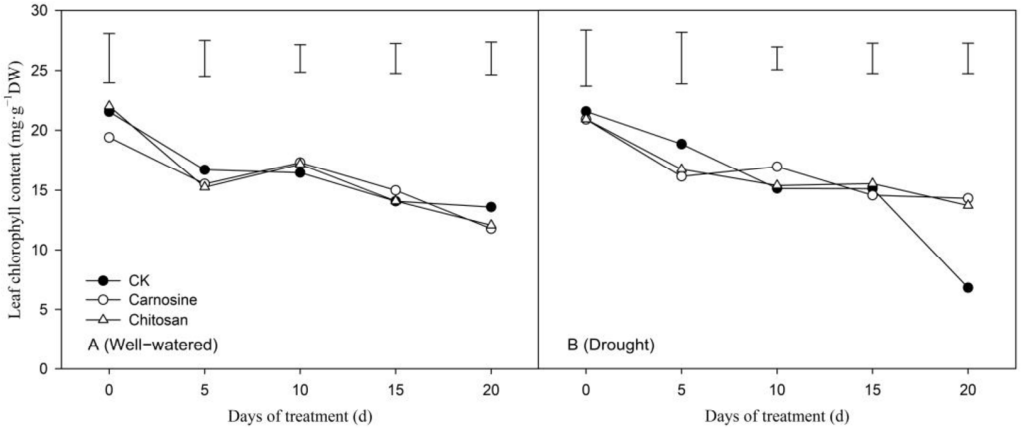

Figure 5. Effects of different concentrations of carnosine and chitosan on leaf chlorophyll content under drought stress in bermudagrass. 'CK' represents control plants sprayed with distilled water under both well-watered and drought stress. Vertical bars represent LSD values where a significant difference ($p < 0.05$) was detected among different treatments at the same time point.

3.4. Effects of Carnosine and Chitosan on Cellular Membrane Stability

Under well-watered conditions, no significant difference in EL was found in plants with foliar application of carnosine and chitosan in comparison with control during the entire experimental period except 5 d (Figure 6A). Under drought stress, EL of both control and treatments with carnosine and chitosan gradually increased with the experimental period. At 20 d of drought stress, exogenous carnosine and chitosan decreased EL by 31.0% and 22.1% compared with drought control. A significant difference was observed in EL in plants with foliar application of both carnosine and chitosan compared with control at 20 d under drought stress, but there was no significant difference between carnosine and chitosan treatments (Figure 6B).

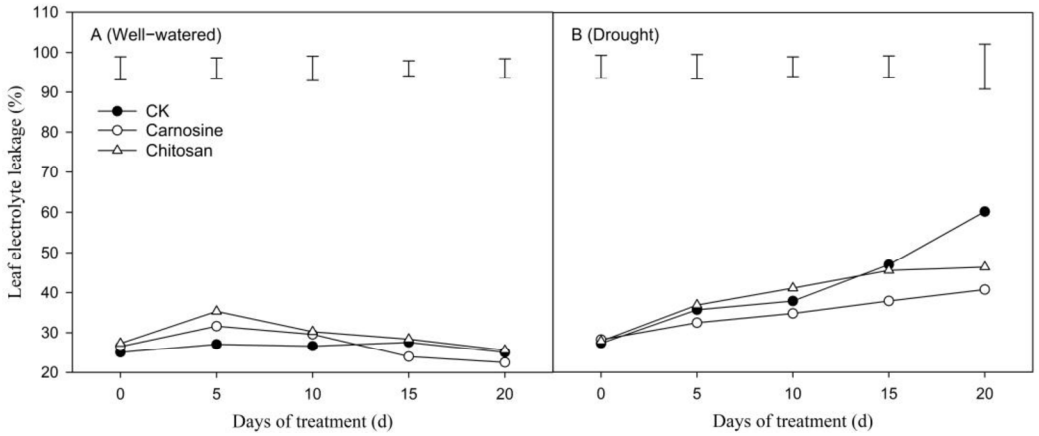

Figure 6. Effects of different concentrations of carnosine and chitosan on leaf electrolyte leakage under drought stress in bermudagrass. 'CK' represents control plants sprayed with distilled water under both well-watered and drought stress. Vertical bars represent LSD values where a significant difference ($p < 0.05$) was detected among different treatments at the same time point.

At 20 d of drought stress, MDA content was 29.7 μmol g^{-1} FW in plants treated with carnosine, which was 43.9% lower than drought control (52.9 μmol g^{-1} FW) (Figure 7). The MDA content of chitosan treatment was 35.3 μmol g^{-1} FW, which was 33.3% lower than drought control.

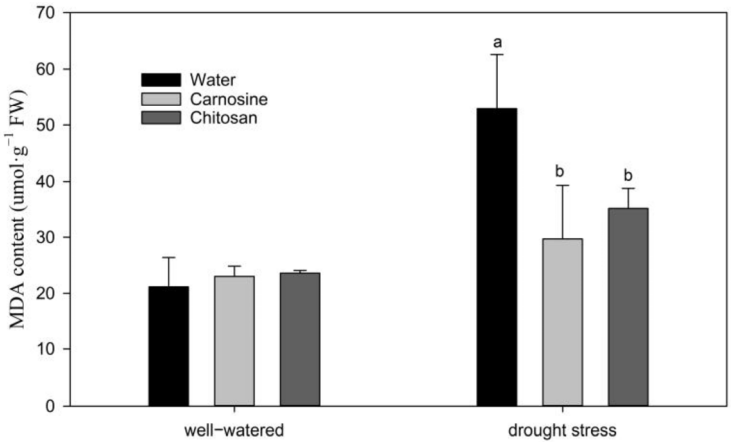

Figure 7. Effects of carnosine and chitosan on MDA content in bermudagrass. 'Water' represents control plants sprayed with distilled water under both well-watered and drought stress. The columns represent the means and standard errors (SE) of 5 replicates. Different letters represent a significant difference among treatments at the same time point at $p < 0.05$.

3.5. Effects of Carnosine and Chitosan on Enzyme Activities of Antioxidants

Activities of four antioxidant enzymes, including SOD, POD, CAT, and APX, were not impacted by the application of both carnosine and chitosan at 20 d of treatments under well-watered control treatment (Figure 8A). Under drought stress, plants with exogenous carnosine and chitosan had higher POD activity by 98.9% and 64.7%, respectively, than untreated control (Figure 8B). CAT activity in plants treated with both carnosine and chitosan was significantly increased by 77.3% and 89.1% compared with the untreated control, respectively.

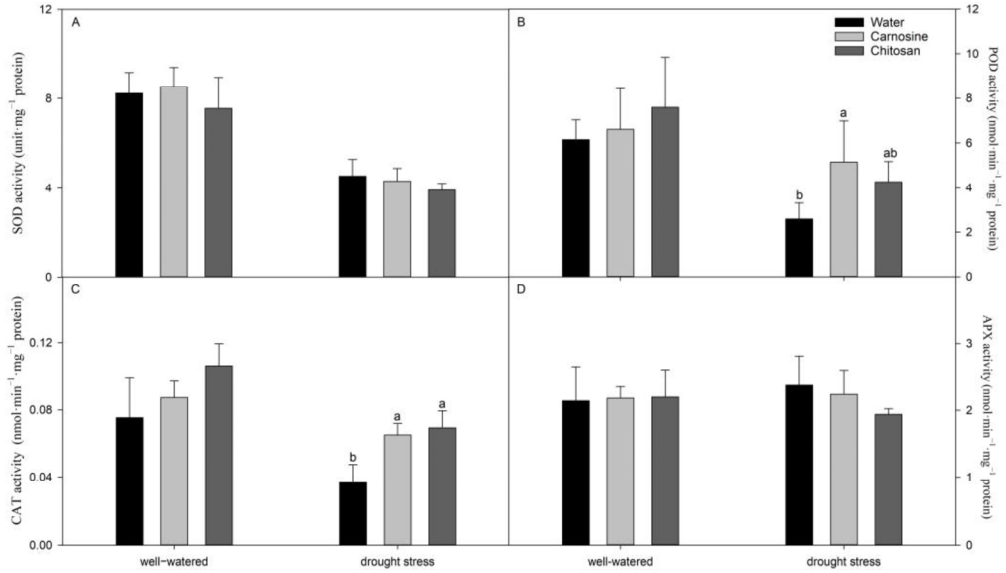

Figure 8. Effects of carnosine and chitosan on the activities of SOD (**A**), POD (**B**), CAT (**C**), and APX (**D**) in bermudagrass. 'Water' represents control plants sprayed with distilled water under both well-watered and drought stress. The columns represent the means and standard errors (SE) of 5 replicates. The different letters represent a significant difference among treatments at the same time point at $p < 0.05$.

At 20 d of well-watered conditions, H_2O_2 content in bermudagrass sprayed with chitosan was significantly lower by 61.2% than the control (Figure 9). After 20 d of drought treatment, H_2O_2 content in carnosine and chitosan was decreased by 56.7% and 41.3%, respectively, compared with the drought control.

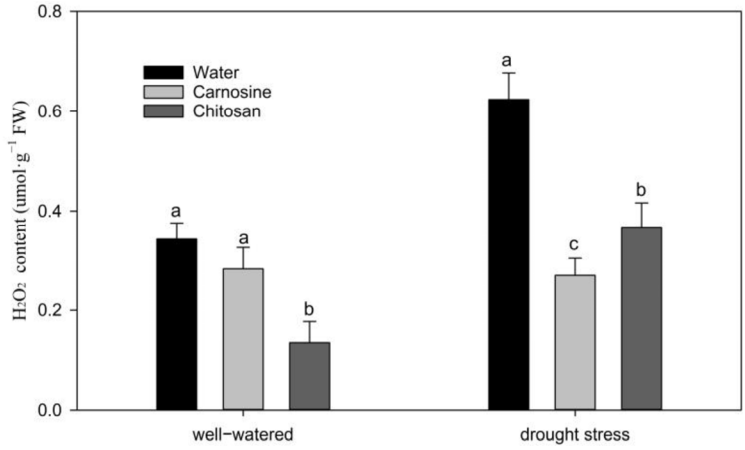

Figure 9. Effects of carnosine and chitosan on H_2O_2 content in bermudagrass. 'Water' represents control plants sprayed with distilled water under both well-watered and drought stress. The columns represent the means and standard errors (SE) of 5 replicates. The different letters represent a significant difference among treatments at the same time point at $p < 0.05$.

4. Discussion

Drought, as one of the most important abiotic stresses, impacted plant growth and development [47]. Carnosine and chitosan have recently been utilized as versatile antioxidants for attenuating cellular oxidative stress in animals [48]. In this study, drought stress caused a significant decrease in TQ, but both 10 mgL^{-1} carnosine and 0.03% chitosan could significantly inhibit the decline in TQ under drought stress, as indicated by higher RWC and Chl content (Figures 4 and 5). Leaf RWC reflected the ability of plants to retain available water under stressed conditions [49]. The greater RWC suggested that

both carnosine and chitosan were beneficial to the maintenance of leaf water status under drought stress. Enhanced RWC by chitosan has also been found in creeping bentgrass (*Agrostis stolonifera*) to improve heat resistance [50]. Chl content was associated with the photosynthetic performance of plants [51], and drought-tolerant rice exhibited higher Chl content [52]. Analogous to our results, Tourian et al. [37] reported the chitosan-responsive alleviation of Chl degradation in wheat grass (*Agropyron repens*). Hence, the larger Chl content in plants treated with foliar application of carnosine or chitosan reflected the protective effects on photosynthetic organs and contributed to the promoted drought tolerance in bermudagrass. The improvement in drought tolerance induced by the application of carnosine or chitosan in bermudagrass, as manifested by TQ, RWC, and Chl, could be due to changes in membrane lipid peroxidation as well as a corresponding antioxidant mechanism as discussed in detail below.

Membrane lipid peroxidation occurs when plants are subjected to various stresses, including drought, and MDA is the byproduct of membrane peroxidation [53]. The accumulation of MDA reflected membrane lipid peroxidation and damaged the structure and function of the cell membrane, thereby disordering the normal activities of a series of physiological and biochemical progresses [54]. Carnosine application on rice seeds was found to reduce the content of MDA under heat stress [55]. Exogenous chitosan was able to delay MDA accumulation in guava (*Psidium Guajava*) in response to low temperatures [56]. In this study, foliar application of both carnosine and chitosan caused a significant decrease in MDA content compared with untreated drought stress (Figure 7), indicating that carnosine and chitosan could inhibit membrane lipid peroxidation and protect membrane integrity. Therefore, as an indicator of cellular membrane stability, EL in plants treated with carnosine and chitosan was significantly lower than control under drought stress at 20 d of the experiment (Figure 6B), suggesting that plants with foliar application of carnosine and chitosan suffered from fewer injuries and maintained relatively better membrane structure compared with untreated control. Other reports also demonstrated that foliar application of chitosan increased cell membrane integrity in *H. verticillate* [57] and apple seedlings [58]. Moreover, the lower levels of both MDA and EL in treatments with carnosine and chitosan application were associated with a better antioxidant defense system, as discussed below.

In order to scavenge ROS caused by drought stress to protect cellular membrane stability in the present case, carnosine application resulted in a significant increase in activities of both CAT and POD under drought stress indicating that carnosine played a crucial role in scavenging H_2O_2 just as indicated by the decreased content of H_2O_2 in bermudagrass (Figures 8B and 9B). Similarly, it was reported that carnosine increased CAT and POD activity in rice seedlings under heat stress [55]. Different from carnosine, chitosan application only enhanced CAT activity among four antioxidant enzymes in plants subjected to drought stress, suggesting that chitosan-induced decline in H_2O_2 in response to drought stress was attributed to the stimulation of CAT activity (Figure 8). In a previous study, an enhancement in CAT activity upon chitosan treatment was also observed in stevia (*Stevia rebaudiana*) under NaCl stress [59]. Antioxidant properties of chitosan were primarily attributed to the abundant active hydroxyl and amino groups, which could react with ROS to form stable and nontoxic macromolecular radicals [60]. Taken together, the exogenous application of carnosine and chitosan protected the oxidative defense system, possibly by scavenging excess ROS that damage the antioxidant defense system, enhancing the activities of antioxidant enzymes, and improving plant growth under drought stress. Further research is still needed to examine the mechanism of the positive effects of carnosine or chitosan on plant growth performance under water deficit conditions at the metabolic and molecular levels.

5. Conclusions

According to our findings, exogenous application of carnosine (0.03%) and chitosan (10 mg L^{-1}) effectively protected plant growth by maintaining the higher Chl and RWC and decreasing EL, MDA, and H_2O_2 content in bermudagrass. The improvement of carnosine

was associated with the increased activities of both CAT and POD, but exogenous chitosan only increased CAT activity. Thus, the application of carnosine and chitosan led to a decrease in the adverse effects of drought stress by regulating the antioxidant defense system, which ultimately enhanced the growth characteristics of bermudagrass. The results of this study would provide contributions to developing new plant growth regulators that could effectively mitigate drought-induced damages in perennial turf grass species. It would bring helpful insights for turf management, especially under drought-stressed seasons.

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