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Research Article

Effects of cytokinin and abscisic acid on heat resistance of *Vetiveria zizanioides*

Jingchao WU[1,2], Yong LU[3], Biyan ZHOU[1]*, Zhiqun HU[1]*

[1]*South China Agricultural University, Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (South China), Ministry of Agriculture and Rural Affairs, College of Horticulture, Guangzhou 510642, China; zhoubiyan@scau.edu.cn (*corresponding author); zhqhu@scau.edu.cn (*corresponding author)*
[2]*Quanzhou Institute of Agricultural Sciences, Quanzhou Fujian 362212, China; 522376867@qq.com*
[3]*Agricultural Science and Technology Extension Center in Shenzhen Baoan District, Shenzhen, Guangdong 518101, China; luyong433@163.com*

Abstract

Vetiveria zizanioides is ideal in maintaining soil and water, and is widely used for remediation of soil contaminated by heavy metals. However, it is affected by high-temperature stress. In this study, *Vetiveria zizanioides* plants were sprayed with 6-BA and ABA in a growth chamber 1 d before heat stress treatment, then the plants were subjected to high-temperature conditions. Relative water content, relative electrical conductivity, contents of ascorbic acid (AsA) and reduced glutathione (GSH) as the antioxidative substances, and content of malondiadehyde (MDA) were determined. Also, the antioxidative enzyme activities and the osmoprotectants levels were detected. Diaminobenzidine (DAB) staining of leaves and roots in *Vetiveria zizanioides* was observed for determination of hydrogen peroxide accumulation. The results showed that relative water content was decreased, relative electrical conductivity and MDA content were increased by the heat stress treatment. Under high-temperature conditions, relative water content was increased and relative electrical conductivity was decreased by 6-BA and ABA treatments. At the middle and the late stages of the heat stress treatment, activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), and the contents of ASA and GSH in leaves of the 6-BA-treated and ABA-treated plants were significantly higher, whereas the MDA content was significantly lower than those in the high-temperature controls. Hydrogen peroxide accumulation levels in the 6-BA-treated and ABA-treated leaves and roots were lower than in the high-temperature controls. Contents of soluble sugar, sucrose, and glucose in the 6-BA-treated and ABA-treated leaves were significantly higher than those in the high-temperature controls. Proline content in the 6-BA-treated and ABA-treated leaves was stable. The results suggested that the enhancement of the heat resistance by 6-BA and ABA treatments was correlated with the activation of the antioxidant system, as well as the sugar-based osmoprotectant.

Keywords: 6-benzylaminopurine; abscisic acid; antioxidant system; high temperature; proline; stress; sugar; *Vetiveria zizanioides*

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Introduction

Vetiveria zizanioides (Linn.) Nash, known as vetiver grass, a perennial plant, is ideal in maintaining soil and water (Mickovski and van Beek, 2009). Also, it is widely used for remediation of soil contaminated with heavy metals (Paz-Alberto *et al*., 2007). The planting areas of vetiver grass are located in tropical and subtropical regions, where temperature is extremely high in summer. The plants may seriously be affected by high-temperature stress. Therefore, it is essential to improve the heat resistance of vetiver grass for its application and promotion.

In recent years, climate change and global warming frequently result in extreme weather, including extremely high temperatures. Plants have developed a set of protective mechanisms during long-term adaptation to environmental stress. Reactive oxygen species (ROS) accumulate when plants are under stressful conditions. Plants can activate their enzymatic defense systems such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and non-enzymatic defense systems such as ascorbic acid (AsA) and reduced glutathione (GSH) to improve ROS scavenging capacity (You and Chan, 2015). Cytokinin (CTK) and abscisic acid (ABA) have been shown to enhance plant tolerance to abiotic stresses (Zhou *et al*., 2005; Dias *et al*., 2014; Gupta and Rashotte, 2017). ABA can improve plant resistance by increasing the activity of antioxidant enzymes and the level of antioxidant substances in elms and *Stylosanthes guianensis* (Zhou *et al*., 2005; Dias *et al*., 2014). Cytokinin can enhance the plant scavenging system, reduce the attack of ROS on photosynthetic membranes and thus maintain high photosynthetic efficiency under stressful conditions (Wang 2000; Cortleven *et al*., 2019). Our previous studies have shown that ABA and CTK effectively improved drought resistance in *Vetiveria zizanioides* (Hu *et al*., 2015). However, the effects of these plant growth regulators on heat tolerance of *Vetiveria zizanioides* have not been reported.

In this paper, *Vetiveria zizanioides* plants were sprayed with 6-benzyladenine(6-BA) and ABA under normal temperature conditions, then subjected to high-temperature stress 1 d later. Physiological and biochemical changes of the *Vetiveria zizanioides* plants under stressful conditions, as well as the effects of 6-benzyladenine(6-BA) and ABA on the plants were studied, with the aim to provide a theoretical basis for improving the heat tolerance of *Vetiveria zizanioides*.

Materials and Methods

Plant material and experiment procedures

'Sunshine' *Vetiveria zizanioides* seedlings were grown in greenhouses in the College of Horticulture, South China Agricultural University. *Vetiveria zizanioides* seedlings with similar number of tillers were transplanted into 15 cm diameter plastic pots with a 5:1 mixture of peat soil and perlite. Each pot was planted with 5 seedlings. Fifteen days after transplanting, 12 pots with 60 seedlings were moved to a controlled growth chamber with high temperature (Chamber I, 06:00-18:00, 43 ℃, 18:00-06:00, 38 ℃), and 4 pots with 20 seedlings were transferred to another room with normal temperature (Chamber II, 06:00-18:00, 28 ℃, 18:00-06:00, 23 ℃). 12 pots were evenly divided into 3 groups, and sprayed with distilled water (CK1), 15 mg L^{-1} 6-BA (6-BA treatment), and 10 mg L^{-1} ABA (ABA treatment), respectively, kept in normal temperature for 24 h. Then the plants were transferred to Chamber I for heat stress treatment. The remaining 4 pots were sprayed with distilled water and transferred to Chamber II as normal temperature control (CK2). The distilled water or solutions contained 0.01% Tween-20. All the seedlings were watered daily to keep the soil moist. The second or third leaves of the seedlings were collected at 1, 3, 5, 7, and 9 d after high-temperature treatment.

Determination of relative electrical conductivity and relative water content

The second mature leaves of *Vetiveria zizanioides* were collected for determination of relative electrical conductivity and relative water content. Relative electrical conductivity was detected according to the method of Blum and Ebercon (1981). Relative water content was determined as described by Zhou *et al.* (2005).

Determination of MDA content

MDA content was detected using the method described by Huang and Guo (2005). The colorimetric method of thiobarbituric acid was used to determine the OD values at the wavelengths of 532 nm and 600 nm. The MDA content (mM) was calculated according to the formula: (OD532 nm -OD600 nm)/(1.55×10^5).

Determination of SOD, CAT, and POD activities

Leaves were ground in phosphate buffer (50 mmol L^{-1}, pH7.8, containing 2% polyvinylpyrrolidone) in an ice bath, centrifuged at 13000×g for 20 min at 4℃. The supernatant was the enzyme extract. SOD activity was determined by the method of Giannoplitis and Ries (1977). CAT activity was determined by the method of Chance and Maehly (1955). POD activity was determined by the method of Kraus and Fletcher (1994).

Determination of AsA and GSH content

Leaves were extracted by grinding with 5 mL of 5% trichloroacetic acid solution, centrifuged for 15 min at 10 000×g at 4 °C, and the supernatant was fixed to 5 mL for the determination of AsA and GSH according to the method of Law *et al.* (1983).

Determination of proline content

Leaves were extracted with 3% sulfosalicylic acid solution, mixed, and extracted in a boiling water bath for 10 min, filtered. The filtrate was proline extract. Proline content was determined according to the method of Xiao and Wang (2005).

Determination of soluble sugar content

Ground leaves 0.25 g were extracted with 5.0 mL 80% ethanol in a boiling water bath for 0.5 h, shaken repeatedly, and centrifuged at 6 000×g for 5.0 min. The residue was extracted again with 3 mL 80% ethanol, combined supernatant, added 0.1 g activated carbon, shaken several times, and filtered. The filtered residue was washed with 2.0 mL of 80% ethanol, and the filtrate was fixed to 8.0 mL, as the soluble sugar extraction solution. Total soluble sugars and sucrose were determined according to the method of Xue (1985). Glucose content was determined according to the method of Yang *et al.* (2014).

Determination of hydrogen peroxide content

Observations were made using diaminobenzidine (DAB, Diaminobenzidine) tissue staining method according to the method of Guan and Scandalios (2000).

Experimental data analysis

Significance of differences was analyzed according to Duncan's new complex polarization method with a significance level of 0.05, using the software DPS (7.05).

Results

Effects of 6-BA and ABA on the growth of Vetiveria zizanioides plants under high temperature stress

As shown in Figure 1, plants of the normal temperature control (CK2) grew well with upright and dark green leaves, while the leaves of the high-temperature control (CK1) wilted and curled. Under high temperature condition, the 6-BA and ABA treated leaves were green, and the yellowish area of the leaves was less than that of the CK1. The results showed that plant growth was inhibited by high-temperature stress, while exogenous application of 6-BA and ABA could reduce the inhibition.

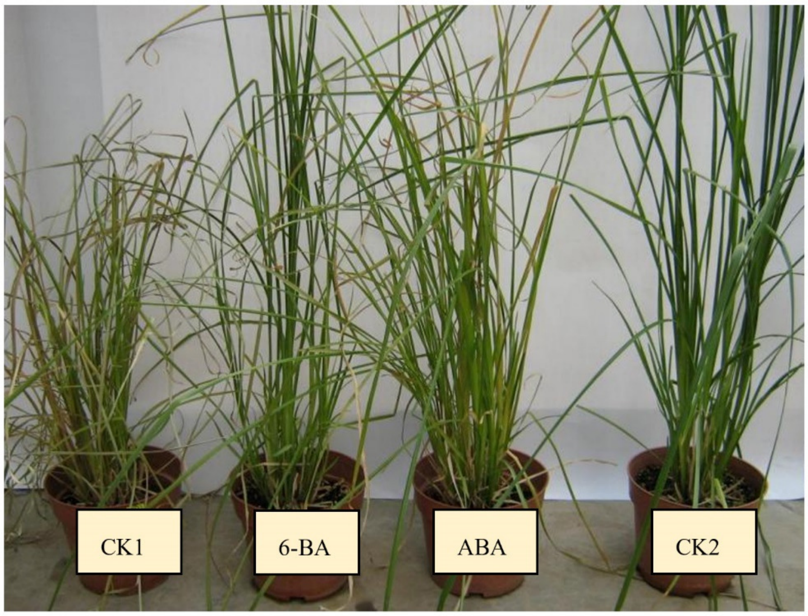

Figure 1. Phenotype of the *Vetiveria zizanioides* plants 9 d after 6-BA and ABA treatments under high-temperature conditions
From left to right are CK1 (high-temperature control) plants, 6-BA treated plants under high-temperature conditions, ABA treated plants under high-temperature conditions, and CK2 (normal temperature control) plants, respectively

Effects of 6-BA and ABA on the relative water content and relative electrical conductivity of leaves

As shown in Figure 2, relative water content (RWC) of *Vetiveria zizanioides* leaves showed a decreasing trend under high-temperature conditions. At day 9, leaf RWC decreased to 17.2%. RWC of the ABA and 6-BA treated leaves maintained at a higher level compared to that of the CK2, and was significantly higher than that of the CK1 after 5 and 9 d of treatment. Relative electrical conductivity of the CK1 leaves showed a sharply increasing trend, while that of the ABA and 6-BA treated leaves increased slowly and remained at a low level and was similar to that of the CK2.

Effects of 6-BA and ABA on SOD, CAT, POD activities and MDA content

As shown in Figure 3A, the SOD activity of the high-temperature (CK1) treated leaves showed a decreasing trend. The activity was significantly lower than that of the CK2, the 6-BA and ABA treated leaves at 7 and 9 d time points. The CAT activity in the CK2 leaves showed a relatively stable trend and remained at a high level, while that of the CK2, the 6-BA and ABA treated leaves showed decreasing trends. Under high-temperature conditions, the CAT activity of the 6-BA and ABA treated leaves was significantly higher than that of the high-temperature control at the 7 and 9 d time points (Figure 3B).

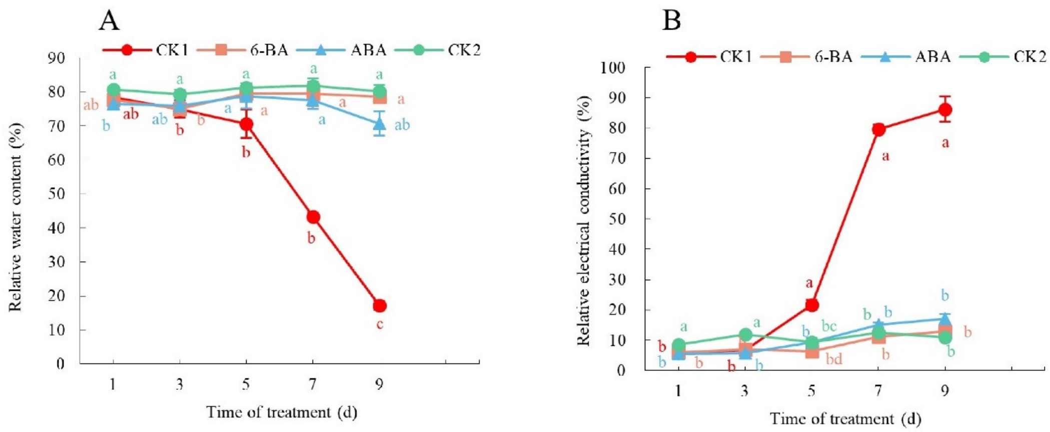

Figure 2. Effects of 6-BA and ABA on relative water content and relative electrical conductivity of *Vetiveria zizanioides* leaves under high-temperature conditions

CK1, high-temperature control. CK2, normal temperature control. Bars on the columns indicate stand errors (n=4). Different letters indicate significant differences at P≤0.05 level among treatments at the same time point according to Duncan's multiple range test.

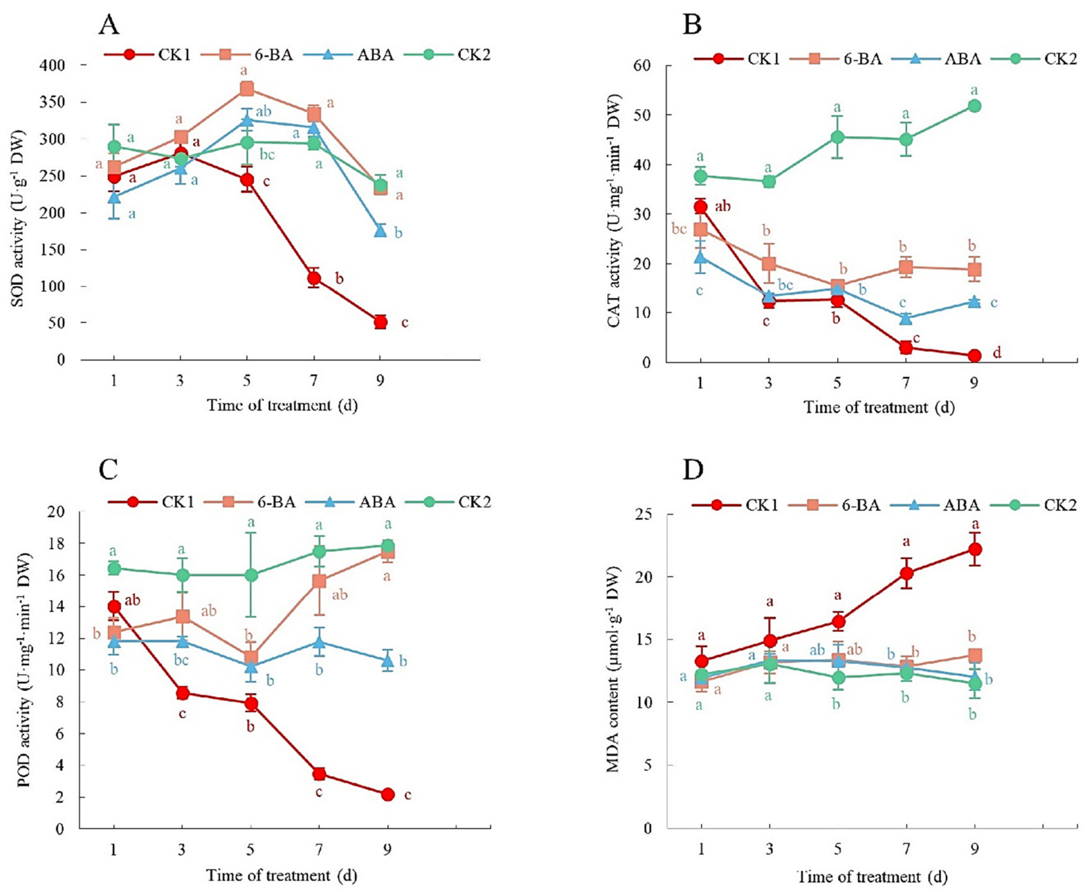

Figure 3. Effects of 6-BA and ABA treatments on SOD (A), CAT (B), POD (C) activities and MDA (D) content of *Vetiveria zizanioides* leaves

CK1, high-temperature control. CK2, normal temperature control. Bars on the columns indicate stand errors (n=4). Different letters indicate significant differences at P≤0.05 level among treatments at the same time point according to Duncan's multiple range test.

POD activity in the CK2 leaves showed a relatively stable trend and remained at a high level, while that of the CK1 showed a decreasing trend. Under high-temperature conditions, the POD activities of the 6-BA and ABA treated leaves were significantly higher than that of the high-temperature control at the 7 and 9 d time points (Figure 3C).

The MDA content of the high-temperature control leaves showed an increasing trend, while that of the CK2, 6-BA, and ABA treated leaves showed a stable trend and remained low. There were no significant differences among the CK2, 6-BA, and ABA treated leaves (Figure 3D).

Effect of 6-BA and ABA on the accumulation levels of H_2O_2 in leaves and roots

DAB staining represents the level of H_2O_2 accumulation. The darker staining indicated more H_2O_2 accumulation. As shown in Figure 4, the accumulation of H_2O_2 in CK2, 6-BA, and ABA treated leaves and roots was less than in the high-temperature control ones (Figure 4, A and B).

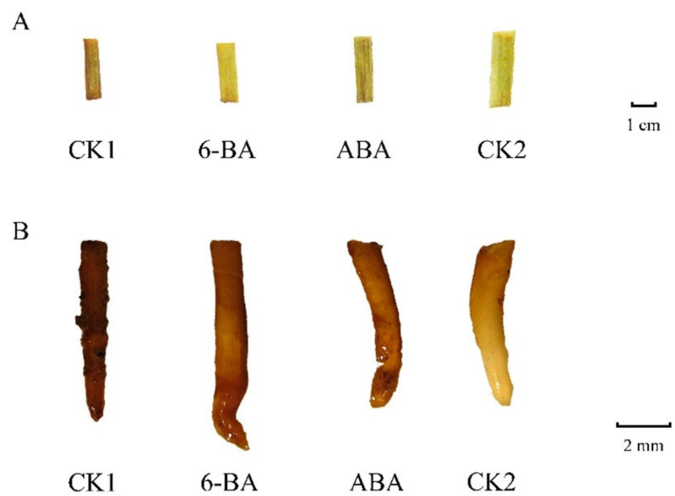

Figure 4. Hydrogen peroxide accumulation of the 6-BA and ABA treated leaves (A) and roots (B) in *Vetiveria zizanioides* indicated by DAB staining

From the left to the right are CK1 (high-temperature control) tissues, 6-BA treated tissues under high-temperature conditions, ABA treated tissues under high-temperature conditions, and CK2 (normal temperature control) tissues, respectively.

Effect of 6-BA and ABA on AsA and GSH contents of Vetiveria zizanioides

As shown in Figure 5A, AsA content in the CK2 leaves showed a relatively stable trend, while that in the high temperature treated leaves increased and then decreased. The AsA content in the 6-BA and ABA treated leaves showed an overall increasing trend. At the 7 and 9 d time points, AsA content in the 6-BA and ABA treated leaves was significantly higher than that in the CK1 and CK2.

GSH content of the CK2 leaves was stable, while that of the high-temperature treated leaves decreased during the treatment. The content in CK1 was only 35.0% of that in CK2 at the 7 d time point. At the 7 and 9 d time points, the GSH content of the 6-BA and ABA treated leaves was significantly higher than that of the CK2 (Figure 5B).

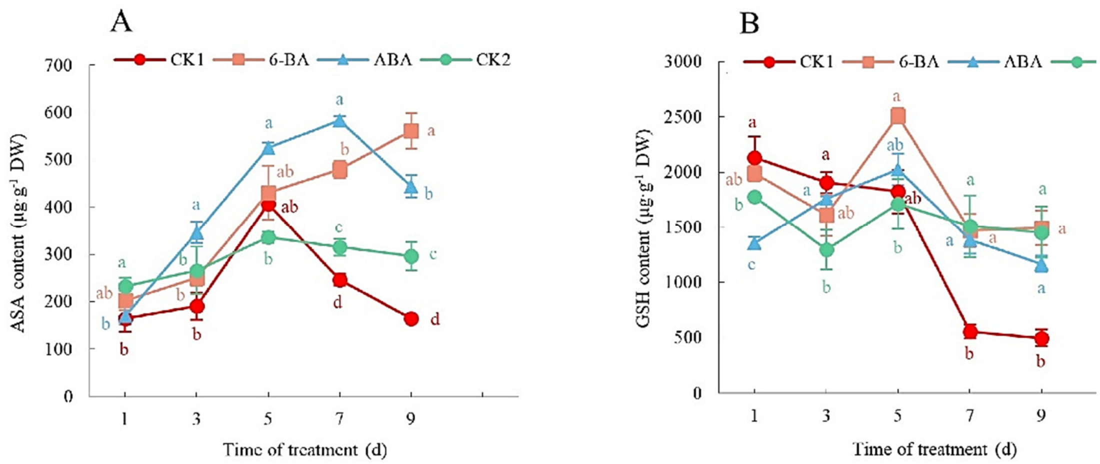

Figure 5. Effects of 6-BA and ABA on AsA (A) and GSH (B) contents of *Vetiveria zizanioides* leaves. CK1, high-temperature control

CK2, normal temperature control. Bars on the columns indicate stand errors (n=4). Different letters indicate significant differences at P≤0.05 level among treatments at the same time point according to Duncan's multiple range test.

Effect of 6-BA and ABA on sugar and proline contents in Vetiveria zizanioides

As shown in Figure 6A, total soluble sugar content of the CK1 leaves increased at the 3 d time point, then decreased, while that of the CK2 was stable. Total soluble sugar content of the 6-BA and ABA-treated leaves showed an increasing trend until 7 d of treatments. Moreover, at 5, 7, and 9 d time points, the content was significantly higher than that of the CK1. Sucrose and glucose contents of the treatments showed similar trends as those of the total soluble sugar contents. Also, at 5, 7, and 9 d time points, they were significantly higher than those of the CK1 (Figure 6, B and C).

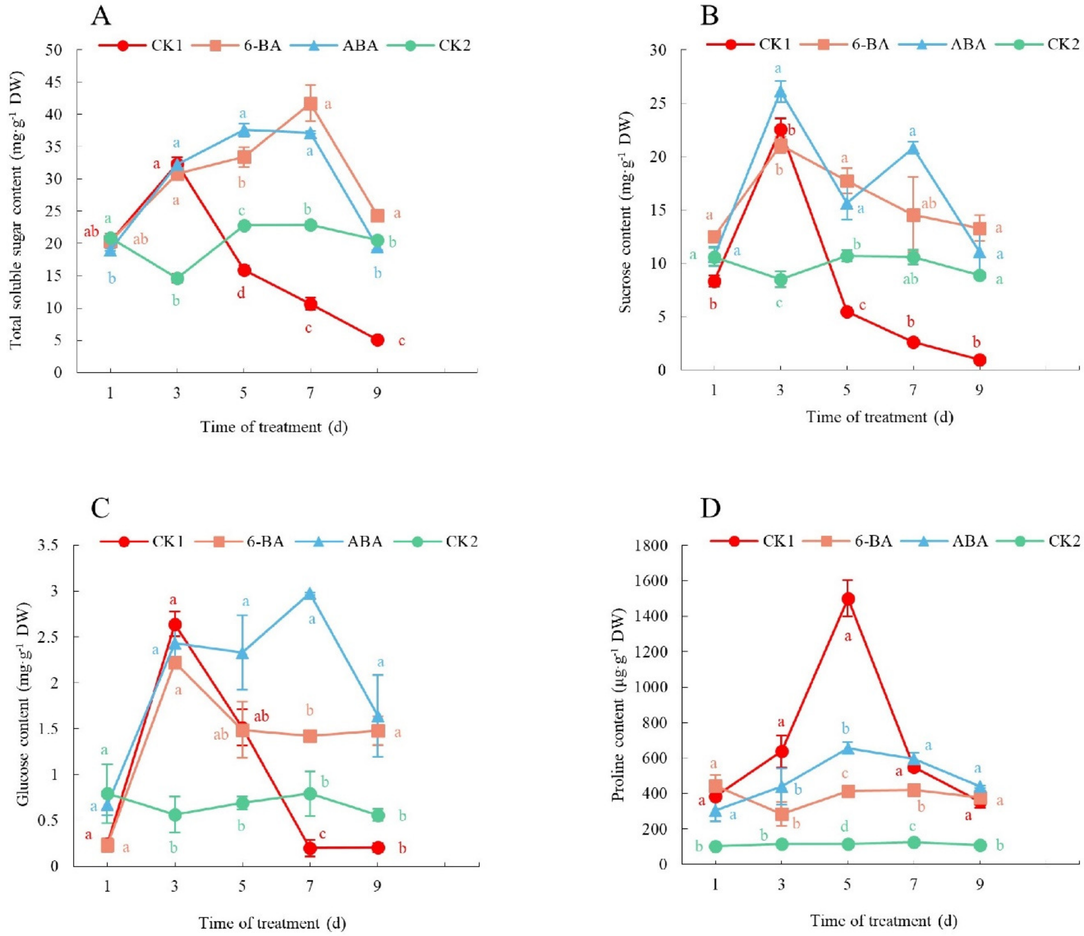

Figure 6. Effects of 6-BA and ABA on sugar and proline contents in *Vetiveria zizanioides* leaves

A, total soluble sugar content. B, sucrose content. C, glucose content. D, proline content. CK1, high-temperature control. CK2, normal temperature control. Bars on the columns indicate stand errors (n=4). Different letters indicate significant differences at P≤0.05 level among treatments at the same time point according to Duncan's multiple range test.

At normal temperature, the proline content of the leaves remained stable. Under high-temperature conditions, that of the CK1 increased to the highest level on day 5 and then decreased. Proline content of the 6-BA and ABA-treated leaves was stable and was significantly lower than that of the CK1 (Figure 6D).

Discussion

When plants are subjected to stressful conditions, membrane structure may change, and membrane permeability increases, resulting in electrolyte leakage. The level of electrolyte leakage is measured by relative electrical conductivity (Takashima *et al*., 2021). Also, lipid peroxidation in the membrane may occur, and the lipid peroxidation product MDA accumulates (El-Beltagi and Mohamed, 2013). In the present study, we determined the relative electrical conductivity and the MDA content in the leaves of the *Vetiveria zizanioides* plants. We found that they were increased by the heat stress, suggested that heat stress could affect membrane by increasing their membrane permeability and lipid peroxidation levels. Interestingly, when the *Vetiveria zizanioides* plants were sprayed with 6-BA and ABA 1 d before subjected to heat stress, their membrane permeability and lipid peroxidation levels were less than those of the heat stress control, indicating that exogenous application of 6-BA and ABA could reduce membrane damage, hence increase heat resistance of *Vetiveria zizanioides* plants.

The plant hormone ABA is known as a signaling substance and is induced by abiotic stresses (Hewage *et al*., 2020). Exogenous application of ABA can promote the recovery of *Zea mays* seedlings from heat stress (Bonham-Smith *et al*., 1988). Cytokinin (CTK) is another crucial hormone involved in plant growth and development, as well as plant responses to stresses (Cortleven *et al*., 2019). Exogenous application of CTK enhanced the heat tolerance of creeping bentgrass (Liu and Huang, 2002). In accordance with these previous studies, our results also showed that exogenous application of ABA, and 6-BA, a kind of CTK, enhanced heat resistance of *Vetiveria zizanioides* by reducing membrane permeability and lipid peroxidation levels. The water status of the plant indicated by RWC was also kept by ABA and 6-BA treatments under high-temperature conditions. Further confirmed that ABA and 6-BA increased the heat resistance of *Vetiveria zizanioides.*

When plants are under heat stress conditions, ROS such as superoxide radical, hydroxyl radical, and H_2O_2 may accumulate. The accumulated ROS may damage the cellular components. Plants can activate their antioxidant system to scavenge ROS (Asthir, 2015). To further investigate the mechanism of the enhancement of heat resistance by the ABA and the 6-BA treatments, we determine the H_2O_2 level and the ROS scavenging system of the treated plants and the control plants, including the antioxidant enzymes SOD, POD, CAT, and the antioxidant substances AsA and GSH. The results showed that the ABA and 6-BA treated *Vetiveria zizanioides* plants had higher activities of SOD, POD, CAT, and contents of AsA and GSH, a lower level of H_2O_2, compared with the untreated plants under heat stress conditions. These results indicated that ABA and 6-BA activated the ROS scavenge system in the *Vetiveria zizanioides* plants, and resulted in a relatively lower level of H_2O_2 under heat stress conditions. The increase in the heat resistance of the *Vetiveria zizanioides* plants was related to the activation of the ROS scavenging system by ABA and 6-BA.

When faced with stresses, plants can accumulate compatible solutes to counteract water efflux to maintain growth and development (Sangu *et al*., 2015; Xia *et al*., 2020). These compatible solutes are regarded as osmoprotectants, e.g., proline, sugars (Chen *et al*., 2007). Under heat stress conditions, the ABA and 6-BA treated *Vetiveria zizanioides* plants had high levels of osmoprotectants, including sucrose and glucose, as well as the total soluble sugar, indicating that exogenous application of ABA and CTK could increase the sugar-based osmoprotectant. However, the proline-based osmoprotectant did not show similar trends. Though we did not find induction of proline by ABA and CTK treatments. An increase in the *Vetiveria zizanioides* plants under heat stress conditions was observed. Similar to the *Sorghum bicolour* plant under heat stress conditions (Gosavi *et al*., 2014). Exogenous application of proline enhances heat resistance of *Vigna radiata* (Priya *et al*., 2019). Our result suggested that the increase in the heat resistance by ABA and CTK was related to the increase in sugar-based osmoprotectant, not the proline-based osmoprotectant.

In the present studies, we focused on the physiological and biochemical mechanism of the ABA and CK induced resistance in *Vetiveria zizanioides* plants. It seems that the activation of the heat resistance by ABA and CTK also involved transcriptomic and proteomic changes. Therefore, future studies should be carried out to investigate the molecular mechanism of the ABA- and CTK-induced resistance in *Vetiveria zizanioides* plants.

Conclusions

6-BA and ABA treatments improved heat resistance of *Vetiveria zizanioides*. The enhancement of the heat resistance in *Vetiveria zizanioides* was related to increase in the activities of the antioxidant enzymes SOD, CAT, POD, the contents of the antioxidant substances AsA, GSH, and the sugar-based osmoregulatory ability of the 6-BA and ABA treated plants.

Authors' Contributions

Methodology, JW, ZH and BZ; formal analysis, JW and YL; writing - original draft preparation, JW and BZ; writing - review and editing, BZ and ZH; project administration, ZH; funding acquisition, BZ.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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