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Exogenous Application of GABA Alleviates Alkali Damage in Alfalfa by Increasing the Activities of Antioxidant Enzymes

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Abstract: Alfalfa (*Medicago sativa* L.) is a widely grown and important forage crop. However, alkali stress is detrimental to alfalfa yield and nutritional quality. Gamma-aminobutyric acid (GABA) is a signaling molecule, it participates in and responds to metabolic regulation related to plant growth and development and stress. In this study, we clarify the effect of spraying alfalfa seedlings with GABA on the alkali tolerance of the seedlings. We determined that exogenous application of GABA at 75 mmol/L improved the resistance of alfalfa seedlings to alkali stress caused by exposure to 100 mmol/L $NaHCO_3$, pH 8.5. Exogenous GABA significantly increased the chlorophyll content, the accumulation of soluble sugars in the plants, significantly decreased their relative electrical conductivity, malondialdehyde (MDA), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) contents, and significantly increased the activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD). Exogenous GABA increased the activities of GAD and GABA-T, increased the content of glutamate and endogenous GABA, and regulated the expression of the genes *MsGAD*, *MsGABA-T* and *MsGDH*. In alfalfa plants that survived for a long time, compared to the control group, root length and fresh weight were significantly higher. Exogenous GABA had a positive effect on the resistance of alfalfa seedlings to alkali stress, and the effect was closely associated with GAD and GABA-T activity, glutamate content and the levels of endogenous GABA and antioxidants. This work provides a new method for the cultivation of early-stage alfalfa seedlings under low or moderate alkali stress conditions through the application of 75 mmol/L GABA.

Keywords: alfalfa (*Medicago sativa* L.); alkali stress; γ-aminobutyric acid (GABA); antioxidant enzymes; GABA metabolism

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1. Introduction

Alfalfa (*Medicago sativa* L.) is a forage legume with a long history of cultivation [1] and is planted all over the world [2]. However, with the increasing area and degree of soil salinization worldwide, alkali stress has become an important factor in restricting the yield and quality of alfalfa.

Abiotic stress responses as well as plant growth and development are strongly influenced by plant growth regulators. GABA, a four-carbon nonproteinogenic amino acid, is widely distributed in both animals and plants. It functions as an endogenous signaling molecule in plants, influencing plant responses to environmental stresses [3,4]. Previous studies have demonstrated that GABA levels change rapidly in response to abiotic stresses such as waterlogging stress, salt stress, cold shock and heat shock, and mechanical stimulation [5–8]. GABA could improve the photosynthesis and chlorophyll(Chl) fluorescence parameters of muskmelon seedlings under normal and hypoxia stress conditions, particularly under hypoxia stress conditions [9]. Under stress conditions, GABA

can also be used as a small molecular protective substance in plants. The content of endogenous GABA in plants increased significantly in a saline-alkali stress environment to improve plant stress resistance [10]. With stress, exogenous GABA can not only be used as a nitrogen source but also to reduce the damage of stress to plants by scavenging reactive oxygen species and alleviating cell acidification [11]. Pretreatment of muskmelon leaves with exogenous GABA could reduce the excessive accumulation of Chl and its precursors caused by salinity and alkalinity stress, so as to avoid photooxidative damage [12]. Other studies showed that application of GABA enhanced the seed germination percentage in white clover (*Trifolium repens* L.) [13], lettuce (*Lactuca sativa* L.) [14], and rice (*Oryza sativa* L.) [15] exposed to various levels of salinity stress. In NaCl-treated perennial ryegrass *(Lolium perenne* L.), exogenous application of 1 mmol/L GABA substantially improved germination, and GABA increased salinity tolerance by improving seedling growth and altering carbohydrate and antioxidant metabolism [16].

GABA also helps to reduce oxidative damage by activating antioxidant enzymes, which form a defense system against ROS. Exogenous GABA has been shown to promote antioxidant enzyme-catalyzed and nonenzymatic reactions as well as membrane stability in a variety of crops [17,18]. Shi et al. found that the addition of GABA induces the expression of genes related to the production of H_2O_2 and ethylene in the roots of *Caragana intermedia* [19]. Nayyar et al. found that exogenous spraying of GABA protects rice seedlings from heat stress by alleviating leaf swelling and upregulating the expression of osmotic protectants and antioxidants [20]. Aminohexenic acid (VGB) is a specific inhibitor of GABA aminotransferase (GABA-T). In the presence of VGB, GABA-T activity is reduced and GABA transformation is inhibited, and this affects mitochondrial respiration and causes ROS accumulation [21]. It has been shown that salicylic acid, proline and GABA synergistically enhance the antioxidant system and the ascorbic acid-glutathione (ASA-GSH) cycle in plants [22]. Proline and GABA were shown to synergically increase the activities of SOD, MDHAR, DHAR, and GR, increase ASA and GSH content, and decrease MDA and H_2O_2 content, thereby alleviating membrane lipid peroxidation damage [23]. Exposure of water-soaked wheat seedlings to exogenous GABA can improve the chloroplast ultrastructure and photosynthetic characteristics, activate the antioxidant defense system by downregulating the enzymes that produce reactive oxygen intermediates (ROI) and improve plant growth under stress conditions [24].

Although the effects of GABA on salinity tolerance have been studied in model plants and crop species [25], the effect of GABA on alkali tolerance in alfalfa is not clear. The current study aimed to provide new insights into what is the role of GABA in alkaline stress and to explore the regulatory aspects of the GABA shunt pathway for ROS production as well as the mechanism of alkali tolerance in alfalfa. Hence, in this study, we first determined the concentration of exogenous GABA that best improves the alkali tolerance of alfalfa seedlings, then devised a study to investigate the effects of GABA on seedling growth, osmotic regulation, and antioxidant metabolism in alfalfa plants subjected to alkali stress. The findings will help improve alfalfa management in alkali-affected areas.

2. Materials and Methods

2.1. Seed Germination and Treatments

Alfalfa seeds (*Medicago sativa* Longmu 806) were purchased from the Institute of Animal Husbandry of Heilongjiang. GABA was purchased from the Coolaber Technology Limited Liability Company of Beijing (Beijing, China).

In this experiment, full alfalfa seeds were selected and evenly seeded in seedling trays; 500 seeds were planted in each seedling tray. Two weeks after germination, each tray was irrigated with 1 L 100 mmol/L pH 8.5 $NaHCO_3$ solution to create an alkali stress environment. The experiment included 4 GABA treatment groups and a non-GABA treatment control group. The four experimental groups were sprayed with 25, 50, 75, and 100 mmol/L GABA solution, respectively, and the control group was sprayed with distilled water. The plants were cultivated in a greenhouse at 25 ± 2 °C under 16 h of light and 8 h

of darkness. After 7 days, spraying was stopped, and the phenotypes of the seedlings were observed and photographed.

2.2. Alkali Stress Treatments

After selecting the optimal spraying concentration of GABA, four groups [control (sprayed with distilled water + Hoagland nutrient solution), GABA (sprayed with 75 mmol/L GABA solution + Hoagland nutrient solution), stress (sprayed with distilled water + 100 mmol/L $NaHCO_3$ (pH 8.5)), and GABA + stress (sprayed with GABA solution + 100 mmol/L $NaHCO_3$ (pH 8.5))] were set up; each treatment group consisted of 3 biological replicates. Samples were taken at 0 d, 1 d, 3 d, 5 d and 7 d after spraying and immediately frozen at −80 °C for determination of physiological indicators. The seedlings were cultured for one month, and the growth index was measured.

2.3. Physio-Biochemical Analysis of Plant Materials

The survival rate of the plants was determined by direct counting. The fresh weight and dry weight of the plants were measured by the weighing method, and the dry weight was weighed when dried in an oven at 80 °C to constant weight for approximately 30 min. Root length and plant height were measured using a ruler. The chlorophyll (Chl) content of the leaves was determined using a chlorophyll analyzer (Tys-B type). Each recorded value represented 3 biological replicates and 3 technical replicates (the average of these replicates was used to calculate the values for each individual group). The relative conductivity of the leaves was measured using a DDS-307 electrical conductivity meter with the vacuum method.

The superoxide dismutase (SOD), catalase (CAT), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), superoxide anion (OFR), and peroxidase (POD) activities were measured with Comin kits (Suzhou Comin Biotechnology Co., LTD,Suzhou,China). Finally, the contents of proline and soluble sugars were measured with Comin kits (Suzhou Comin Biotechnology Co., LTD,Suzhou,China), and the optical density was read at 520 and 620 nm.

2.4. Determination of GAD Enzyme Activity by a Colorimetric Method

Leaf samples were taken after 3 and 7 days of treatment, and the GAD enzyme activity in the leaves was determined by the colorimetry method. We took 50 mg of the material and added 2 volumes of PBS solution, then stirred and ground the tissue evenly. After standing for 7 h, the supernatant solution was centrifuged at 4000 r/min for 5 min, which was the enzyme solution to be tested. We took 0.2 mL 50 mmol/L phosphate buffer (pH 5.7), which contained 1% sodium glutamate and 0.2 mmol/L PLP(Pyridoxal-5-phosphatemonohydrate), added 0.1 mL enzyme solution to be tested and 0.1 mL distilled water to a 37 °C water bath for 30 min, then quickly put it in an ice bath, added 0.2 mL 0.2 mmol/L boric acid buffer (pH 9.0), 1 mL of 6% phenol solution and 0.4 mL of 9% effective chlorine sodium hypochlorite solution were fully mixed. After 10 min of a boiling water bath, they were immediately iced for 20 min and continuously mixed until blue-green compounds appeared. Then, 2 mL of 60% ethanol was added and 10 mmol/L L-Glu was used as a blank control to measure the absorbance value at 645 nm. With A645 as the ordinate and GABA concentration as abscissa, the linear regression equation and correlation coefficients were obtained by Excel.

2.5. Determination of Organic Acid Content by High-Performance Liquid Chromatography

Extraction of organic acids from the samples was performed according to Shen Hong et al. [26], and improvements were made under these basic conditions. The preserved samples were removed from storage at −80 °C and ground into powder in liquid nitrogen. Samples (100 mg) were accurately weighed and placed in 2 mL EP tubes; 2 mL ultrapure water was then added, and the samples were extracted in a 75 °C water bath for 15 min. After being allowed to stand at room temperature, the supernatant was centrifuged at 10,000 r/min for 30 min. The filtrate was passed through a 0.22 μm filter and placed in a

2 mL sample bottle for chromatographic analysis. Five concentrations of standard product formulations of oxaloacetic acid, malic acid, citric acid, succinic acid and ketoglutaric acid were used to construct standard curves for chromatographic identification, with the *Y*-axis as the area and the *X*-axis as the mass concentration. Linear regression equations and correlation coefficients were obtained using an Excel table.

2.6. Determination of GABA-T Activity, Glu and GABA Content by the Microplate Method

The frozen samples were ground into a fine powder using liquid nitrogen, and the endogenous GABA-T activity, glutamate (Glu) and GABA contents of the plants were determined by the microplate method according to the instruction manual provided with the kit manufactured by Suzhou Comin Biotechnology Co., LTD (Suzhou,China).

2.7. RNA Extraction and qRT–PCR Analysis

Total RNA was extracted using an UItrapure RNA kit (CoWin Biotech, Beijing, China). A PCR template was prepared using reverse-transcribed RNA. A quantitative real-time PCR experiment was performed in 96-well plates (20 µL reactions) using Trans Start Top Green qPCR SuperMix (Vazyme Biotech, Nanjing, China); standardization was performed using GAPDH. RNA was extracted from three different plant materials and used for all reactions in biological triplicates. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers are listed in Supplementary Table S1.

2.8. Statistical Analysis

Three biological replicates were given to each group, and Microsoft Excel 2010 was used to analyze the test results. With GraphPad Prism 9.0, significant differences were analyzed using one-way ANOVA or Student's *t*-test. $p < 0.01$ indicates that the differences are extremely significant, while $0.01 < p < 0.05$ denotes that the difference is significant.

3. Results

3.1. Determination of the Optimal Concentration of GABA for Exogenous Application

To determine which concentration of GABA is the most suitable for improving the alkali tolerance of alfalfa through exogenous spraying, we set up four experimental groups, each of which was sprayed with GABA at a different concentration. Exogenous GABA was applied for 7 days, and 100 mmol/L $NaHCO_3$ (pH 8.5) treatment was performed at the same time. On the 10th day, the alfalfa seedlings in the different groups began to show slight differences. By the 21st day, the phenotypic differences were significant. Although all of the treatment groups had withered and yellowed, the 75 mmol/L GABA group displayed a certain level of alkali resistance and had the highest survival rate (Supplementary Figure S1). Based on this, the exogenous application optimal concentration of GABA was identified as 75 mmol/L.

3.2. Effect of Exogenous GABA on Alkali Tolerance in Alfalfa

Based on the changes in survival rate, chlorophyll content and relative conductivity, exogenous GABA enhanced the alkali tolerance of the alfalfa seedlings (Figure 1). After exposure to 100 mmol/L $NaHCO_3$ (pH 8.5) alkali stress for 7 days, most of the alfalfa seedlings wilted and died; however, compared to the stress group, the survival rate of the stress + GABA group was significantly higher (Figure 1A). Alkali stress also decreased the chlorophyll content of the alfalfa seedling leaves, but in the stress + GABA group, the Chl content was more than that in the stress group. It is worth mentioning that compared to the control group, the Chl content in the GABA group was significantly higher ($p < 0.05$) (Figure 1B). Similarly, the relative conductivity of the plants in the GABA group was also significantly lower than that of the control group ($p < 0.05$). After alkali stress, the relative conductivity of the leaves increased in the stress + GABA group, and the trend of this change was consistent with that of the control group (Figure 1C). Exogenous GABA increased the chlorophyll content of the alfalfa seedling leaves and reduced their relative conductivity.

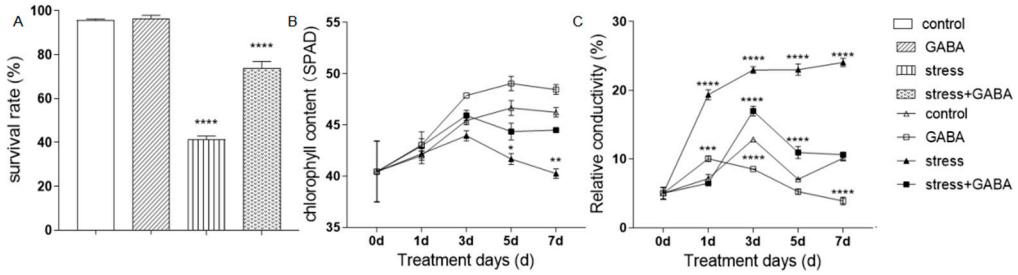


Figure 1. Survival rate (**A**), chlorophyll content (**B**), and relative conductivity (**C**) in the leaves of alfalfa seedlings in the control and alkali treatment with or without GABA for 7 days. Note: A group-group significance analysis was performed, and each treatment day's GABA (Hoagland nutrient solution+exogenous GABA), stress (alkali), and stress + GABA (alkali + exogenous GABA) were compared to the control group(Hoagland nutrient solution + distilled water). Each value is the mean ± SD of three independent experiments. One star indicate difference at $p < 0.05$ by Duncan's test, more stars indicate significant difference at $p < 0.01$ or $p < 0.001$.

3.3. Effect of Exogenous GABA on the Levels of Osmotically Active Substances

The proline and soluble sugar contents of the alfalfa seedlings at 7 days after GABA treatment are shown in Figure 2. During the growth of the alfalfa seedlings, their proline content increased. After 7 days of alkali stress, the proline content decreased compared with normal conditions. Whether or not GABA was sprayed had no significant effect on the proline content (Figure 2A). However, the change in the number of soluble sugars was different (Figure 2B). There was a certain difference in soluble sugar content on the first day of alkali stress. In the stress and stress + GABA groups, the soluble sugar content was higher than in the control ($p < 0.001$) and GABA groups, and the soluble sugar content of the plants in the alkali stress group decreased slowly. After 7 days of GABA treatment, the soluble sugar content of the plants in the control and GABA groups was significantly higher than that in the stress group ($p < 0.001$), and the soluble sugar content of the alfalfa leaves supplied with exogenous GABA was significantly higher than those without GABA treatment ($p < 0.001$).

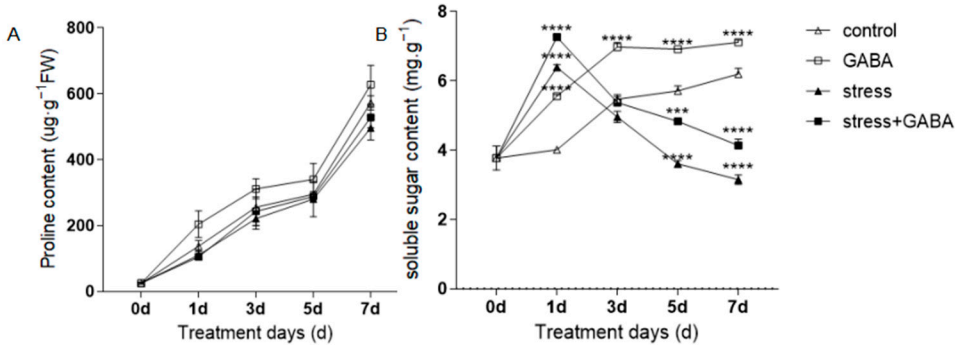


Figure 2. Proline (**A**) and soluble sugar content (**B**) in the leaves of alfalfa seedlings subjected to normal and alkali treatment with or without GABA. Note: A group-group significance analysis was performed, and each treatment day's GABA, stress , and stress + GABA were compared to the control group. Each value is the mean ± SD of three independent experiments. One star indicate difference at $p < 0.05$ by Duncan's test, more stars indicate significant difference at $p < 0.01$ or $p < 0.001$.

3.4. Effects of Exogenous GABA on the Activity of Antioxidant Enzymes and the Production of Reactive Oxygen Species under Alkali Stress Conditions

Exogenous GABA has a complex effect on the antioxidant enzyme system of alfalfa seedlings, and a variety of changes in superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities were observed (Figure 3) in the alfalfa leaves after alkali treatment. The SOD activity in the leaves of the stress group was significantly higher than that of the control group. the stress + GABA group showed the highest SOD activity; it was higher

than the stress group after 5 days of alkali treatment, with increases of 42% and 27% at 5 and 7 d, respectively (Figure 3A).

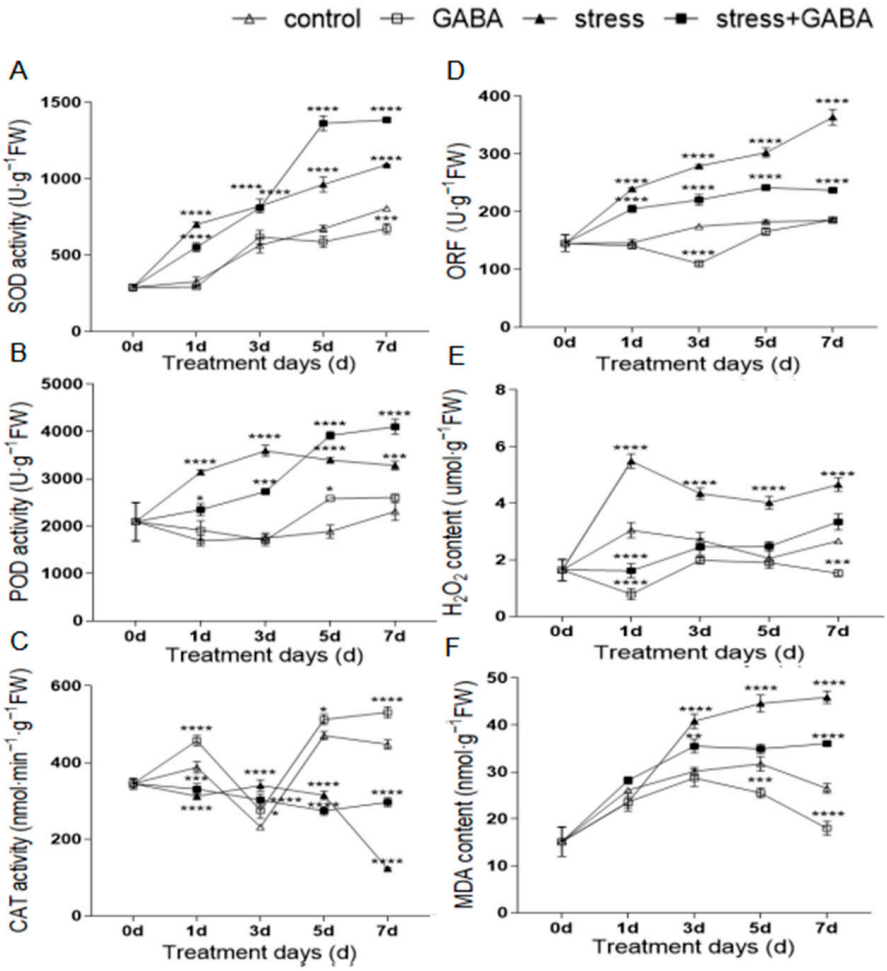


Figure 3. SOD activity (**A**), POD activity (**B**), and CAT activity (**C**), ORF (**D**), H_2O_2 content (**E**), MDA content (**F**) in the leaves of alfalfa seedlings subjected to normal condition and alkali treatment with or without GABA. Note: A group-group significance analysis was performed, and each treatment day's GABA, stress, and stress + GABA were compared to the control group. Each value is the mean ± SD of three independent experiments. One star indicate difference at $p < 0.05$ by Duncan's test, more stars indicate significant difference at $p < 0.01$ or $p < 0.001$.

The POD activity in the alfalfa leaves followed a pattern similar to that of the SOD activity. After 7 d of alkali treatment, the POD activity was highest in the stress + GABA group, with increases of 25% and 77% compared with the stress group and the control group, respectively. It is worth mentioning that at the early stage of alkaline stress (1 and 3 d), the POD activity in the stress group was significantly higher than that in the stress + GABA group. After 5 days of alkaline stress treatment, the POD activity in the stress + GABA group increased significantly, while in the stress group, the POD activity was stable and did not show a significant increase but was also higher than that in the control group (Figure 3B).

After 7 days of alkali stress and exogenous GABA, CAT enzyme activity in the leaves of the four groups differed significantly ($p < 0.05$), and CAT enzyme activity decreased after alkali stress treatment. However, CAT enzyme activity was significantly higher in the GABA and stress + GABA groups than in the control and stress groups, respectively (Figure 3C).

In alfalfa plants exposed to alkali stress, the content of superoxide anion (O_2^-) in the leaves was markedly higher than that in the control group (Figure 3D). When GABA was

sprayed on plants exposed to alkali stress conditions, the content of O_2^- was significantly lower than that in the stress group, showing a reduction of 35%. After 7 days of GABA treatment, there was no difference in the O_2^- content of the control group and those in the GABA group, but the O_2^- content of the leaves of the plants in the GABA group decreased significantly when GABA was sprayed for 3 days, and it then increased on the 5th day.

The H_2O_2 content of the leaves of the plants in the stress group increased significantly after 1 d of alkali stress treatment and then slowly decreased (Figure 3E). After 7 d of alkaline stress, the H_2O_2 content of the leaves was 74% higher than that of the leaves of the plants in the control group. Because of the application of GABA, the H_2O_2 content of the leaves of the plants in the stress + GABA group did not change significantly after alkali treatment. On the 7th day of alkali stress, it was significantly lower than that of the stress group and higher than that of the control group. Moreover, without $NaHCO_3$ treatment, the H_2O_2 content of the leaves of the plants in the GABA group was lower than that of the leaves of the plants in the control group within 7 days of the application of exogenous GABA. GABA application significantly inhibited H_2O_2 accumulation, resulting in reductions of 8–74% compared to the control group and reductions of 52–85% compared to the stress group.

As shown in Figure 3F, the malondialdehyde (MDA) content of the leaves of plants treated with $NaHCO_3$ was significantly higher than that of the leaves of the control plants. After 7 days of alkali treatment, the MDA content of the leaves of the plants in the stress + GABA and GABA groups was significantly lower than that of the stress group ($p < 0.001$) and the control group ($p < 0.001$), with reductions of 21% and 32%, respectively.

3.5. Effects of Exogenous GABA on the Content of Metabolites and the Activity of Enzymes in the GABA Pathway under Alkali Stress

Exogenous application of GABA would be expected to result in changes in endogenous GABA levels as well as in the content of enzymes and metabolites in the GABA pathway. The levels of endogenous GABA and glutamate (Glu) and the activities of the enzymes GABA-aminotransferase (GABA-T) and glutamate decarboxylase (GAD) after exogenous spraying of GABA were measured (Figure 4). The levels of endogenous GABA in the leaves of the plants in the GABA and stress + GABA groups increased as the spraying time increased. After exposure to alkali stress for 7 days, endogenous GABA content, especially in the stress + GABA group, was slightly higher than that found in the controls, indicating that alkali stress induced GABA accumulation (Figure 4A).

The change in Glu content was similar to the change in GABA content but slightly different. There was no significant difference between the control group and the stress group in Glu content, and exposure of the plants to alkali stress did not change the Glu content. The Glu content in the GABA and stress + GABA groups was significantly increased after the plants were sprayed with GABA for 7 d; at that time, the Glu content in the stress + GABA group was significantly lower than that in the GABA group (Figure 4B). It meant that the spraying of GABA increased the content of glutamate.

It was found that the activity of GABA-T was inhibited by alkali stress with reductions of 40% and 64% compared with the control after $NaHCO_3$ treatment for 3 d and 7 d, respectively, while GABA application significantly enhanced GABA-T activity, especially in plants that had been exposed to alkali stress for 7 d; an increase of 314% in GABA-T activity compared with the stress group was observed (Figure 4C). Increased GABA-T enzymatic activity also results in the entry of increased amounts of succinate into the Calvin cycle and enhanced carbon metabolism. The contents of succinic acid, citric acid and α-ketoglutaric acid were measured (Supplementary Figure S2). Although the contents of these three organic acids were very low, exogenous GABA indeed changed the organic acid content of the leaves and enhanced carbon metabolism.

There was no significant difference in GAD enzyme activity between the exogenous GABA and stress + GABA groups, but there was a significant difference between with and without GABA groups. Alkali stress did not significantly affect GAD enzyme activity,

and the GAD enzyme activity in all groups was consistent with their endogenous GABA content (Figure 4D).

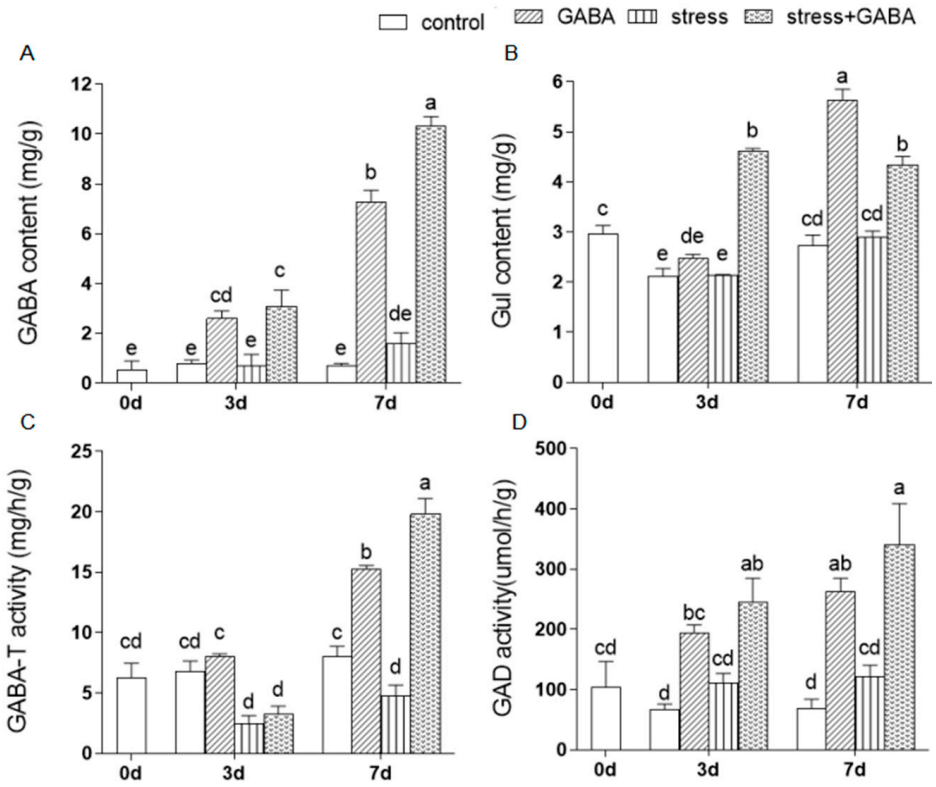


Figure 4. GABA content (**A**), Glu content (**B**) and GABA-T activity (**C**), GAD activity (**D**) in the leaves of alfalfa seedlings subjected to normal condition and alkali treatment with or without GABA. Note: There were four treatment groups: control, GABA, stress, and stress + GABA. Each value is the mean ± SD of three independent experiments. Different lowercase letters indicate significant differences $p < 0.05$.

3.6. Effects of Exogenous GABA on the Levels of Expression of Four Genes Related to GAD and Those of Other Related Genes in Leaves under Alkali Stress

We screened four highly expressed *GAD* genes in the *Medicago sativa* genome; the conserved regions of these genes showed high homology. The sequence alignments and renaming are shown in Supplementary Table S2. The patterns of expression of four GAD paralogs in the leaves were analyzed based on the RNA-seq data of the *Medicago sativa* Xinjiang Daye cultivar (Supplementary Figure S3). After alkali treatment, *MsGAD4* expression increased slightly, while that of *MsGAD3* decreased significantly. It can be seen that there were differences in the expression of the four *MsGAD* genes under alkaline stress.

The expression levels of the four *MsGAD* genes after alkali treatment and exogenous application of GABA were determined by qPCR (Figure 5). Exogenous GABA significantly induced the expression of *MsGAD1* and *MsGAD2* and reduced the expression of *MsGAD3* compared with the control. Expression of *MsGAD4* was slightly increased after 1 d and significantly decreased after 3 d of treatment with exogenous GABA. Alkali stress treatment also has different effects on the expression of the *MsGAD* genes. *MsGAD4* expression increased significantly after 3 d of alkali treatment, but *MsGAD3* showed the opposite trend, and *MsGAD1* and *MsGAD2* expression showed no significant change. After exogenous GABA and alkali treatment, the trend in the expression of these genes was consistent with that observed in plants under alkali stress and in plants that received exogenous GABA alone; *MsGAD1* and *MsGAD4* expression was significantly increased.

The differential expression of the *MsGABA-T* and *MsGDH* genes in the leaves of plants that received alkali treatment was examined (Figure 5). Both exogenous applications of GABA and alkali treatment decreased the expression of *MsGABA-T*. *MsGABA-T* had a lower

expression level after stress + GABA treatment than that in the control condition, but it also showed a trend of first decreasing and then increasing. After 3 days of GABA application, the expression of *MsGDH* decreased slightly. However, its expression was significantly upregulated after alkali treatment for 3 days, at which time it was approximately 2.2-fold the level observed in the control group. The expression levels of *MsP5CS* and *MsPDH* were significantly downregulated after GABA application. In the stress + GABA group, *MsP5CS* expression increased significantly after 3 days of treatment, while *MsPDH* expression continuously decreased.

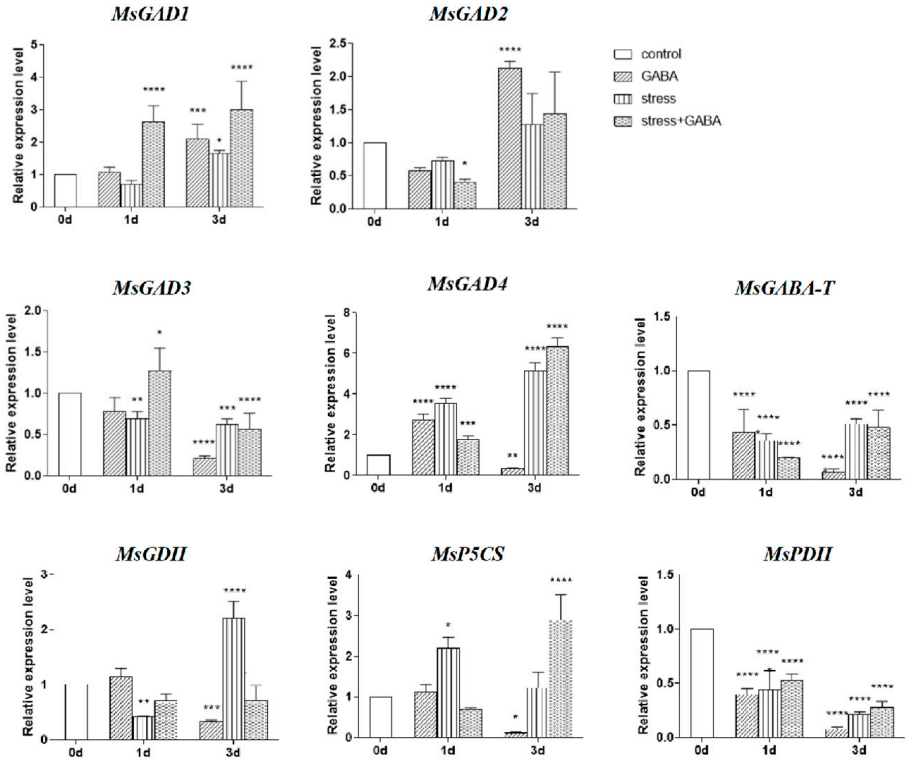


Figure 5. Dynamic change in the relative expression of four alfalfa GAD genes and other relative genes in the leaves in the normal condition and alkali treatment with or without GABA. Note: Using the gene expression level of 0 d as 1, and the ratio of the gene expression level of the other time point to 0 d as the ordinate. There were four treatment groups: control, GABA, stress, and stress + GABA. The mean ± SD of three independent experiments was used. One star indicates a significant difference by Duncan's test at $p < 0.05$, and more stars indicate significant differences by $p < 0.01$ or 0.001.

3.7. Effect of GABA Application on the Growth of Alfalfa under Alkali Stress Conditions

To study the effects of exogenous GABA on plant growth and alkali tolerance, the survival rate, chlorophyll content, plant height, root length, fresh weight and dry weight of the alfalfa seedlings were investigated (Figure 6). Under normal conditions, exogenous GABA did not affect the survival rate of the seedlings while alkali stress reduced their survival rate; however, in the stress + GABA group, there was a higher survival rate than that in the stress group ($p < 0.05$). After one month of growth of the seedlings, there were no significant differences between the GABA group and the unsprayed group in terms of chlorophyll content, plant height or dry weight, but the plants in these two groups displayed significant differences in root length and fresh weight.

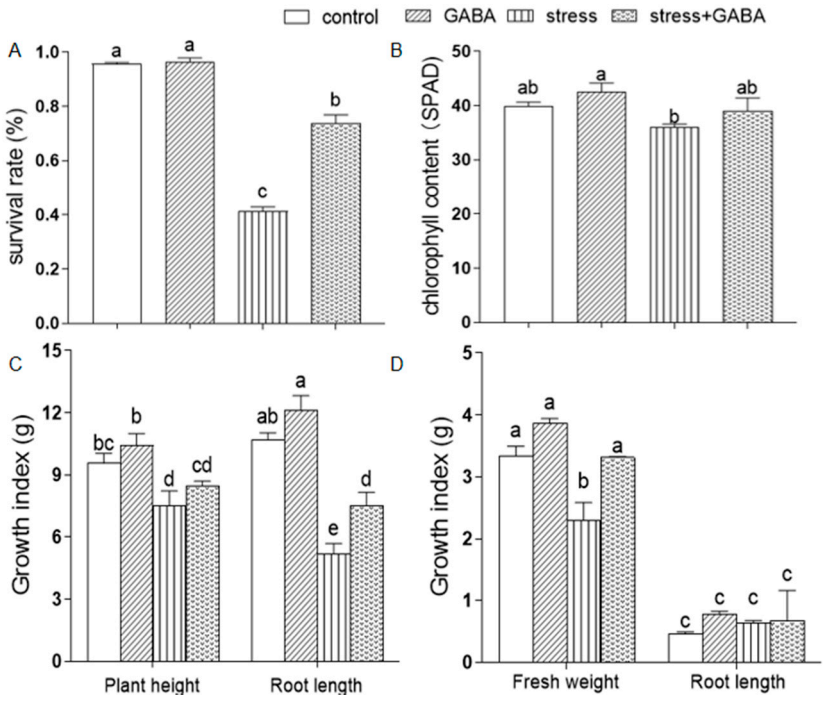


Figure 6. Survival rate (**A**), chlorophyll content (**B**), plant height and root length (**C**) and fresh weight and dry weight (**D**) in the leaves of alfalfa seedlings subjected to normal culture and alkali treatment with or without GABA, Note: There were four treatment groups: control, GABA, stress, and stress + GABA. Each value is the mean ± SD of three independent experiments. Different lowercase letters indicate significant differences $p < 0.05$.

4. Discussion

Alkali stress is a relatively complex type of abiotic stress that involves ionic stress and the subsequent induction of water deficiency, damage to the plasma membrane and oxidative stress; thus, it affects the normal physiological activities of plants [22]. In this study, it was found that the application of exogenous GABA improves the alkaline resistance of alfalfa seedlings, mainly by increasing the survival rate of the seedlings. In seedlings exposed to alkali stress conditions, chlorophyll content and soluble sugar content were significantly higher in the group that was sprayed with GABA than in the non-sprayed group. MDA, H_2O_2 and O_2^- levels were significantly decreased, and antioxidant enzyme activity was significantly increased. Thus, exogenous GABA can be applied to improve the alkaline tolerance of alfalfa seedlings.

4.1. Exogenous GABA Improved Alkali Stress Tolerance by Alleviating the Phenotypic Symptoms of Alkali Damage

Compared to plants not treated with GABA, GABA-treated plants showed better growth, fresh and dry weights, and chlorophyll levels after exposure to saline water [9,27]. It has not been reported that GABA application can alleviate the effects of alkali stress, however. In this study, after $NaHCO_3$ treatment for 7 d, alfalfa seedlings slowly began to display the classic signs of alkali damage, such as a decreased survival rate and chlorophyll content, and an increase in relative conductivity. GABA applicated plants also showed an increase in survival rate and chlorophyll content and a decrease in relative conductivity in response to alkali stress (Figure 1). Based on these results, growth reductions of alfalfa plants could be alleviated by adding exogenous GABA, which is consistent with the results of other studies on salt and drought stress [28,29].

Spraying with GABA for 7 days significantly increased the survival rate of alfalfa seedlings after alkali stress treatment for a period of up to one month after treatment. In the stress + GABA group, there was no significant difference in survival between plants that received 7 days of alkali treatment and those that received 1 month of alkali treatment;

their survival rates were 73% and 79%, respectively (Figures 1 and 6), indicating that the death of alfalfa seedlings due to alkali stress occurred mainly at the early stage of alkali stress. The first week of alkali stress can be called the "critical period" for plant growth. Application of exogenous GABA during this critical period effectively improved the survival rate of alfalfa seedlings exposed to alkali stress, and sustained alkali tolerance was achieved by spraying the plants for only 7 days. Therefore, exogenous GABA is beneficial for improving the survival rate and yield of alfalfa in saline-alkali grassland during sowing and seedling growth.

4.2. Exogenous GABA Regulated the Content of Osmotically Active Substances in the Leaves of Plants Exposed to Alkali Stress

Salt or alkali accumulation around the root areas of plants leads to osmotic stress, and this increases the risk of injury to the plants [30]. Osmotic regulation is one of the most basic characteristics of plant salt and alkali tolerance, and the improvement of osmotic regulation ability is an important mechanism through which plants enhance their salt and alkali tolerance [26,31]. In this study, it was observed that 1 day after alkali treatment, the soluble sugar content of the plants increased significantly, and the content of proline was not significantly different from that of the control group (Figure 2). While GABA application had little effect on the proline content, it significantly increased the soluble sugar content (Figure 2). However, in studies of salt and drought, it was found that the application of exogenous GABA can significantly increase the proline content of plants and improve their tolerance to salt and drought [6,32], a finding that is not completely consistent with our result. The observed changes in proline content are not completely consistent in different plants under alkali stress conditions [33,34]. It is still uncertain whether there is a correlation between alkali stress and proline accumulation.

Proline is also involved in GABA metabolism: glutamate is the substrate for the synthesis of GABA catalyzed by GAD, and it is also the substrate for the synthesis of proline. Plants can also synthesize GABA from polyamines (PAs), such as spermidine and putrescine, as well as from pyrroline. Proline can also be converted to GABA by pyrroline. This study investigated the expression of the *P5CS* and *PDH* genes, which encode key enzymes involved in the synthesis and degradation of proline (Figure 5). The results showed that the expression of *MsP5CS* and *MsPDH* decreased significantly after GABA application. In the stress + GABA group, *MsP5CS* expression increased significantly after 3 days of treatment, while *MsPDH* expression continuously decreased. Therefore, the application of exogenous GABA might alter proline synthesis and metabolism. It is uncertain whether the proline content of the plants was related to the exogenous application of GABA.

4.3. Exogenous GABA Improved Alkali Stress Tolerance by Regulating the Antioxidant System

Stress can induce the production of reactive oxygen species (ROS) and lead to membrane damage and the accumulation of lipid peroxidation [35]. Li et al. found that exogenous application of GABA increased the photosynthetic and antioxidant enzyme activities of wheat seedlings and that it improved their growth and decreased their MDA content and electrolyte conductivity under salt stress [29]. In this study, the content of H_2O_2 and O_2^-, molecules that lead to membrane lipid peroxidation, was significantly lower in the group that received GABA treatment than in the non-sprayed group (Figure 3D,E). This is related to the enhancement of SOD, POD and CAT activities (Figure 3A–C), and the results are consistent with those reported in other studies [6,15,18]. Our results clearly indicate that exogenous GABA improves the activity of antioxidant enzymes and increases the plants' ability to scavenge ROS, thus reducing membrane lipid peroxide levels, alleviating the oxidative stress caused by alkali stress and thereby strongly protecting alfalfa seedlings from oxidate damage and thus enhancing alkaline tolerance.

4.4. Regulation of Exogenous GABA via the GABA Metabolic Pathway in Alfalfa Seedlings under Alkali Stress

The decomposed products of GABA that accumulate in the cytoplasm are transported to mitochondria, where they enter the TCA cycle and form other metabolic intermediates [15]. Carbon metabolism and nitrogen metabolism are linked by GABA, which plays a key role in plant resistance. Studies have shown that in wheat plants exposed to salt stress, key metabolic enzymes in the TCA cycle are inhibited by salt, and GABA branches are activated to supplement metabolism [36]. GDH (glutamate dehydrogenase) is a key enzyme in the synthesis of GABA and catalyzes the conversion of α-ketoglutarate to glutamate. Glutamate is decarboxylated by GAD to GABA, which is then transformed by GABA-T to ammonia, generating succinic acid hemaldehydes; succinic acid then enters the TCA cycle. This pathway, which is known as the GABA branch pathway, is thought to be the main pathway for the synthesis of GABA and can also be used as a compensatory pathway for the TCA cycle in plants under stress. The Glu content of alfalfa leaves increased rapidly after exogenous GABA was sprayed on plants exposed to alkali stress conditions, and the change in Glu content was related to the content of endogenous GABA (Figure 4). Under stress conditions, exogenous GABA acts as a signaling molecule. GAD is activated by Ca^{2+}/CAM and increases the endogenous GABA content in the cytosol [37]. In this study, in addition to the significant increase in endogenous GABA, the activities of GAD and GABA-T were also significantly increased (Figure 4), and this was accompanied by changes in metabolite content in the TCA cycle (Supplementary Figure S2). This means that the GABA metabolic pathway was activated. Exogenous GABA can accelerate the metabolic cycle of the GABA branch and provide a carbon source for the TCA cycle in plants exposed to stressful environments, thereby helping maintain a sufficient energy supply for plants under stress.

Although the enzymatic activities of GAD, GABA-T and GDH showed a uniform increasing trend in plants that were exposed to alkali stress and sprayed with exogenous GABA, the expression of the genes that encode these enzymes (*MsGAD1/2/3/4*, *MsGABA-T* and *MsGDH*) was not completely consistent with the observed changes in enzymatic activities. For the *MsGAD* genes, each of which encodes a specific isoenzyme, the changes in expression of the corresponding four genes were different. Among them, the expression of *MsGAD1* and *MsGAD4* was more significantly regulated by alkali stress or GABA spraying, while the changing trend of gene expression was not consistent with that of GAD enzyme activity. However, despite the fact that there is a gap between the observed transcription levels of specific genes and the metabolic pathways involved, our results confirm that the exogenous application of GABA improves the alkali tolerance of alfalfa by regulating gene expression, enzyme activity and metabolite content in the GABA metabolic pathway.

5. Conclusions

Spraying alfalfa plants with 75 mmol/L GABA for 7 d significantly increased the soluble sugar content and the SOD, CAT and POD activities of the plants; it also significantly decreased their relative conductivity and their MDA, superoxide anion free radical and hydrogen peroxide content, enhanced the osmotic regulation and antioxidant capacity of alfalfa seedlings, decreased the membrane lipid oxidation level, and increased the activities of GAD and GABA-T and the content of endogenous GABA and Glu in the GABA pathway. The survival rate and fresh weight of plants grown under alkali stress conditions were significantly increased, and long-term survival was possible.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12071577/s1, Figure S1: Phenotypic chart of the effect of exogenous GABA on the alkali tolerance of Alfalfa Seedlings. The spraying concentrations of GABA were 0 mmol/L, 25 mmol/L, 50 mmol/L, 75 mmol/L and 100 mmol/L observations on 10 d and 21 d; Figure S2: Succinic acid,citric acid and ketoglutaric acid in the leaves of alfalfa seedings subjected to normal condition and alkali treatment with or without GABA, The seedings were shown to one of the following four teatments: control, GABA (control + exogenous GABA), stress (alkali),

and stress + GABA (alkali + exogenous GABA). Each value is the mean ± SD of three independent experiments.(Duncan test: $p < 0.05$); Figure S3: Expression pattern analysis of predicted GAD genes in *M. sativa* leaves under different alkaline stress stages. Two-month-old *M. sativa* seedlings were precultured in a 1/2 Hoagland nutrient solution,then treated with 100 mmol/L $NaHCO_3$ (pH = 8.5) 0 h, 3 h, 6 h, 12 h and 48 h.Immediately, the collected samples were frozen and stored at −80 °C for RNA-seq. For all the above samples, three biological replicates were employed for each sample. Table S1: Gene-specific primers designed for qRT-PCR; Table S2: Naming of predicted GAD genes in *M. sativa* and their orthologs in *M. truncatula*. The characterized proteins having highest homology to the *M. sativa* proteins in alignment analyses are included for reference.

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