

BRASSINOLIDE ALLEVIATES GLUFOSINATE-AMMONIUM TOXICITY BY ENHANCING PHOTOSYNTHESIS AND NITROGEN METABOLISM IN *TRICHOSANTHES KIRILOWII*

YAN WU¹, XIANGXIANG WANG², XIAOYAN QIAO² AND QINGHAI GAO^{2*}

¹Anhui Science and Technology University, College of Architecture, 233000, Bengbu, Anhui, China

²Anhui Science and Technology University, College of Agriculture, 233000, Bengbu, Anhui, China

*Corresponding author's email: Gaoqh1977@163.com; Tel: +86-550-6732806

Abstract

This paper explored the alleviation of brassinolide (BR) to *Trichosanthes kirilowii* Maxim cv. WanLou No.9 (WL9) seedlings under the stress of herbicide glufosinate-ammonium (GA). The results showed that under GA stress, a reduction in chlorophyll content, photosynthetic rate, and light energy utilization rate of WL9 seedlings has occurred. In addition, a significant decline in the activities of nitrogen metabolism-related enzymes, such as nitrate reductase (NR), glutamine synthetase (GS) and glutamate synthase (GOGAT), were also observed. Furthermore, amino acid synthesis in the leaves was inhibited, resulting in a large accumulation of ammonium nitrogen. Application of BR, however, could significantly enhance chlorophyll content, photosynthetic rate, and light energy utilization efficiency of WL9 seedlings under GA stress, besides promoting the key enzymatic activities of nitrogen metabolism. Among them, leaf NR and GS activities were 27.5 $\mu\text{gNaNO}_2 \cdot \text{g}^{-1}\text{FW} \cdot \text{h}^{-1}$ and 5.6 $\mu\text{molGHA} \cdot \text{g}^{-1}\text{FW} \cdot \text{min}^{-1}$ respectively, which were 74.8% and 60.0% higher than that of GA treatment. In addition, BA promoted the synthesis of amino acids, and the ammonium nitrogen content in leaves was remarkably reduced. Application of BA alleviated the inhibition of GA on the growth of WL9 seedlings, which was 1.34 and 1.21 times higher than that of GA treatment in shoot and root respectively. In conclusion, GA reduced the light energy utilization efficiency and nitrogen metabolic capacity of WL9 seedlings, while application of BA could alleviate the toxicity of GA on light energy utilization and nitrogen metabolism of WL9 seedlings.

Key words: Brassinolide; Glufosinate-ammonium; *Trichosanthes kirilowii*; Nitrogen metabolism; Photosynthesis.

Introduction

As an imperative traditional Chinese medicine, *Trichosanthes kirilowii* Maxim has been extensively cultivated in Anhui, Jiangsu, Shandong, and other provinces due to its good economic sense. Glufosinate-ammonium (GA), a widely used herbicide all over the world, is a highly active, fast-acting, and broad-spectrum herbicide, which is often used for weed control due to its killing effect on both shoot and root systems of weeds (Geng *et al.*, 2017; Hack *et al.*, 1994). The use of GA, however, has adverse effects on crop production due to improper use or other external factors like wind. Studies have revealed that glutamine synthase (GS) activity in plant leaves is significantly reduced by GA toxicity, which affects ammonia metabolism in plants, resulting in the accumulation of a large amount of ammonia in cells (Manderscheid *et al.*, 2005; Merkel *et al.*, 2004). It's reported that GA treatment could reduce transmembrane H^+ , disrupt cell membrane structure and chlorophyll, decrease photosynthetic rate of plants, and finally lead to death (Wendler *et al.*, 1990; Kapoor *et al.*, 2014).

Brassinolide (BR) is a new plant growth regulator, which can regulate plant metabolism, such as stomatal movement and photosynthesis, consequently promoting plant growth and development (Wendler *et al.*, 1990). It was reported that BR could effectively alleviate the toxicity of heavy metals to radish and maize seedling (Kapoor *et al.*, 2014; Soares *et al.*, 2016). Interestingly, studies have shown that BR could alleviate the stress of herbicides on plants. It was found that BR could not only improve the activity of PS II by increasing stomatal conductance and content of photosynthetic pigments in

millet leaves, but also maintain the balance of sucrose metabolism as well as promote the release of carbohydrates from the "source", consequently alleviating herbicide toxicity to the grain (Yang *et al.*, 2017). BR could alleviate the harm of herbicide (metsulfuron) to rice, and the alleviation is closely associated to its concentration and the degree of harm (Mu *et al.*, 2002). Seed soaking with BR could also improve the chlorophyll content and root vigor of rice seedlings, promote the metabolism of herbicide (ethametsulfuron) in crops, and alleviate its inhibition on rice root length (Zhou *et al.*, 2003). As a commonly used broad-spectrum and effective herbicide, improper use of GA would cause damage to *Trichosanthes kirilowii*, whereas application of BA could alleviate the damage. Currently, there have been no reports about the alleviation of herbicide damage by BA. The effects of exogenous BA on the growth, photosynthesis, and nitrogen metabolism of WL9 were studied under GA treatment. The aim of this paper was to elucidate the physiological mechanism of BA in alleviating the GA damage to *Trichosanthes kirilowii* from the perspectives of photosynthetic fluorescence and nitrogen metabolism.

Materials and Methods

Materials: The variety of Wanlou 9 (WL9) was provided by the Horticultural Laboratory of Anhui Science and Technology University. The herbicide is a 20% Glufosinate-ammonium (GA) solution, which is produced by Zhejiang Yongnong Biotechnology Co., Ltd. Brassinolide (BA, 0.003%) is produced by Zhongnong Agricultural Chemicals Co., Ltd.

Treatments: The experiment was conducted in a greenhouse from February to August 2019. The experimental field is located in Fengyang, Anhui Province, with 117°56' east longitude and 32°86' north latitude, belonging to the warm temperate semi-humid monsoon climate area. The seeds were immersed in warm water at 28°C for 48 h. The water was changed every 4 h. The seeds were then put in 28-30°C for 5 d for germination. Then the seeds were sown into a 30-hole tray. After germination, conventional temperature and water management was carried out. When the seedlings grew to 4-5 leaves, the seedlings with uniform growth were picked out and transplanted into the plastic pots of 15 cm × 15 cm. The cultivation matrix was a composite matrix (peat: vermiculite = 1:1). Seedlings with 4-6 leaves were prepared for treatments. The experiment was divided into four treatments: control group (-GA-BR): control treatment, spraying with distilled water; (-GA+BR): 0.05 mg·L⁻¹ brassinolide foliar spraying treatment; (+GA-BR): matrix surface spraying 200 times 20% glyphosate treatment; (+GA+BR): matrix surface spraying 200 times 20% glufosinate, 0.05 mg·L⁻¹ brassinolide foliar spraying and root irrigation treatment. The experimental treatment started on May 6 with an average temperature of 25°C and a maximum optical quantum flux density of 960 μmol·m⁻²·s⁻¹. While treating with GA, it was sprayed evenly on the matrix surface around the seedling of WL9 after 4 pm of the day, more than 20cm away from the seedling. While treating with exogenous BA, the leaves and roots of WL9 seedling were sprayed and irrigated once in the afternoon one day before GA treatment. Fifty seedlings of each treatment were prepared, and the entire process was repeated three times.

After 7 days of treatment, the leaves of WL9 were taken to determine the activity of nitrogen metabolizing enzyme and the content of amino acid etc. The photosynthesis was measured on 10th day of treatment, and the fresh weight of shoots and roots of WL9 seedlings was recorded on 15th day of treatment. All data measurements were repeated four times.

Determination of biomass indicators: At the 15th day of treatment, 10 seedlings of each group were randomly taken, washed, and dried. The fresh weight of the seedlings was measured. The data of each group were recorded, and then the average value was calculated.

Determination of chlorophyll content and photosynthetic rate: The chlorophyll content was determined according to Lichtenthaler & Buschmann (2001) method. The mature leaves in the middle of seedlings were selected, and the net photosynthetic rate (Pn) was measured by CIRAS-3 portable photosynthesis/fluorescence measuring system. The measuring time was 9:30-10:30 in the morning on a sunny day, and each treatment was repeated 5 times.

Determination of physiological indexes related to nitrogen metabolism: At the 7th day of treatment, 8 seedlings of each group were randomly selected, and the leaves were cut off, cleaned, and dried. The veins were removed, and the related physiological indexes were

determined after cutting and mixing. Each group was treated 3 times. The activities of nitrate reductase (NR) and glutamine synthetase (GS) were determined according to the method of Debouba *et al.*, (2006). And the activities of glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) were determined according to the method of Debouba *et al.*, (2007).

Leaf total nitrogen, ammonium nitrogen and nitrate nitrogen were determined according to the method of Karoojee *et al.*, (2021). The determination method of amino acid content refers to Gao *et al.*, (2006).

Fluorescence detection and chlorophyll imaging: Chlorophyll fluorescence was measured using a pulse amplitude modulation fluorometer (PAM-2100, Walz, Effeltrich, Germany). All the chlorophyll fluorescence measurements and the various parameter calculations followed the procedures of Lichtenthaler *et al.*, (2005) and Maxwell & Johnson (2000). The minimal (F_o) and maximal (F_m) fluorescence emissions in the leaves were assessed after 30 min of dark adaptation.

The third leaf from the top of seedlings was randomly selected, and FluorCam chlorophyll fluorescence imaging system (Ecotech Co., Ltd.) was applied to it.

Statistical analysis: SPSS 20.0 software was used to carry out analysis of variance and significance test of difference (Duncan's multiple range tests, $p < 0.05$). Excel 2010 software was used to carry out data statistics as well as to make charts.

Results

Effect of exogenous BA on fresh quality of WL9 under GA stress: From Figs. 1 and 2, it can be seen that GA treatment significantly inhibited the growth of WL9 seedlings, and the shoot and root fresh weight were significantly reduced ($p < 0.05$), merely 18.5 g·plant⁻¹ and 3.42 g·plant⁻¹ respectively.

Exogenous BA could effectively alleviate the inhibition of GA on the growth of WL9 seedlings, and the shoot and root fresh weight reached 24.8 g·plant⁻¹ and 4.13g·plant⁻¹ respectively, thus indicating that exogenous BA can effectively alleviate the toxicity of GA to WL9 seedlings. In control group, exogenous BA treatment had no significant effect on the growth of WL9 seedlings.

Effect of exogenous BA on chlorophyll content and photosynthetic rate of WL9 under GA stress: It can be seen that GA treatment significantly reduced the chlorophyll content and net photosynthetic rate of WL9 seedlings (Figs. 3 and 4; $p < 0.05$). The chlorophyll content and photosynthetic rate of the leaves of WL9 treated with GA were only 0.86 mg·g⁻¹ FW and 4.8 μmol·m⁻²·s⁻¹ respectively. The photosynthetic rate of WL9 seedlings under GA stress could be significantly increased by BA treatment, with the chlorophyll content and photosynthetic rate reaching 1.04 mg·g⁻¹FW and 7.9 μmol·m⁻²·s⁻¹ respectively, indicating that exogenous BA could effectively alleviate the effect of GA on photosynthesis of WL9 seedlings.

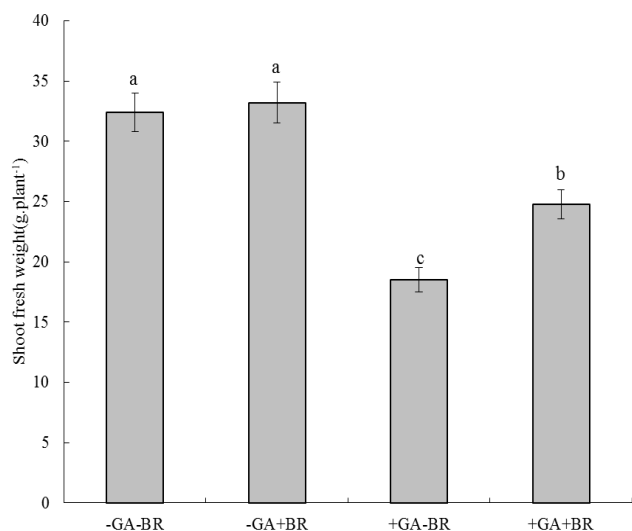


Fig. 1. Effect of exogenous BR on shoot fresh weight of *Trichosanthes kirilowii* under herbicide stress.

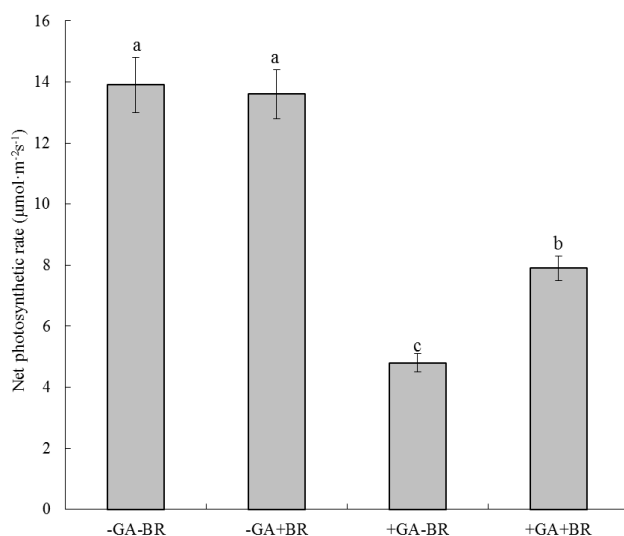


Fig. 4. Effect of exogenous BR on net photosynthetic rate of *Trichosanthes kirilowii* under herbicide stress.

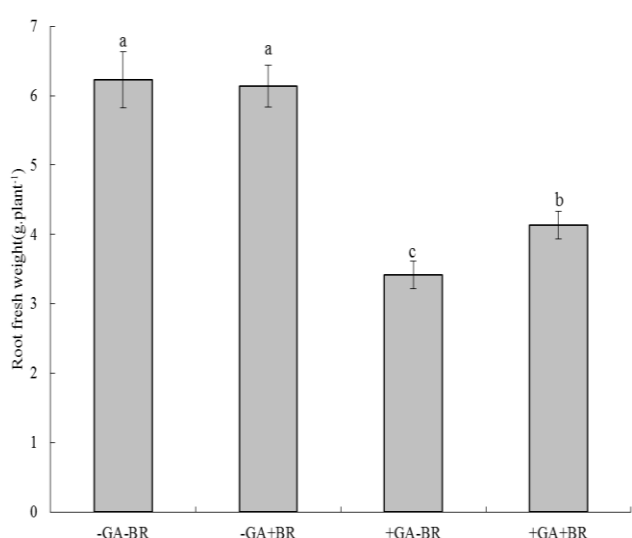


Fig. 2. Effect of exogenous BR on root fresh weight of *Trichosanthes kirilowii* under herbicide stress.

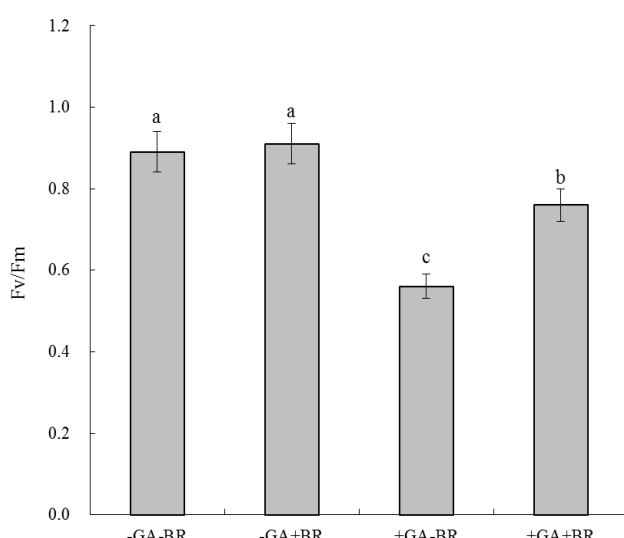


Fig. 5. Effect of exogenous BR on Fv/Fm of *Trichosanthes kirilowii* under herbicide stress.

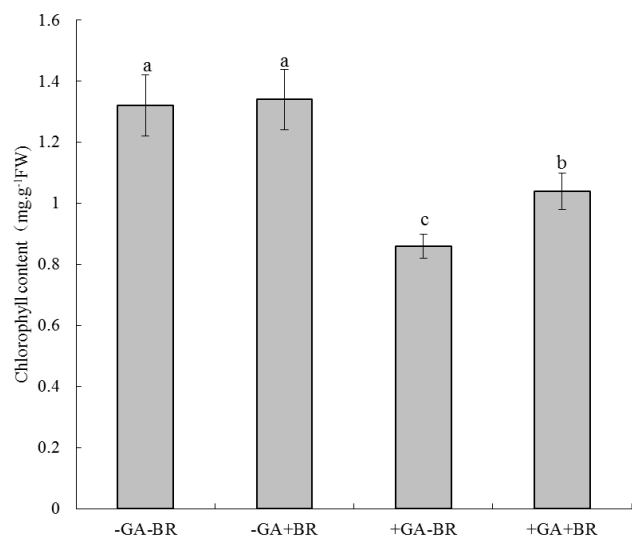


Fig. 3. Effect of exogenous BR on chlorophyll content of *Trichosanthes kirilowii* under herbicide stress.

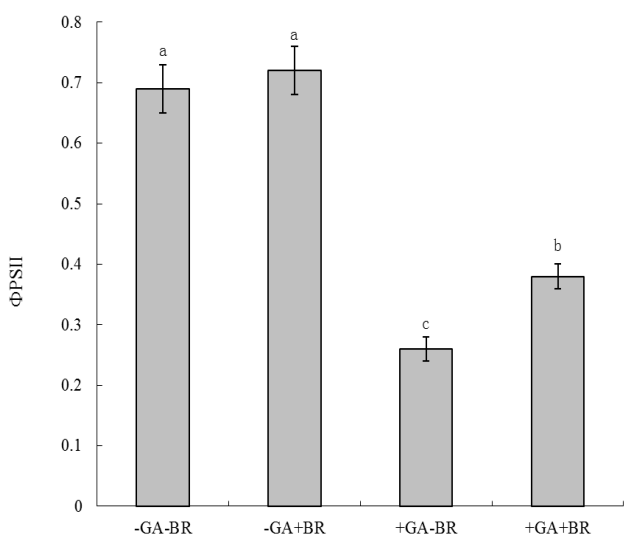


Fig. 6. Effect of exogenous BR on ΦPSII of *Trichosanthes kirilowii* under herbicide stress.

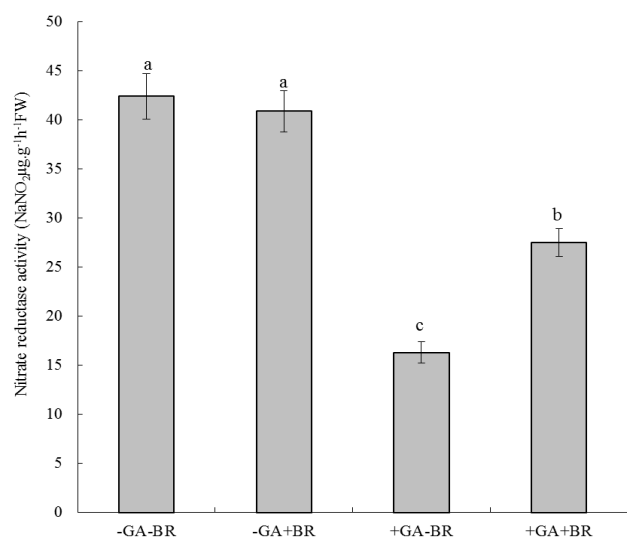


Fig. 7. Effect of exogenous BR on nitrate reductase activity of *Trichosanthes kirilowii* under herbicide stress.

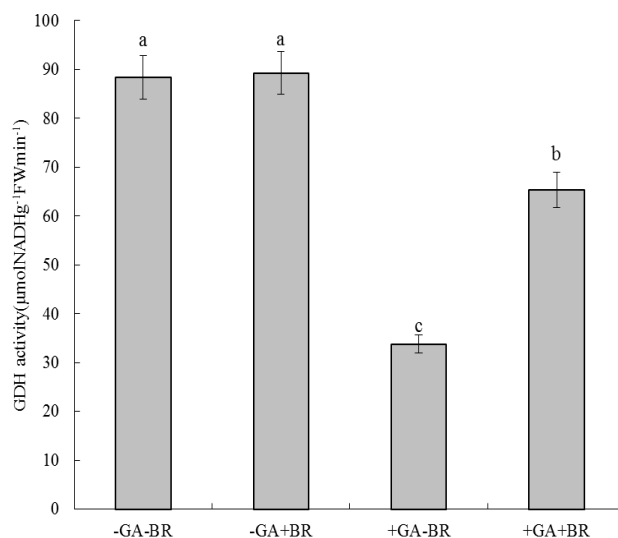


Fig. 10. Effect of exogenous BR on leaf GDH activity of *Trichosanthes kirilowii* under herbicide stress.

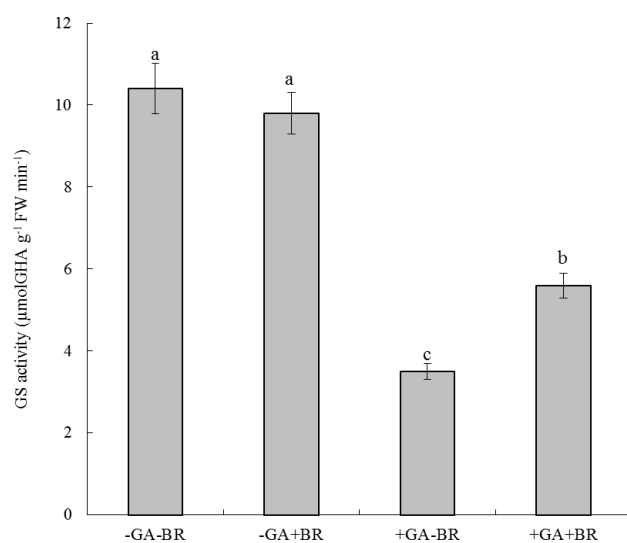


Fig. 8. Effect of exogenous BR on leaf GS activity of *Trichosanthes kirilowii* under herbicide stress.

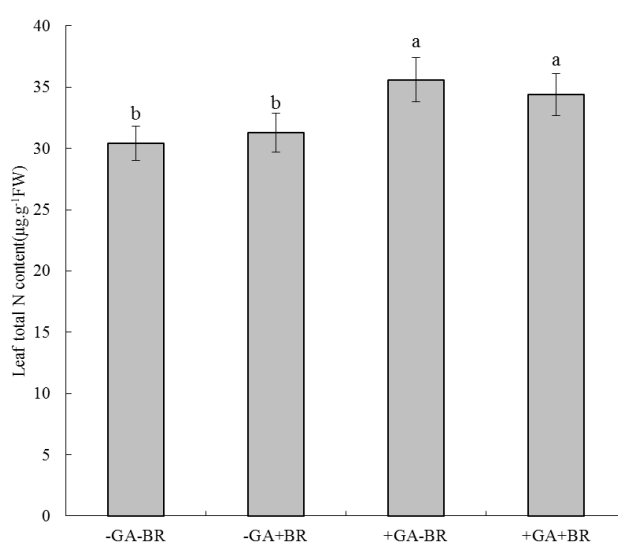


Fig. 11. Effect of exogenous BR on leaf total N content of *Trichosanthes kirilowii* under herbicide stress.

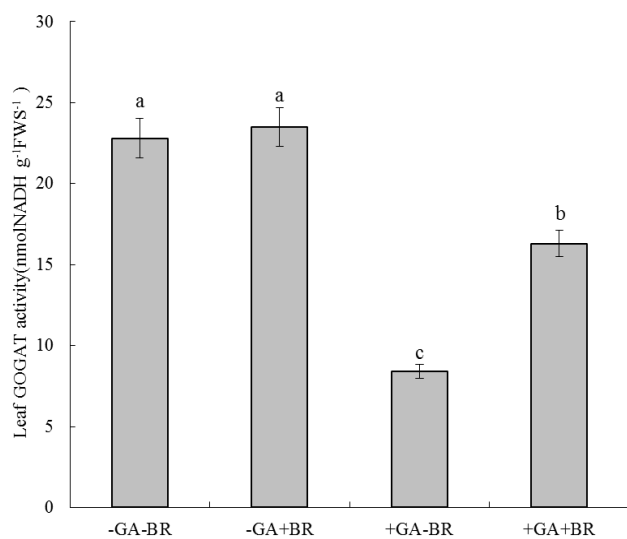


Fig. 9. Effect of exogenous BR on leaf GOGAT activity of *Trichosanthes kirilowii* under herbicide stress.

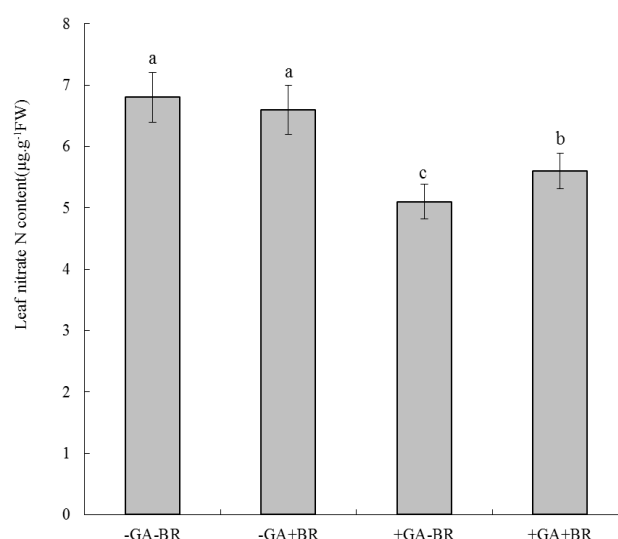


Fig. 12. Effect of exogenous BR on leaf nitrate N content of *Trichosanthes kirilowii* under herbicide stress.

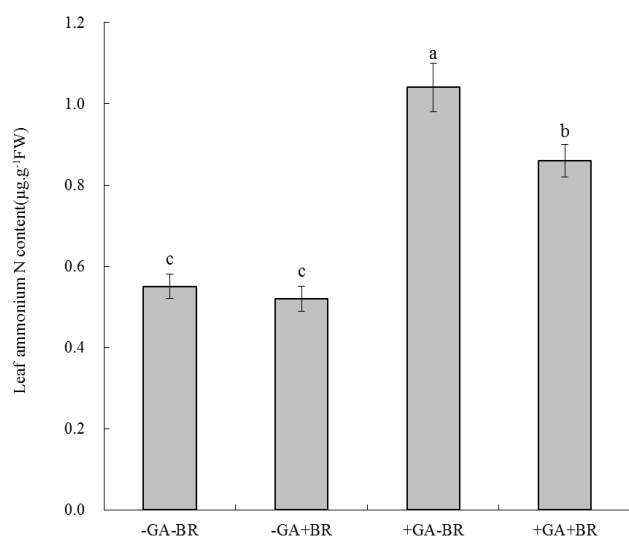


Fig. 13. Effect of exogenous BR on leaf ammonium N content of *Trichosanthes kirilowii* under herbicide stress.

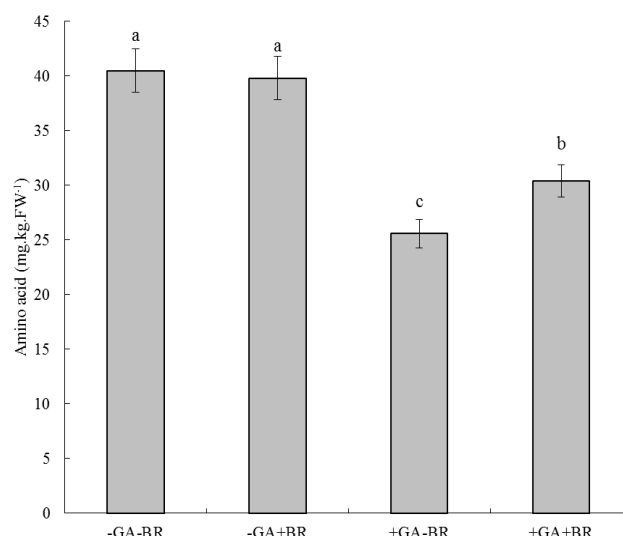


Fig. 14. Effect of exogenous BR on Amino acid content of *Trichosanthes kirilowii* under herbicide stress.

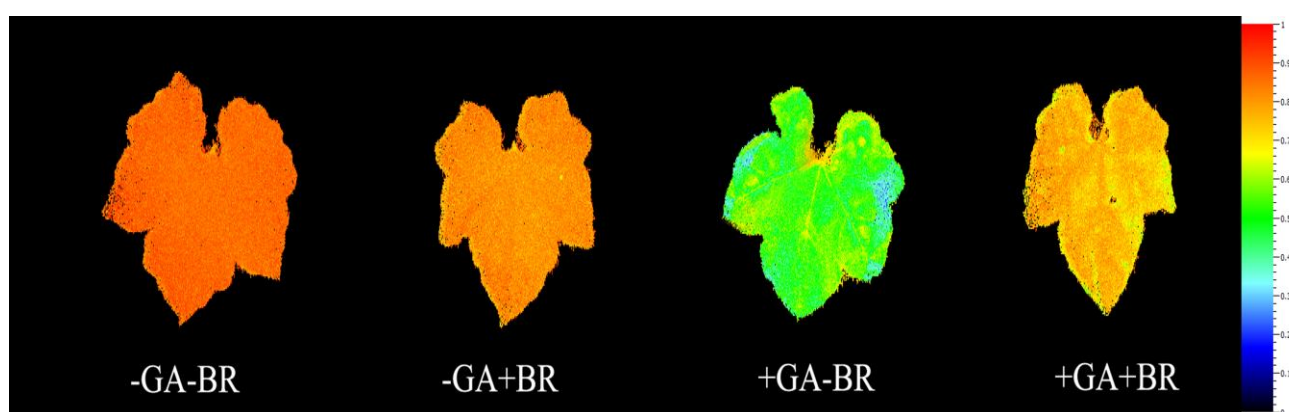


Fig. 15. Effect of exogenous BA on chlorophyll fluorescence imaging of WL9 leaves under GA stress.

Effect of exogenous BA on the utilization rate of light energy in leaves of WL9 under GA stress: As can be seen from Figs. 5 and 6, compared with the control treatment, GA treatment significantly inhibited the light energy utilization rate of WL9 seedlings, and both the maximum photochemical efficiency and the actual photochemical efficiency were significantly reduced ($p < 0.05$). Its actual photochemical efficiency was only 0.26, while the exogenous BA treatment could significantly improve the light energy utilization rate of WL9 seedlings under GA stress. The actual photochemical efficiency of the leaves reached 0.38, which was higher than that of GA treatment. The actual photochemical efficiency increased by 46.15%, which indicated that foliar spraying of BA alleviated the inhibition of GA on light energy utilization of WL9 seedlings, and the effect was significant.

Effects of BA on NR and GS enzyme activities in leaves of WL9 under GA stress: Compared with the control treatment (-GA-BR), the GA treatment significantly reduced the NR and GS enzyme activities of WL9 seedlings, and their activities were only $16.3 \mu\text{gNaNO}_2 \cdot \text{g}^{-1}\text{FW} \cdot \text{h}^{-1}$ and $3.5 \mu\text{mol GHA} \cdot \text{g}^{-1}\text{FW} \cdot \text{min}^{-1}$

respectively; especially the GS enzyme activity was only 33.6% of the control treatment (Figs. 7 and 8; $p < 0.05$). The enzymatic activities of NR and GS, the key enzymes of nitrogen metabolism, were significantly increased by exogenous BA treatment, which were 68.7% and 60% higher than GA treatment respectively. Therefore, it was suggested that the application of BA could maintain the higher NR and GS enzyme activities of WL9 seedlings under GA stress.

Effects of BA on the activities of GOGAT and GDH enzymes in leaves of WL9 seedlings under GA stress:

Compared with the control treatment (-GA-BR), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) activities of WL9 seedlings were significantly decreased under GA treatment, only $8.4 \text{ nmol NADH} \cdot \text{g}^{-1}\text{FW} \cdot \text{S}^{-1}$ and $3.8 \mu\text{mol NADH} \cdot \text{g}^{-1}\text{FW} \cdot \text{min}^{-1}$ (Figs. 9 and 10; $p < 0.05$). The activities of GOGAT and GDH enzymes were significantly increased by exogenous BA treatment under GA stress, which were 94.0% and 93.5% higher than that of GA treatment respectively. Therefore, GA treatment significantly reduced the activities of GOGAT and GDH enzymes in the leaves of WL9 seedlings, and exogenous BA could alleviate the activities of GOGAT and GDH enzymes.

Effect of BA on total N and nitrate N content in leaves of WL9 under GA stress: Compared with the control (-GA-BR), the effect of BA was not significant (Figs. 11 and 12; $p < 0.05$). The total N content in leaves of WL9 seedlings increased, but the nitrate N content decreased under GA treatment. The total N content in the leaves of WL9 seedlings treated with GA was $5.2 \mu\text{g} \cdot \text{g}^{-1} \text{FW}$ higher than that of the control treatment, and the nitrate N content was $1.7 \mu\text{g} \cdot \text{g}^{-1} \text{FW}$ lower than that of the control treatment. Under GA treatment, the application of exogenous BA could reduce the total N content and increase the nitrate N content.

Effects of BA on ammonia N and amino acid contents in leaves of WL9 under GA stress: Compared with the control treatment (-GA-BR), the ammonium N content in the leaves treated with GA increased significantly to $1.68 \mu\text{g} \cdot \text{g}^{-1} \text{FW}$, which was 2.05 times higher than that of the control treatment (-GA-BR) ($p < 0.05$). Exogenous BA could significantly reduce the ammonium N content in the leaves, whose content was only $1.05 \mu\text{g} \cdot \text{g}^{-1} \text{FW}$. There was no significant difference between BR treatment (-GA+BR) and control treatment (-GA-BR) (Fig. 13). As shown in Fig. 14, GA treatment inhibited the synthesis of amino acids in leaves of WL9. Compared with control treatment, the content of amino acids in the leaves under GA treatment was significantly reduced, and the content of amino acids in the leaves was only $25.6 \text{ mg} \cdot \text{kg}^{-1} \text{FW}$, which was 63.2% of the control treatment. Exogenous BA treatment could significantly increase the content of amino acids in the leaves to $30.4 \text{ mg} \cdot \text{kg}^{-1} \text{FW}$. Therefore, GA treatment increased the ammonium N content in the leaves of WL9 and decreased the amino acid synthesis. Exogenous BA significantly reduced the ammonium N content and promoted the amino acid synthesis.

Effect of exogenous BA on chlorophyll fluorescence imaging in leaves of WL9 under GA stress: It can be seen that the leaves of WL9 in control treatment (-GA-BR) were dark red, while the effect of exogenous BA treatment was not significantly different. The leaves treated with GA (+GA-BR) were mainly green, and the addition of BA (+GA+BR) significantly changed the chlorophyll fluorescence imaging, and the color changed to red-yellow. Compared with the standard, the maximum photochemical efficiency of leaves of WL9 treated with GA was significantly reduced, and the toxicity of GA to the leaves could be effectively alleviated under BA treatment (Fig. 15).

Discussion

Trichosanthes kirilowii is an important traditional Chinese medical plant. In order to prevent weed growth, GA is often used as a herbicide in the production of *Trichosanthes kirilowii*. GA usually drifts to *Trichosanthes kirilowii* plants in the prevention and control of weeds, which causes certain harm to the growth of *Trichosanthes kirilowii*. Previous studies on cucumber showed that under GA treatment, chlorophyll content and photosynthetic rate significantly decreased, and chlorophyll fluorescence can be used as a detection

standard for herbicide toxicity (Dayan & Zaccaro, 2012). Our results showed that the chlorophyll content and photosynthetic rate were significantly decreased, and the maximum photochemical efficiency and actual photochemical efficiency were also significantly lower than those of the control treatment. The application of exogenous BR could improve the chlorophyll content, photosynthetic rate, and actual photochemical efficiency of WL9 seedlings under GA stress, thereby alleviating the photosynthetic effect of GA on WL9 seedlings. GA can inhibit chlorophyll synthesis and destroy photosystem II (Ziegler & Wild, 1989). Exogenous BA can improve photosynthetic capacity of plants under stress and alleviate the stress on photosystem (Ali *et al.*, 2008).

The harm of herbicides to plants is manifested not only in photosynthesis (Reddy *et al.*, 2011), but also in nitrogen metabolism (Tsai *et al.*, 2006; Tan *et al.*, 2006). The study disclosed that the expression levels of nitrate reductase and glutamine synthetase, the key enzymes of nitrogen metabolism, were significantly decreased, and the enzyme activities were significantly decreased in GA treated plants as compared to the control treatment (Xie *et al.*, 2014). The present results showed that GA treatment significantly reduced the activities of nitrate reductase, glutamine synthetase, glutamate synthetase, and other key enzymes of nitrogen metabolism in leaves of WL9, especially glutamine synthetase, as compared to the control treatment, and then affected the nitrogen metabolism in the cells of WL9, which was manifested by the significant increase in the intracellular ammonia content and the significant decrease in amino acid content. Exogenous BA could significantly improve the level of nitrogen metabolism and the key enzymes of nitrogen metabolism, among which nitrate reductase and glutamine synthase were 68.7% and 60% higher than GA treatment respectively. Glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) were also significantly increased. Therefore, GA treatment affected nitrogen metabolism by affecting the key enzyme activities of nitrogen metabolism in WL9 seedlings, and exogenous BA could effectively alleviate nitrogen metabolism. This is due to the fact that glutamine synthetase is a detoxifying enzyme in plants, which can remove the toxicity of ammonium released from nitrate reduction, amino acid degradation, and photo respiration during nitrogen metabolism (Sellers *et al.*, 2004; Bernard *et al.*, 2009). The target enzyme for degrading GA is glutamine synthetase. Exogenous BA can change the effect of GA on glutamine synthetase. It can reduce ammonia accumulation and alleviate GA toxicity by increasing glutamine synthetase activity in leaves of WL9.

Conclusions

We conclude that GA had a certain harmful effect on seedlings of WL9, reducing chlorophyll content and photosynthetic rate, and the actual photochemical efficiency was also significantly reduced. The contents of key enzymes and amino acids in nitrogen metabolism were also significantly reduced; inhibiting the growth of WL9 seedlings. Under GA stress, exogenous BR treatment could significantly increase chlorophyll content and photosynthetic rate of leaves. The activities of nitrate

reductase and glutamine synthase, which are key enzymes of nitrogen metabolism, effectively reduced ammonia accumulation, promoted amino acid synthesis, and then promoted the growth of seedlings under GA stress.

Acknowledgments

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