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EFFECTS OF EXOGENNOUS EBR ON PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF SOYBEAN UNDER CADMIUM STRESS

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Abstract

Cadmium (Cd) is one of the highly toxic, non-essential heavy metals that inhibit plant growth and development. The differences in physiological indicators and leaf microstructure between cadmium-tolerant and cadmium-sensitive soybean under Cd stress were studied. The results showed that the photosynthetic efficiency of cadmium-sensitive soybeans was higher after cadmium stress so as to maintain the normal growth of the plants. Compared with that of cadmium-tolerant soybeans, the photosynthetic rate of cadmium-sensitive soybeans under cadmium stress had a higher correlation with each physiological indicator. Overall, EBR affected cadmium-tolerant soybean under cadmium stress mainly by changing the leaf cell structure and maintaining the integrity of cell structure, thereby increasing the net photosynthetic rate of soybean leaves, ultimately maintaining the normal growth of plants.

Introduction

According to *Summary of national ecological and environmental quality in 2020* issued by the Ministry of Ecology and Environment of China, Cadmium (Cd) is the most common heavy metal pollutant in farmland. The World Health Organization has listed Cd as a key food pollutant, which poses a great threat to human health and food safety. The accumulation of Cd negatively affects the water status, disturbs photosynthesis and indirectly induces oxidative stress (Matraszek *et al.* 2016). Brassinosteroid (BR) is a new class of plant hormones in the plant kingdom, and 24-epibrassinolide (EBR), one of the most active BRs, is widely applied in experimental studies. Numerous studies have shown that EBR plays multiple roles in regulating the plant growth and development, including cell division and expansion (Belkhadir *et al*. 2015), photomorphogenesis and photosynthesis (Sharma *et al*. 2022), and responses to various environmental conditions (Vriet *et al*. 2013). Therefore, EBR can induce the performance of plants under a broad range of abiotic stresses such as chilling, drought, salt, alkali, and heavy metal by increasing photosynthesis capacity and strengthening antioxidation and detoxifcation potentials (Bajguz *et al.* 2010).

Grain legumes are vital and dominant crops next to cereals such as wheat, rice, and maize, which contribute immensely to human nutrition. Among the legumes, soybean (*Glycine max* (L.) Merr.), is a unique and distinct crop belonging to the *Fabaceae (Leguminosae)* family, order *Fabales*, and sub-family *Papilionaceae*. The different soybean varieties have different tolerance to cadmium stress. However, the mechanisms by which EBR is tolerant to Cd are still poorly understood. In this study, two soybean varieties with high and low accumulation of cadmium were used to explore the underlying mechanisms by which EBR reduced Cd toxicity. The purpose of this study is to investigate the effects of EBR on physiological, biochemical, and cytological characteristics of soybean seedlings with different cadmium tolerance.

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Materials and Methods

The experiment was performed at Hubei Province Engineering Research Center for Legume Plants, China. Healthy soybean seeds germinated in a growth chamber to the stage of two leaves and one heart. The seedlings with consistent growth status were transferred to 1/2 Hoagland's hydroponic solution. There were three replicates for each treatment in the experiment. The minimum and maximum temperatures were 20.0 and 25.0ºC, respectively. The relative humidity during the experimental period was 75%. The photoperiod was 14 hrs light/10 hrs dark. The cadmium-tolerant variety was m6, and cadmium-sensitive variety was m20.

The EBR (24-Epibrassinolide) stock solution was prepared by ethanol dissolution method (Hu *et al.* 2023), and 0.02% Tween 20-distilled water was used as a surfactant. 0.1 μmol/l EBR was prepared from this stock solution by dilution. The plant leaves were sprayed with EBR at 7:00 - 9:00 am and 16:00 - 18:00 pm for three consecutive days**.** After foliar EBR spray, cadmium stress was implemented by adding $CdCl_2·2.5\ H_2O$ to 1/2 Hoagland's solution to reach a final concentration was 10 mg/l. After 3 days of cadmium treatment, leaves were harvested for the analyses of physiological and biochemical indicators. There were four treatments as followed: T1, control (CK), spraying water instead of EBR; T2 (Cd), 10 mg/l cadmium; T3 (Cd + EBR), 0.1 μmol/l EBR + 10 mg/l cadmium; T4 (CK + EBR), control + 0.1 μmol/l EBR; m6- cadmium tolerant variety and m20- cadmium sensitive variety.

The plant height (mm) was measured by using a ruler, and stem diameter was measured with vernier calipers. Plant tissue was dried in an oven at 80ºC until reaching constant weight for dry weight measurement. The plant fresh weight and dry weight were measured for five times to take the average value. The relative water content was calculated according to the following formula: RWC = [(FW(fresh weight of leaves)-DW(the leaf dry weight)) / (TW(the blade expansion weight)-DW)]×100.

The leaf pigments and other parameters of leaf were analyzed following the method mentioned below:

The 0.1 g leave samples were put into 10 ml mixed extract solution (at the ratio of ethanol: acetone: water = 4.5: 4.5: 1), soaked in dark conditions, and pigment was extracted until the leaf fragments completely turned white. The absorbance of samples was measured at 440 nm, 645 nm, and 663 nm using a spectrophotometer (Hu *et al*. 2023).

EC was measured according to the method of principles and techniques of plant physiological biochemical experiment (Wang *et al*. 2015).

The 100 mg leaf tissue was added into 50 mmol/l phosphate buffer and mixed, and then the mixed solution was centrifuged at 15000 ×g for 15 min. The catalase (CAT) activity, and peroxidase (POD) activity in the supernatant were then determined.

The portable photosynthetic system LI-6400XT was used to measure the photosynthetic characteristics of leaves at 9:00-11:00. Three soybean plants with similar leaf growth status and leaf area were selected for each group, and the average value was calculated. The net photosynthetic rate (P_N), intercellular CO_2 concentration (C_i), stomatal conductance (g_s), and transpiration rate (E) of soybean leaves under different treatments were measured.

After 3 days of Cd exposure, the 1 cm^2 leaves (square) were collected from the middle part of fully expanded leaves. Subsequently, all the collected leaf material was fixed in FAA (Formalin–acetic acid–alcohol) for 24 hours, dehydrated in ethanol, embedded in historesin, and cut into thin slices. After staining with fast Green, the sections were observed under a microscope. The images were captured and analyzed with Caseviewer software.

Analysis of variance (ANOVA) was performed with one-way ANOVA using SPSS 19.0 software. Treatments were compared for significant differences ($p < 0.05$ level) using DMRT. All the data are expressed as the mean± SE of three biological replicates (n=3). Grey correlation analysis was conducted by previously reported method (Guo *et al.* 2019).

Results and discussion

It could be seen from Fig. 1 that under cadmium stress, the fresh weight and dry weight of cadmium-tolerant soybean m6 decreased significantly by 23.00 and 32.00% ($p < 0.05$), respectively, while the fresh weight and dry weight of cadmium-sensitive soybean m20 respectively decreased by 15.26 and 16.00% with no significant differences before and after cadmium stress. The application of 0.1 μmol/l EBR increased the fresh weight and dry weight of m6 by 7.00 and 16.00%, respectively, and decreased the fresh weight and dry weight of m20 by 12.87 and 19.00%, respectively, with no significant difference before and after EBR application. The plant heights of both m6 and m20 decreased significantly under cadmium stress, but there was no significant change in plant height before and after spraying EBR. Therefore, it could be concluded that cadmium stress reduced the fresh weight and dry weight of m6 and m20. The short-term application of 0.1 μmol/l EBR could not effectively alleviate the decline of dry weight and fresh weight of soybean seedlings under cadmium stress, and it had no alleviation effect on plant height, stem diameter, and leaf length/width ratio.

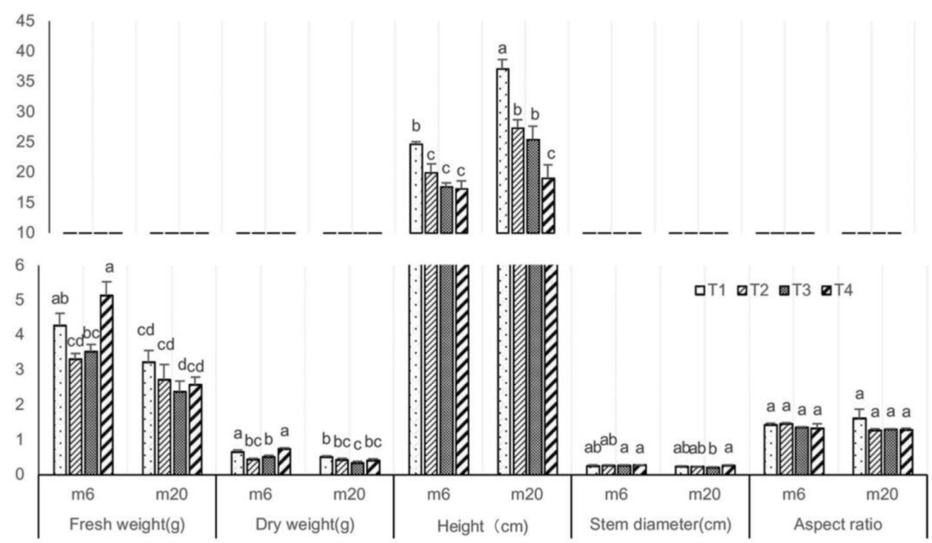

Fig. 1. Effects of 24-epibrassinolide on fresh weight, dry weight, plant height, stem diameter, leaf length/width ratio of soybean under cadmium stress.

Studies have shown that oxidative stress in plants is one of the most common symptoms of Cd poisoning (Romero-Puertas *et al.* 2019). Antioxidant enzymes such as SOD, POD and CAT can remove oxygen free radicals in plant cells and accelerate ROS clearance. Liu *et al.* (2023) point out that the important reason for the increase in enzyme activity in cadmium-tolerant tobacco leaves under cadmium stress is that the antioxidant capacity of cadmium-tolerant tobacco is higher than that of cadmium-sensitive tobacco (Liu *et al.* 2023). EBR promotes seedling growth and alleviates Cd toxicity by increasing the activity of antioxidant enzymes (Kapoor *et al.* 2016). In this study, in order to resisting the damage of cells caused by cadmium, the POD and CAT activities of cadmium-tolerant soybean seedlings were significantly increased compared with the control under cadmium stress, the results were consistent with Xu (2022). POD activity increased significantly and CAT activity decreased significantly of cadmium-sensitive soybean under cadmium stress. After EBR spraying, the CAT enzyme activity of cadmium-sensitive soybean was in order to remove excess ROS and alleviate cadmium stress. The difference is that m6 itself has a strong cadmium resistance, and EBR does not significant improve CAT and POD activity. In addition, EBR can significantly reduce the EC of cadmium-tolerant soybean and cadmium sensitive soybean (Fig. 2).

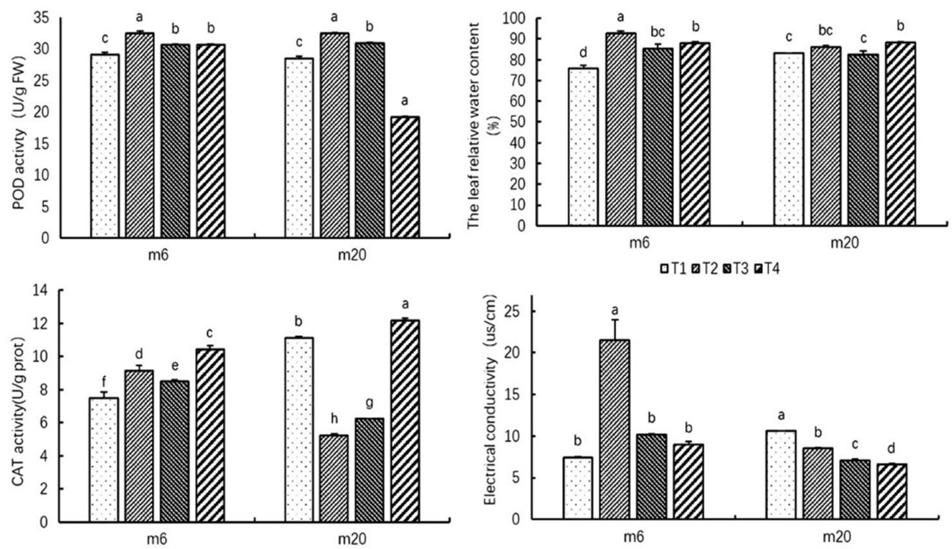

Fig. 2. 0.1 μmol/l EBR affects Peroxidase (POD), catalase (CAT), Electrical conductivity (EC), and leaf relative water content (RWC) in soybean plants under cadmium stress.

In period 2, the P_N value of cadmium-tolerant soybean seedling leaves under T2 decreased by 2.53 %, compared with that in CK group, but the difference was not significant. However, the P_N value in Cd + EBR (T3) group was significantly increased by 285.00%, compared with that in Cd (T2) group, and the P_N value in CK+EBR (T4) group was significantly increased by 77.46%, compared with that in CK group, indicating that spraying EBR could significantly improve the P_N value of cadmium-tolerant soybean seedlings under cadmium stress, and it could also significantly increase the P_N value of the control group ($P<0.05$). However, the P_N value of cadmium-sensitive soybean seedlings under the treatments of Cd (T2) and Cd + EBR (T3) was significantly decreased by 52.14% and 59.26%, compared with that in CK group. In addition, the P_N value in Cd + EBR (T3) group was significantly (14.88%) lower than that in Cd (T2) group. These results showed that spraying EBR could effectively alleviate the Cd stress-induced the decrease of P_N value of cadmium-tolerant soybean, but it could not effectively alleviate the decrease in P_N value of cadmium-sensitive soybean. Moreover, in period 2, the P_N values of m20 under CK (T1), Cd (T2), and CK + EBR (T4) treatments were all higher than those of m6, indicating the photosynthetic efficiency of cadmium-tolerant soybean seedling leaves was higher that of cadmium-sensitive soybean seedling leaves under the same treatment.

Photosynthesis is the basis of crop yield and crop quality. Photosynthesis is closely associated with bean plants' growth, and it can be considered as a determinant factor for Cd tolerance

(Bahmania *et al*. 2020). External stress can reduce net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate, and exogenous substances can effectively maintain photosynthetic characteristics well under cadmium pollution (Liu *et al*. 2019). According to the Shu (Shu *et al*. 2016), the increase or decrease in the net photosynthetic rate of cadmium-tolerant soybean and cadmium-sensitive soybean is accompanied by the increase or decrease of the intercellular CO_2 concentration (Ci) and stomatal conductance (g_s), respectively, and thus the net photosynthetic rate is mainly limited by stomatal conductance. Data obtained in this study showed that EBR significantly alleviated the decrease in net photosynthetic rate and stomatal conductance of cadmium-tolerant soybean under cadmium stress, but those of cadmium-sensitive soybean failed to be alleviated (Fig. 3).

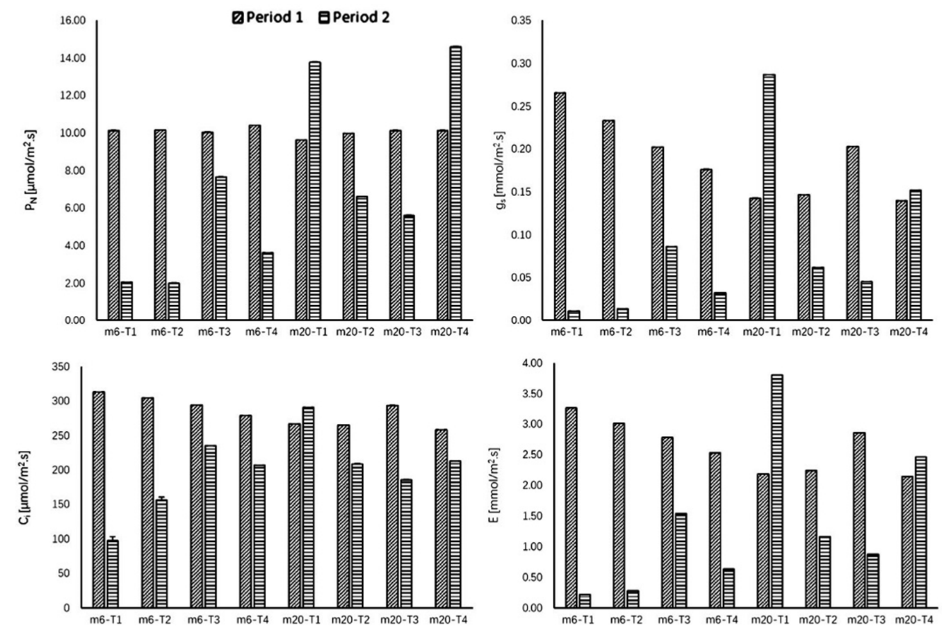

Fig. 3. Effects of cadmium stress and EBR treatment on photosynthetic parameters of soybean seedlings. Photosynthetic parameters were successively measured in 2 different periods, namely, after EBR application (before cadmium stress, period 1), and after cadmium stress (period 2).

The fundamental parameter affecting photosynthesis is chlorophyll, and photosynthesis is positively correlated with the content of the Chlorophyll content (Chen *et al*. 2023). Foliar EBR application of significantly increases the total chlorophyll content of chickpea under salt and/or cadmium stress (Wani *et al*. 2017). EBR significantly increases the content of Chl a and Chl a + b under low temperature and low light conditions, and the Chl a/b ratio also increases (Shu *et al*. 2016). In this study, cadmium stress did not cause significant changes in various pigment indexes (chlorophyll a, chlorophyll b, carotenoid content, chla/chlb, chla +b), but unexpectedly, some pigment contents were decreased by EBR. After EBR spraying, the chlorophyll a content and carotenoid content of cadmium-sensitive soybean seedlings were significantly decreased by 46.3 and 45.4%, respectively, while the chlorophyll b content and total chlorophyll content of cadmium-tolerant soybean seedlings were significantly decreased by 29.4 and 19.9%, respectively (Fig. 4).

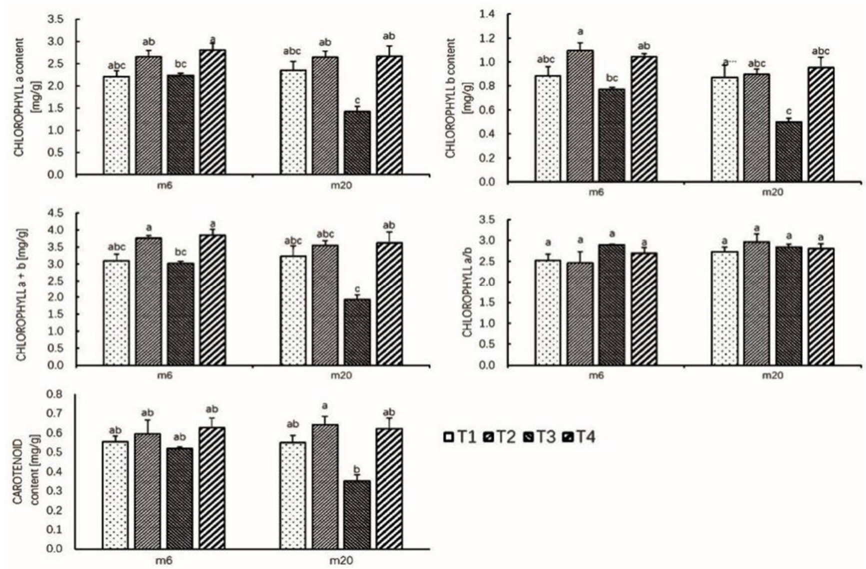

Fig. 4. Effects of 0.1 µmol/l EBR on Chl a(Chlorophyll a), Chl b (Chlorophyll b), Chl a/b (Chlorophyll a/ Chlorophyll b), Chl a + b (Total chlorophyll), Car (Carotenoids) of soybean under cadmium stress

In the process of plant evolution, the changes of leaf morphology and structure will inevitably affect the change of plant physiological and biochemical characteristics, and the leaf thickness, palisade tissue and spongy tissue differentiation degree are related to the environment (Chartzoulakis *et al*. 2002, Manoj *et al*. 2010). The reduction in the thickness of the palisade tissue and the spongy tissue will inevitably lead to a decrease in the number of chloroplasts and chlorophyll content and a decrease in the photosynthetic capacity of the leaves, thus resulting in excess light energy, a large amount of active oxygen free radicals, and the damage to the photosynthetic mechanism of plant leaves, eventually causing photoinhibition or even photodestruction (Zhang *et al*. 2011, Junker *et al*. 2016). This study found that under cadmium stress, the leaves of cadmium-tolerant and cadmium-sensitive soybeans formed anatomical structure characteristics adapted to the environment. The irregular shape and arrangement of cells might be attributed to the fact that cadmium entered the plant tissue cells, further strongly inhibiting the normal growth and metabolism of the plant body, finally resulting in changes in the microscopic structure of the cells and significantly reduced cell integrity.

The anatomical structure types of soybean seedling leaves all exhibited the typical characteristics of C3 plant anatomical structure, with epidermis and 2 layers of palisade cells. Fig.5 showed that the palisade tissue cells of the cadmium-tolerant soybean leaves in CK group were arranged closely and neatly in a long columnar shape. After cadmium stress (Cd), some of the leaf palisade tissue cells appeared to be shortened, the arrangement was uneven, and the space between cells was large. After EBR application, the spacing of palisade tissue cells narrowed, and the arrangement of palisade cells was closer than that of cadmium-stressed cells. In addition, after EBR treatment (T3), cell microstructure images of cadmium-tolerant soybean, the thickness of the palisade tissue was increased, which was beneficial to the photosynthesis of the leaves; after EBR treatment (T3), cell microstructure images of cadmium-sensitive soybean, the thickness of the palisade tissue was decreased, which was not beneficial to the photosynthesis of the leaves. Therefore, the changes of leaf cell structure under EBR treatment were consistent with the changes of P_N.

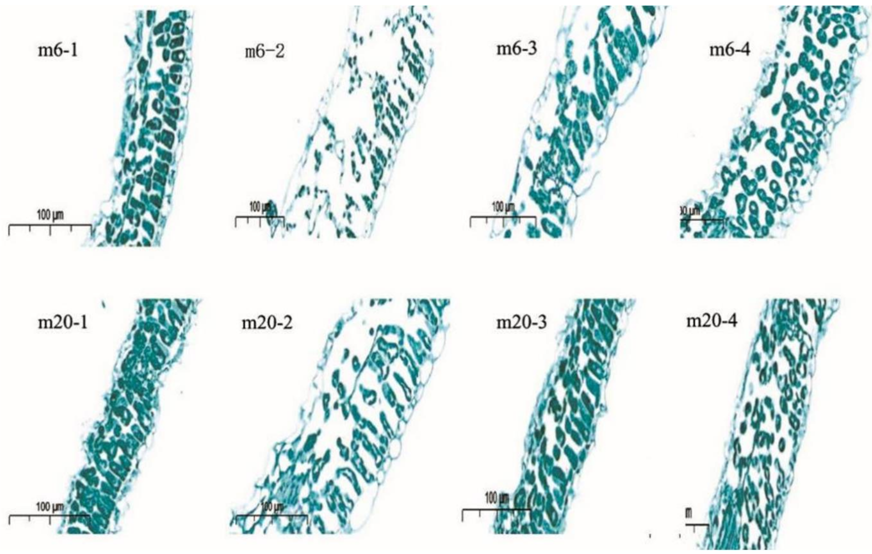

Fig. 5. Leaf cross sections of soybean plants treated with EBR and subjected to 3-day cadmium stress. Black bars= 100 μm.

Table 1. Correlation between photosynthetic rate and various indicators of two types of soybean seedlings.

Indexes	Cd-tolerant soybean				Cd-sensitive soybean			
	T1	T2	T3	T4	T1	T2	T3	T4
Stem diameter	0.623	0.551	0.619	0.923	0.930	0.912	0.669	0.547
leaf length-width ratio	0.618	0.603	0.608	0.926	0.917	0.860	0.711	0.520
EC	0.717	0.572	0.420	0.875	0.887	0.876	0.740	0.470
Chl a	0.651	0.558	0.600	0.839	0.885	0.823	0.642	0.562
Chl b	0.627	0.559	0.565	0.891	0.856	0.808	0.639	0.554
Chl a/b	0.654	0.560	0.665	0.855	0.933	0.808	0.710	0.540
Chl a+b	0.644	0.559	0.590	0.852	0.877	0.818	0.641	0.560
Car	0.631	0.558	0.612	0.820	0.884	0.838	0.659	0.560
RWC	0.664	0.555	0.610	0.898	0.909	0.843	0.693	0.542
POD	0.631	0.555	0.600	0.842	0.912	0.798	0.684	0.471
CAT	0.677	0.630	0.614	0.757	0.872	0.882	0.642	0.631
g_s	0.844	0.484	0.878	0.812	0.917	0.790	0.866	0.764
Ci	0.827	0.693	0.702	0.875	0.860	0.862	0.690	0.549
E	0.858	0.396	0.896	0.881	0.869	0.841	0.913	0.781
Height	0.616	0.737	0.655	0.817	0.806	0.788	0.807	0.497

Note: T1, control; T2 (Cd), 10 mg/l cadmium; T3 (Cd +EBR), 0.1 μmol/l EBR + 10 mg/l cadmium; T4 (CK + EBR), control + 0.1μmol/l EBR; Control, sparying water instead of EBR.

Grey correlation analysis is an effective method to analyze the relationship between the photosynthetic rate and physiological traits of soybean seedlings under short-term cadmium stress and exogenous EBR treatment. According to Table 1, the order of correlation between each physiological indicator and leaf photosynthetic rate was E > gs > Ci > Stem diameter > Aspect ratio > Chl a/b > Height > RWC > CAT > Car > Chl a > EC > Chl a+b > Chl b > POD. Compared with cadmium-tolerant soybean, cadmium-sensitive soybean under cadmium stress exhibits a higher correlation between the photosynthetic rate and each indicator. Under Cd+ EBR treatment (T3), the correlation between photosynthetic rate and each physiological indicator of cadmium-tolerant soybean was higher than that of cadmium-sensitive soybean.

In summary, the effects of EBR application on cadmium-tolerant and cadmium-sensitive soybeans under cadmium stress are different. When conducted the comprehensive analyses of paraffin sections, and photosynthetic parameters of seedling leaves and found that compared with cadmium-tolerant soybeans, cadmium-sensitive soybeans exhibited closer cell arrangement and a higher net photosynthetic rate, indicating that there might be a direct relationship between leaf cell structure and photosynthetic rate. By grey correlation analysis, the correlation between various physiological indexes and photosynthetic rate of cadmium-sensitive soybean was stronger compared with cadmium-tolerant soybean.

Acknowledgments

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