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·Exogenous Betaine Enhances Salt Tolerance of *Glycyrrhiza uralensis* through Multiple Pathways

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Abstract

Background: *Glycyrrhiza uralensis* Fisch. is a medicinal plant with high economic value. Despite the relatively strong salt tolerance of perennial individuals, its salt tolerance at the seedling growth stage is low. Improving its salt stress resistance at the seedling stage is the key to the cultivation of the plant using salinized land. In this study, we investigated the enhancement effect of exogenous glycine betaine (GB) on the growth of *G. uralensis* seedlings under NaCl stress conditions and analysed its physiological mechanism.

Results: The root growth of *G. uralensis* seedlings was severely inhibited under NaCl stress conditions, and the addition of GB could effectively alleviate the adverse effects of the salt stress; the application of 20 mM GB had the most significant alleviating effect. Compared with the NaCl-only treatment group, the application of 20 mM GB under NaCl stress conditions significantly increased total root length, total root surface area, and total root volume by 80.38%, 93.28%, and 175.61%, respectively, and obviously increased the GB content in its roots, stems, and leaves by 36.88%, 107.05%, and 21.63%, respectively, and their activity of betaine aldehyde dehydrogenase 2 ($BADH_2$) was increased by 74.10%, 249.38%, and 150.60%, respectively. The GB-addition treatment significantly increased content of osmoregulatory substances (soluble proteins, sluble sugars and proline) in the roots and leave, markedly enhanced the activity of antioxidant enzymes and the content of antioxidant, and dramatically promoted salt secretion capacity of the leaves, which especially promoted the secretion of Na^+.

Conclusions: In summary, the exogenous addition of GB is an effective way to significantly improve the salt tolerance of *G. uralensis* seedlings, and promotion of the synthesis of osmoregulatory substances, enhancement of antioxidant enzyme activities, reduction of membrane lipid peroxidation products, and discharge of excess salts in the body (especially the significant promotion of the secretion of Na^+) are the critical reasons why exogenous addition of GB was able to effectively alleviate the NaCl damage of *G. uralensis* seedlings.

Keywords: Liquorice; NaCl stress; Salt secretion; Salt tolerance; Glycine betaine

Background

Soil salinization is a major factor limiting crop yields globally (especially in drylands)[1]. According to the Food and Agriculture Organization of the United Nations, the global salinized land area was approximately 8.33×10^8 hm^2 in 2021, and there was a salinized land area of approximately 9.9×10^7 hm^2, in China. Salt stress (mainly NaCl stress) triggers osmotic imbalance, excessive accumulation of reactive oxygen species (ROS) and Na^+ retention through osmotic stress, oxidative stress, and ionic toxicity. They disrupt plant metabolic systems, lead to reduction in crop yield and quality crop, and substantial losses in the agricultural economy. Due to limited arable land, exploiting salt-tolerant plants and improving their salt tolerance is important to develop the economy of saline soils and to increase farmers' income, which is also a hot topic in research presently.

G. uralensis is a halophyte of Leguminosae and has a high commercial value. Its dried roots and rhizomes are traditionally used as herbal medicines due to its flavonoids and triterpenoids components. Its stems and leaves are high in crude protein and crude fat and low in crude fibre; thus, the liquorice is an excellent forage or supplementary grass for cattle and sheep[2]. Notably, as increasing of people's health awareness, natural medicine use has risen rapidly worldwide, and the demand for *G. uralensis* in the world has increased; thus, the dwindling wild liquorice resources cannot fulfil market demand[3]. Therefore, artificial cultivation of the plant should be implemented to solve the imbalance between supply and demand in the liquorice industry.

Most liquorice planting areas are threatened by soil salinization, especially in the northern part of China. Although adult

individuals of *G. uralensis* have strong salt tolerance, the tolerance is relatively weak in their seedling stage. Planting of *G. uralensis* in salinized land, dead seedlings often occur red[4], the medicinal material's production was reduced sustainable sharply, which restricted the development of the liquorice industry. Notably, the history of liquorice from a wild to a cultivated plant is relatively short compared to other crops, and the breeding for germplasm with strong salt tolerance has not occurred. Therefore, improving the salt tolerance of *G. uralensis* by cultivation techniques is urgently necessary.

Addition of exogenous substances has become one of the most effective cultivation techniques to improve salt tolerance of crops in a short time. Glycine belaine (GB) is a kind of growth regulator widely present in organisms. Exogenous GB is chemically and physically stable, and is easily absorbed by plants, and its synthesis process has been well developed. The literature demonstrated that exogenous GB can enhance the activity of enzymatic and non-enzymatic systems, reduce ROS (reactive oxygen species) accumulation, and increase the content of osmoregulatory substances to enhance the salt tolerance of plants. Exogenous GB can significantly enhance salt tolerance and significantly increase fruit or seed yield of cotton (*Gossypium hirsutum* L.)[5], maize (*Zea mays*)[6], rice (*Oryza sativa*)[7], and tomato (*Lycopersicon esculentum*)[8-9]. However, further research is necessary on whether the GB may improve the root yield of a plant.

Thus, we investigated the effects of GB addition on salt tolerance indices of *G. uralensis* seedlings under NaCl stress conditions, such as growth, osmotic adjustment, reactive oxygen metabolism system, and salt secretion, so as was to provide a theoretical basis and technical support for the cultivation of high-yield and high-quality liquorice herbs in salinized land.

Material and methods

Reagent and Plant material

The betaine ($C_5H_{11}NC_2$) required for the test was purchased from McLean Company (Shanghai, China) and had a relative molecular mass of 118.15 and a purity greater than 98%; all the other reagents required for the test were analytically pure. The *G. uralensis* seeds were provided by the Institute of Liquorice in Shihezi University. The experiment was conducted from April to October in 2021 on the campus of Shihezi University, China.

Experimental treatments

Healthy and full seeds with the same size were selected and soaked in 98% sulfuric acid solution at room temperature

(25℃) for 30 min, washed with tap water until there was no sulfuric acid residue on their surface, and then soaked in distilled water for 12 h. Next, the seeds were spread evenly in the plastic pot (bottom diameter × top diameter × height = 20 cm × 30 cm × 20 cm; gauze mesh was laid at the bottom). Each pot contained approximately 8 kg sandy soil, and the ratio of river sand to loam was 7:3. The soil background details are presented in Table 1, 15 seeds were evenly sown in each pot at a sowing depth of 1 cm.

Fertilizing sscheme were similar to those of *G. uralensis* field management (urea [N ≥ 46%] 14.99 g/m^2, calcium superphosphate [$P_2O_5 \geq 46\%$] 23.99 g/m^2, potassium sulfate [$K_2O \geq 50\%$] 10.49 g/m^2)[10], of which calcium superphosphate was applied as a basal fertilizer, one-third of the urea and potassium sulfate were also applied as basal fertilizers. The remaining two-thirds of them were applied at the seedling stage. When the *G. uralensis* seedlings had six true leaves, four seedlings of uniform growth were retained in each pot by converting the planting density of cultivated liquorice fields (usually 6.0×10^5 plants/hm^2) with the area of the upper calibration of the plastic pots.

Tab. 1 Physical and chemical properties of sandy soil

Total nitrogen g/kg	Total phosphorus g/kg	Total potassium g/kg	Available N mg/kg	Available P mg/kg	Available K mg/kg	Organic matter g/kg
0.315	0.131	5.47	52.59	5.23	50.04	6.64

NaCl concentration was set by referring to the salinity range of *G. uralensis*[11] and the grading of saline soils by Bausdan (2000), 200 mL NaCl solution was used for watering every other day for a total of 15 times. Once the salt treatment was over, 200 mL GB solution of the corresponding concentration (10, 20, 40, and 80 mM GB) was applied to the roots every 5 d, for a total of three times; an equal amount of distilled water was applied to the control group and NaCl-only treatment group[12].

The detailed formulation of each treatment group is shown in Table 2. Each treatment was replicated five times. Except for the experimental treatments, the other management measures used were the same as those of the *G. uralensis* field. Samples were collected at 9:00 a.m. every day on the seventh day after the last treatment. Next, the corresponding indices were measured. The third fully expanded functional leaf (from the top of the plant) was used to determine the physiological

indices. Plants harvested and measured their biomass after all physiological indices had been measured.

Tab. 2 Experimental treatments of seedling growth stage

Code	Treatments abbreviations	NaCl concentration （mmol/L）	GB concentration （mmol/L）
（1）	CK	0	0
（2）	NaCl	160	0
（3）	NaCl+GB10	160	10
（4）	NaCl+GB20	160	20
（5）	NaCl+GB40	160	40
（6）	NaCl+GB80	160	80

Plant growth parameters

Height: the height of the plant was measured from the surface of the potting soil to the terminal bud of the plant. Ten plants were randomly selected for each treatment.

Root morphological indices: the root systems of above plants were placed in an acrylic tray (25 cm × 15 cm) of a root scanner (WinRHIZO La 2400, Epson, Japan), and was scanned using EPSON Scan scanning software. After obtaining the scanned images of the root system, the root system analysis system WinRHIZO was used to measure the root morphology indices such as total root length (TRL), total root surface area (TRSA), and total root volume (TRV) and other morphological indicators of the root system.

Biomass: 10 individual plants were randomly selected from each treatment. The roots were removed from the plastic pot, and the sandy soil on the surface of the roots was gently washed with tap water. The roots were divided into three parts (roots, stems, and leaves), they were placed into a paper bag, heated in an oven at 105 ℃ for 30 min, and then dried at 80 ℃ to a constant weight. The dry weight was determined by an analytical balance with an accuracy of 0.001 g (BS423S, Sartorius, Germany).

Determination of endogenous betaine content and betaine aldehyde dehydrogenase activity

The content of GB (endogenous betaine) and the activity of $BADH_2$ (betaine aldehyde dehydrogenase) in roots, leaves, and stems of *G. uralensis* were determined by using a Solarbio kit (GB-BC3130, Beijing Solarbio Science and Technology

Co., Ltd., China) and a botanical betaine aldehyde dehydrogenase 2 ($BADH_2$) ELISA kit (Jiangsu Jingmei Biological Technology Co., Ltd., China), respectively.

Determination of osmoregulatory substances

Proline (Pro), soluble sugar (SS), and soluble protein (SP) contents in the roots and leaves of *G. uralensis* were determined using the acidic indinotrione method[13], anthrone method[14], and Coomassie brilliant blue (G-250) method[15], respectively.

Determination of conductivity and malondialdehyde content

The relative conductivity of the roots and leaves of *G. uralensis* was determined using the conductivity method[16]. One gram of fresh leaves was placed in a 15 mL centrifuge tube, to which 10 mL deionised water was added. The leaves were then soaked at room temperature for 12 h. The conductivity of the solution was measured using a conductivity metre (Bante 5, Shanghai Bante, China) and recorded as R_1. Next, it was heated in a water bath at 100 °C for 30 min. Its conductivity was measured after the solution was cooled to room temperature and recorded as R_2. The formula for calculating the relative electrical conductivity is as follows:

$$EL = R_1/R_2 \times 100\%$$

The determination of MDA in roots and leaves was performed using the thiobarbital acid method[17].

Determination of reactive oxygen scavenging systems

Hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-) content and superoxide dismutase (SOD), POD, peroxidase, catalase (CAT) and ascorbate peroxidase (APX) activity in the leaves and roots of *G. uralensis* were measured using Solarbio kits (H_2O_2-BC3590, O_2^--BC1290, SOD-BC0170, POD-BC0095, CAT-C017, APX-BC0220, Beijing Solarbio Technology Co., Ltd., China). Ascorbic acid content (ASA) was quantitatively determined by using the 2,6-dichlorophenol indidophenol (DCPIP) method[18].

Observation of salt discharge behaviour and ionic measurements

The stomata and salt glands on the lower leaves of *G. uralensis* have strong salt secretion ability[19]. Therefore, some lower healthy and fully expanded leaves were selected, cut into 0.5 cm × 0.5 cm pieces (over the main vein) near the apical one-third of the leaf, and fixed in FAA for 48 h. Next, the fixed material was glued to the sample stage and subjected to ion

sputtering plating. An Electron microscope (SU8010, Hitachi High-tech Company, Japan) was used to observe secreted salt by the salt glands and stomatal of *G. uralensis* and capture images. Each treatment was repeated for five materials.

Referring to Newete[20], the experimental material was placed under a rain-proof shed, and 15 fully expanded lower leaves were randomly selected. The upper and lower surfaces of the leaves were cleaned with deionised water and then marked. After 7 d, the leaves were placed in a centrifuge tube containing 20 mL ultrapure water, centrifuged at 2000 r/min for 10 min, and removed. The leaf area was scanned using a scanner (WinRHIZO La 2400, Epson, Japan), and the contents of K^+, Na^+, and Ca^{2+} in the leachate were determined using atomic absorption spectrophotometry (Agilent 240DUO, Thermo Fisher, USA). Each treatment was repeated thrice.

Data analysis

Data were statistically analysed using SPSS 20.0 (IBM Corp., New York, USA) software. The significance of differences between treatments was tested using One-way ANOVA (One-way ANOVA) and the least significant difference (LSD) metod (P<0.05). Data are presented as mean ± standard deviation, the OriginPro 2022b (Electron ic Arts Inc, New York, USA) software was used for plotting. The relationships between the measured indicators were analysed using principal component analysis (PCA) to obtain principal components based on eigenvalues >1.

Results

Effects of exogenous GB on the growth of salt-stressed *G. uralensis* seedlings

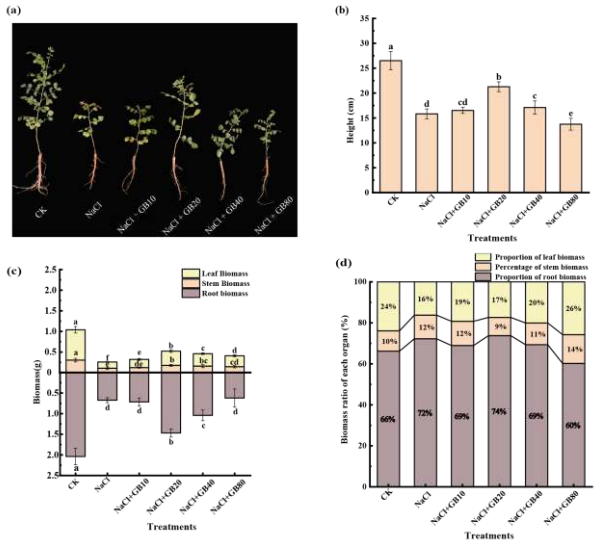


Fig. 1 Effects of exogenous GB (0, 10, 20, 40 and 80 mM) on morphological characteristics of *G. uralensis* seedlings under salt stress (Mean ± SD)

When the experiment was completed, phenotypic observations and images were collected for each treatment group. Plant height and root, stem, and leaf biomass were measured and counted. NaCl stress significantly inhibited the growth of *G. uralensis*, with significantly more yellow leaves and weaker individual growth on *G. uralensis* seedlings under NaCl stress than those in the control (Fig. 1a). Under the salt stress (NaCl) condition, the plant height (Fig. 1b) and the biomass of roots, stems, and leaves (Fig. 1c) of *G. uralensis* decreased by 40.31%, 66.99%, 64.76%, and 79.42%, respectively, compared with those CK, however the root biomass percentage increased from 66.21% in CK treatment to 72.23% in NaCl-treated group (Fig. 1d). GB significantly ameliorated the leaf yellowing and growth inhibition of *G. uralensis* seedlings caused by NaCl stress and promoted the accumulation of biomass in various organs. After the application of 20 mM GB, all the growth indices of *G. uralensis* reached their maximum value, and the plant height and the biomass of roots, stems, and leaves increased by 34.42%, 118.24%, 64.78%, and 128.22%, respectively, comparaed with those of the NaCl-only treatment (P<0.05). In conclusion, exogenous application of GB significantly alleviated the inhibitory effect of NaCl stress on the growth and yield of roots, stems, and leaves of *G. uralensis*.

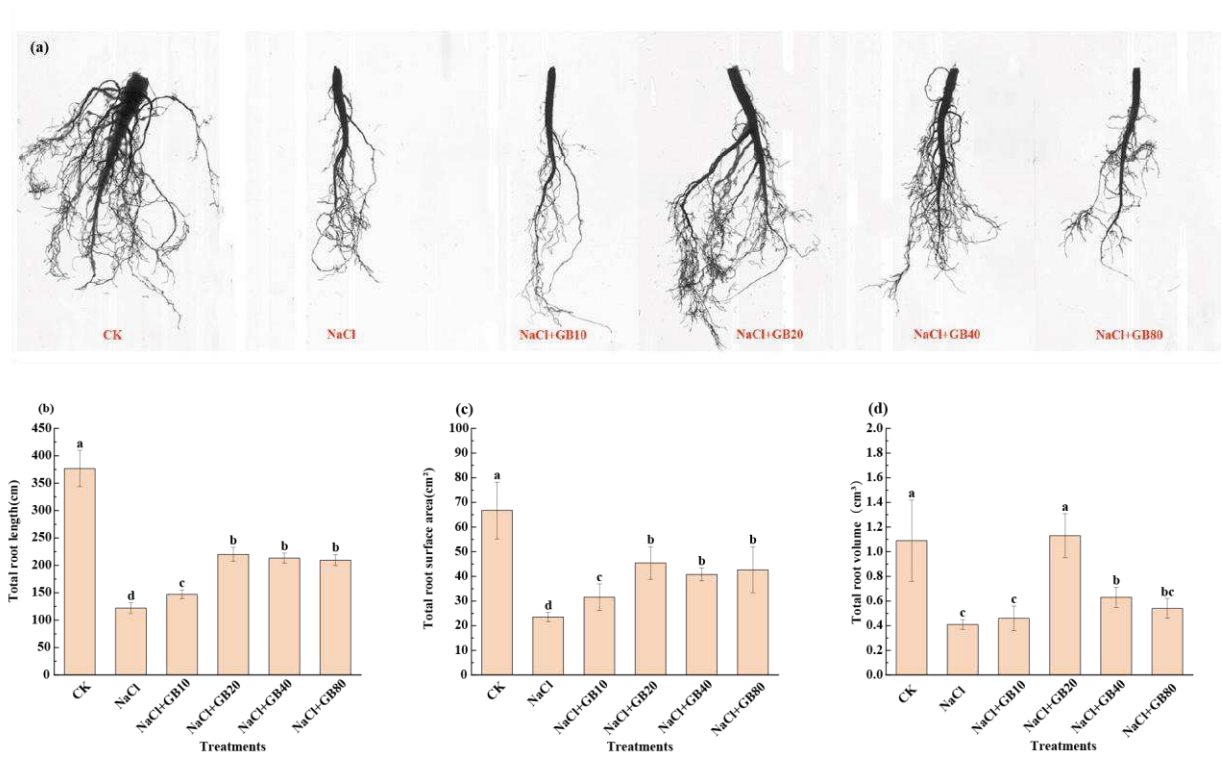


Fig. 2 Effects of exogenous GB (0, 10, 20, 40 and 80 mM) on root characteristics of *G. uralensis* seedlings under salt stress (Mean ± SD)

Salt in the soil inhibited the root growth of *G. uralensis* by reducing its root length (TRL) (Fig. 2b), root surface area

(TRSA) (Fig. 2c), and root volume (TRV) (Fig. 2d), resulting in the root indices of NaCl-only treatment being significantly lower than those of CK (Fig. 2a) (P<0.05). The application of GB at an appropriate concentration was able to effectively alleviate the inhibitory effect of salt damage on the root growth of *G. uralensis*. The TRL, TRSA, and TRV of *G. uralensis* increased by 80.38%, 93.28%, and 175.61%, respectively, with the application of 20 mM GB compared to the NaCl-only treatment group, which indicated that the GB treatment was able to promote the root growth of *G. uralensis* and thus alleviate the damage caused by the NaCl stress to the seedlings.

Effects of exogenous GB on endogenous GB content of salt-stressed *G. uralensis* seedlings

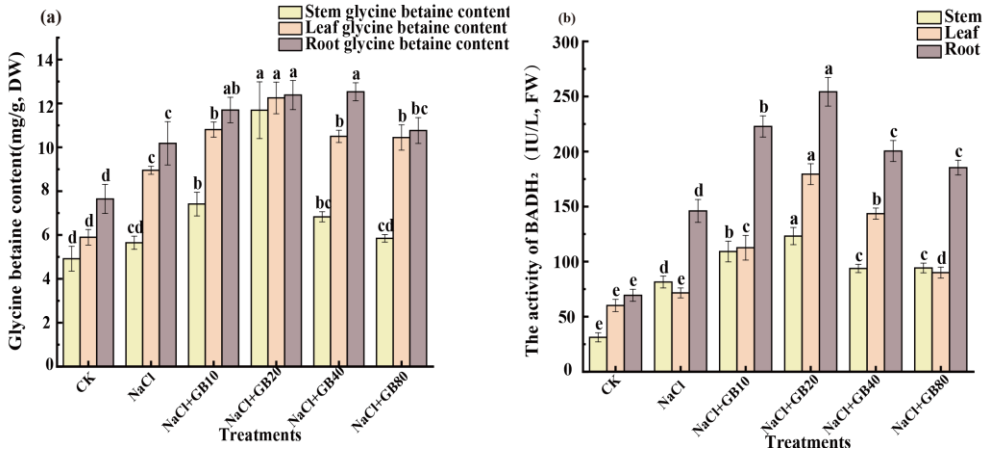


Fig. 3 Effects of exogenous GB (0, 10, 20, 40 and 80 mM) on endogenous GB of *G. uralensis* seedlings under salt stress (Mean ± SD)

Endogenous GB content in roots, stems, and leaves of *G. uralensis* were obviously increased after salt stress treatment (Fig. 3a), and $BADH_2$ activity was enhanced (Fig. 3b). The GB content and $BADH_2$ enzyme activity were significantly increased in roots, stems, and leaves of *G. uralensis* after the application of exogenous GB. Especially, the GB content and $BADH_2$ enzyme activity reached the maximum value after addition of 20 mM GB, in which the GB content in roots, stems, and leaves increased by 36.88%, 107.05% and 21.63%, respectively, compared with the NaCl-only treatment; the activity of the $BADH_2$ enzyme increased by 74.10%, 249.38% and 150.60% compared with the NaCl-only treatment.

Effects of exogenous GB on soluble protein, soluble sugar, and proline contents of salt-stressed *G. uralensis* seedlings

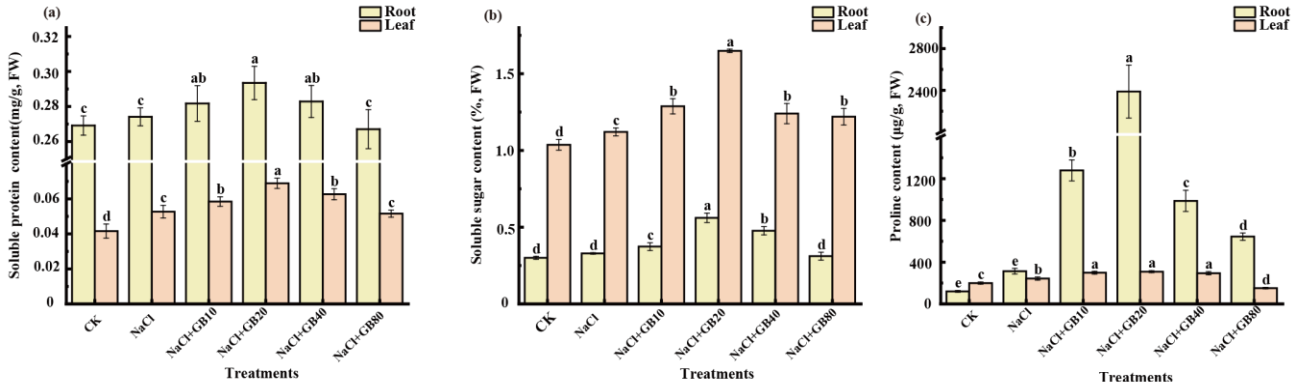


Fig. 4 Effects of exogenous GB (0, 10, 20, 40 and 80 mM) on soluble protein, soluble sugar, and proline contents of *G. uralensis* seedlings under salt stress (Mean ± SD)

The contents of soluble protein (Fig. 4a), soluble sugar (Fig. 4b), and proline (Fig. 4c) in the roots and leaves of *G. uralensis* showed different degrees of increase after NaCl stress. Under salt stress conditions, the contents of SP, soluble sugar, and proline in the roots and leaves of *G. uralensis* reached the maximum value after application of 20 mM GB; the contents of SP, soluble sugar, and proline in roots increased by 7.05%, 70.52% and 661.06%, respectively, compared with those NaCl-only stress; and the three aforementioned osmotic regulators in the leaves increased significantly by 30.74%, 47.11%, and 26.88%, respectively. Thus, the GB treatment was able to significantly promote the synthesis of these osmoregulatory substances and thus alleviate the damage of NaCl stress on *G. uralensis* seedlings.

Effects of exogenous GB on reactive oxygen metabolism systems of salt-stressed *G. uralensis* seedlings

The H_2O_2 (Fig. 5a), O_2^- content (Fig. 5b) and rate of O_2^- production (Fig. 5c) in the roots and leaves of *G. uralensis* seedlings significantly increased under NaCl-only treatment compared with CK. Here, the three indices in roots increased by 825.07%, 221.26%, and 221.26%, and those in leaves increased by 213.5%, 177.12%, and 266.38%, respectively ($P < 0.05$). In contrast, the application of exogenous GB under salt stress decreased the H_2O_2 and O_2^- content and reduced the rate of O_2^- production in roots and leaves. After the application of 20 mM GB, the H_2O_2, O_2^- content and O_2^- production rate of the *G. uralensis* root system decreased by 80.49%, 63.41%, and 63.41%, respectively, compared with that of NaCl-only treatment, and the aforementioned three indices in the leaves decreased by 53.47%, 53.86%, and 53.86%, in that order. The results indicated that NaCl stress caused a large accumulation of ROS in *G. uralensis*, and the application of GB reduced the ROS content, with the most obvious effect of 20 mM GB treatment.

The MDA (Fig. 5d) content of the roots and leaves increased by 44.97% and 214.29% ($P < 0.05$), respectively, and the

electrical conductivity (Fig. 5e) increased by 168.14% and 137.13% in the NaCl-treated group as compared with CK. The application of 20 mM GB significantly reduced the MDA content and conductivity of roots and leaves by 27.62% and 45.20% ($P < 0.05$) for MDA content and 60.23% and 59.30% ($P < 0.05$) for conductivity content as compared with NaCl-only treatment. The results indicated that NaCl stress caused a significant increase in MDA content and relative conductivity in *G. uralensis*, and GB application was able to reduce MDA content and leaf relative conductivity, with the most pronounced reduction in the 20 mM GB treatment group.

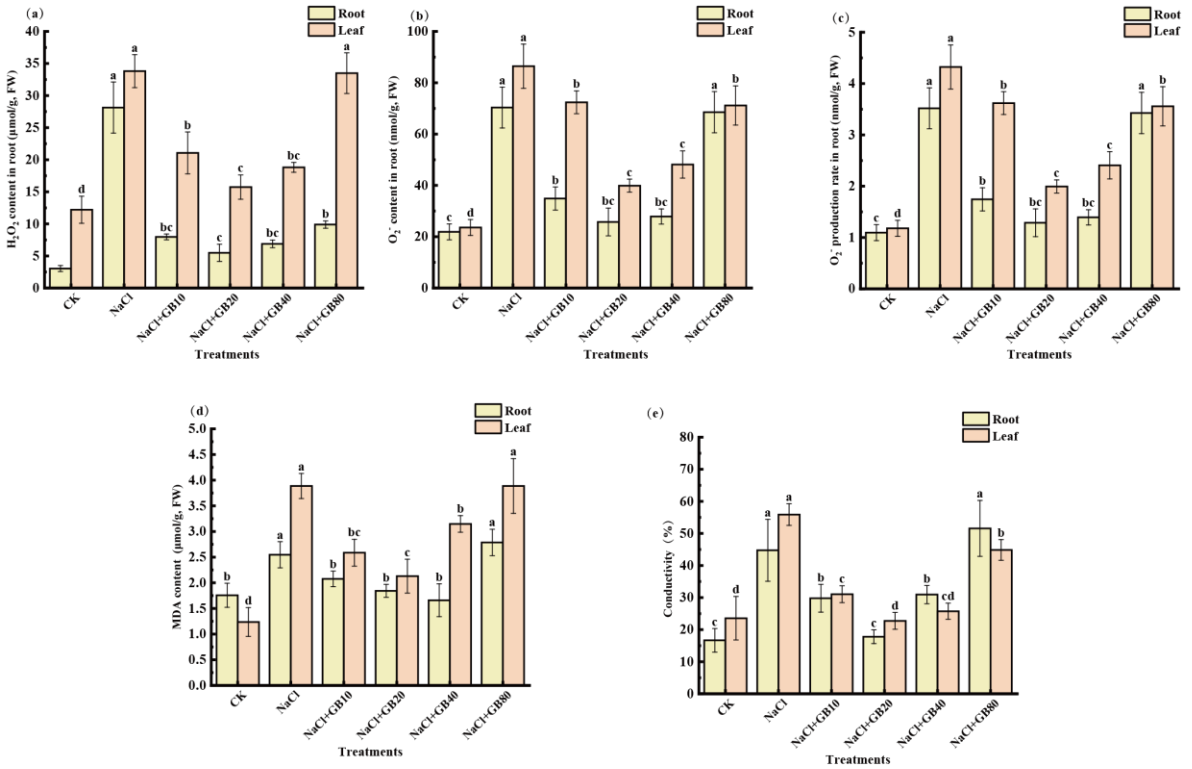


Fig. 5 Effects of exogenous GB (0, 10, 20, 40 and 80 mM) on ROS, MDA and conductivity of *G. uralensis* seedlings under salt stress (Mean ± SD)

SOD (Fig. 6a), CAT (Fig. 6b), POD (Fig. 6c), APX (Fig. 6d) activities, and ASA (Fig. 6e) contents in the roots of *G. uralensis* were significantly higher than those of the control (increased by 36.86%, 103.55%, 52.60%, 46.15%, and 36.37%, respectively) under NaCl stress. The activities of SOD, CAT, and POD in leaves of *G. uralensis* were significantly lower than those of the control (decreased by 38.88%, 42.53%, and 47.33%, respectively); the APX activity and ASA content in the leaves were significantly higher than those of the control (increased by 109.65% and 37.04%, respectively). The maximum values of antioxidant enzyme activities and antioxidant contents were reached in roots and leaves of *G. uralensis* after the application of 20 mM GB, where SOD, CAT, POD, APX and activities and ASA contents in roots were elevated by 59.55%,

413.07%, 225.91%, 300.00% and 73.33%, respectively, as compared with the NaCl-only treatment. The aforementioned five indices in leaves increased by 877.51%, 359.89%, 199.15%, 144.35%, and 108.11%, respectively.

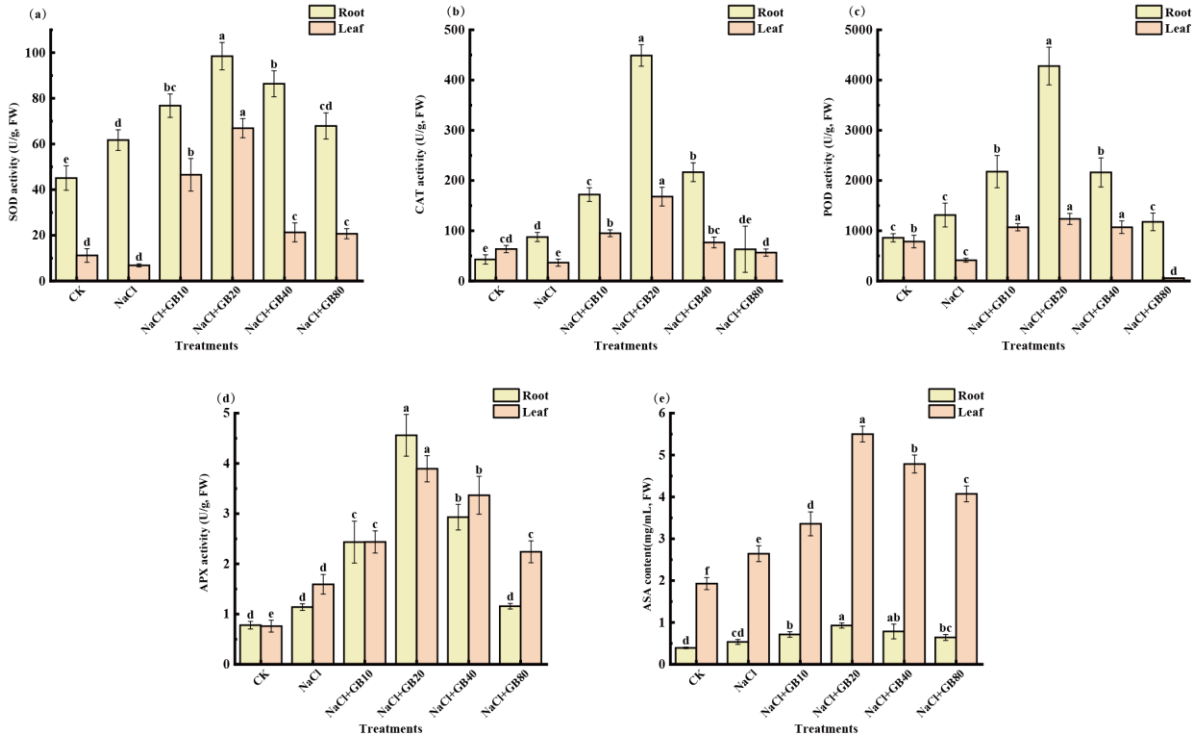


Fig. 6 Effects of exogenous GB (0, 10, 20, 40 and 80 mM) on antioxidant enzymes and antioxidant of *G. uralensis* seedlings under salt stress (Mean ± SD)

Effects of exogenous GB on salt secretion capacity of salt-stressed *G. uralensis* seedlings

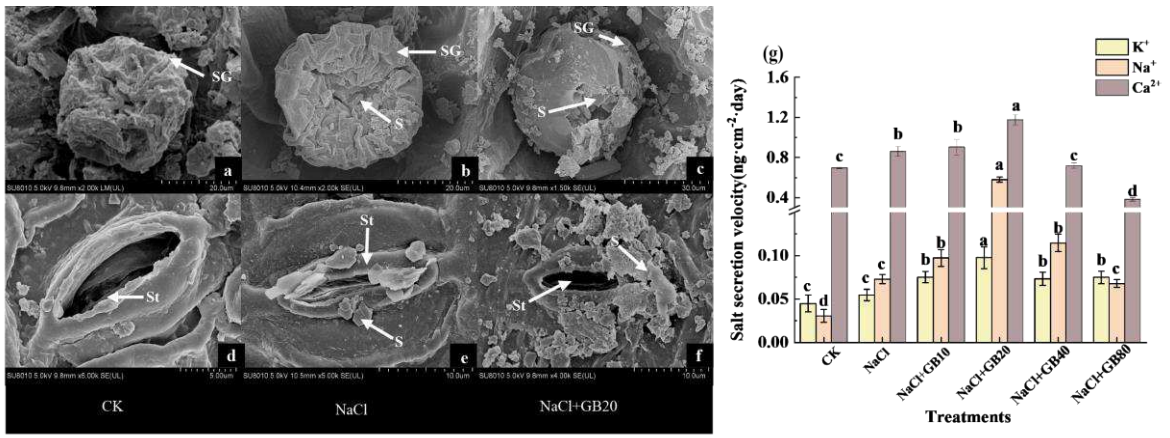


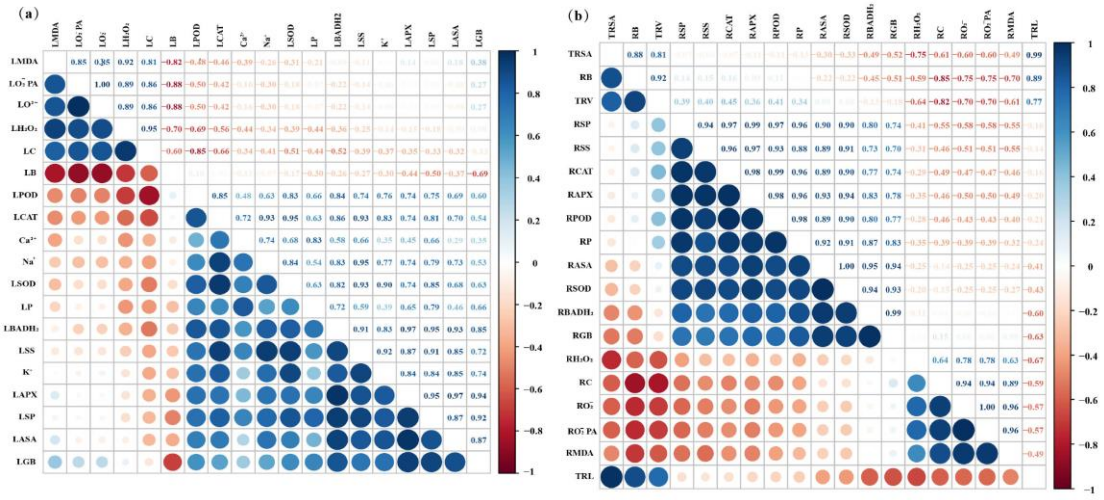
Fig.7 The effect of exogenous GB on salt secretion from saline glands and stomata of *G. uralensis* (Mean ± SD)

Note: SG: Salt gland; St: Stoma; S: Salt

The salt glands and stomata on the leaves were observed, we did not found any salt crystals on the glands or around the stomata in the CK group (Fig. 7a, Fig. 7d). Under the NaCl stress conditions, salt secretion behaviour was observed in salt glands (Fig. 7b) and stomata (Fig. 7e). Also under the NaCl stress condition, the salt secretion capacity of the stomata and salt glands was significantly enhanced (P<0.05) after the application of 20 mM GB (Fig. 7c, Fig. 7f), and the secretion rates of

240 Ca^{2+}, Na^{+}, and K^{+} were 1.79, 7.93, and 1.37 times higher than those of the NaCl-only treatment group, respectively (Fig. 7g).

241 **Trait correlations and variations**



242 Fig. 8 The matrix of the Spearman's correlation coefficients and 95% confidential circle among aboveground and
243 abovebelow traits.

244 Note: The significance level of correlations is indicated; abbreviations stand for: PH: plant height, RB: root biomass, SB:
245 stem biomass, LB: leaf biomass, TRL: total root length, TRSA: total root surface area, TRV: total root volume, RGB: root GB
246 content, SGB: stem GB content, LGB: leaf GB content, $RBADH_2$: root betaine aldehyde dehydrogenase, $SBADH_2$: stem
247 betaine aldehyde dehydrogenase, $LBADH_2$: leaf betaine aldehyde dehydrogenase, RSP: root soluble protein, LSP: leaf soluble
248 protein, RSS: root soluble sugar, LSS: leaf soluble sugar, RP: root proline, LP: leaf proline, RH_2O_2: root hydrogen peroxide,
249 LH_2O_2: leaf hydrogen peroxide, RO_2^-: root superoxide anion, LO_2^-: superoxide anion, RO_2^-PA: root superoxide anion
250 production rate, LO_2^-PA: leaf superoxide anion production rate, RMDA: root malondialdehyde, LMDA: leaf malondialdehyde,
251 RC: root conductivity, LC: leaf conductivity, RSOD: root superoxide dismutase, LSOD: leaf superoxide dismutase, RCAT:
252 root catalase, LCAT: leaf catalase, RPOD: root peroxidase, LPOD: leaf peroxidase, RAPX: root ascorbate peroxidase, LAPX:
253 leaf ascorbate peroxidase, RASA: root ascorbic acid, LASA: leaf ascorbic acid, K^+: potassium ion secretion, Na^+: sodium ion,
254 Ca^{2+}: calcium ion.

255 To effectively examine the response of *G. uralensis* leaves and roots to salt stress, we conducted Spearman's correlation
256 analysis for each of the 19 representative traits of its leaves and roots, respectively. The results shows the correlations among

the indicators of *G. uralensis* leaves. Here, endogenous GB was significantly and positively correlated with $BADH_2$, antioxidant enzymes (SOD, CAT, POD, and APX) activities, osmoregulatory substances (soluble sugars, soluble proteins, and proline), and salt secretion rate (Na^+, K^+, and Ca^{2+}) (Fig. 8a).

Fig. 8b shows the correlation among the indicators of *G. uralensis* roots, where endogenous GB in the root system was significantly and positively correlated with $BADH_2$, antioxidant enzymes (SOD, CAT, POD, and APX) activities, and osmoregulators (soluble sugars, soluble proteins, and proline).

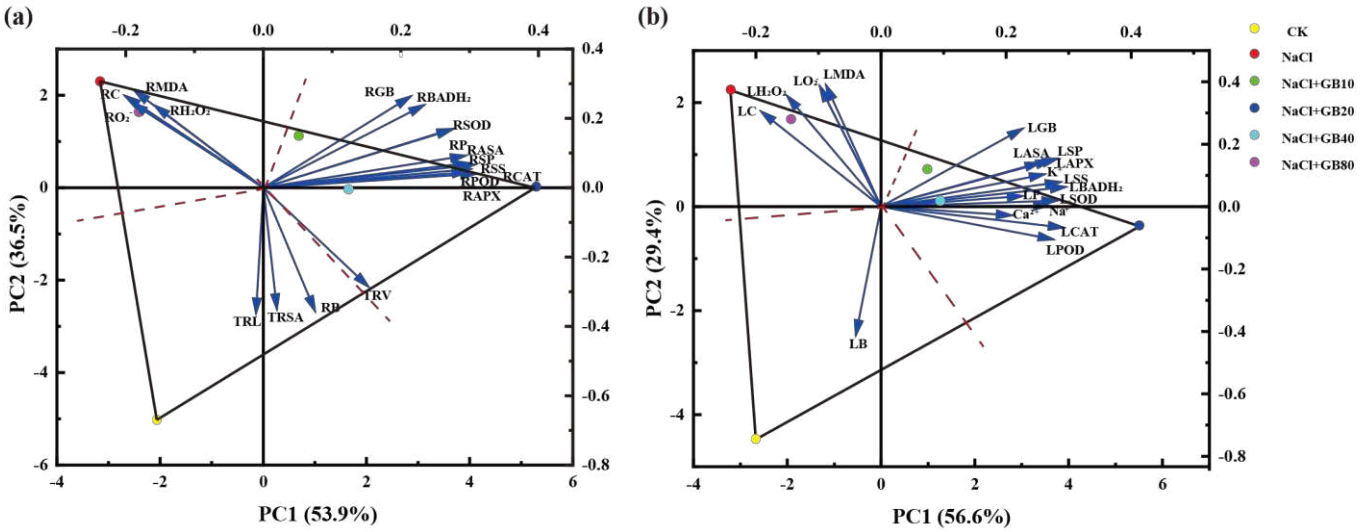


Fig. 9 Principal component analysis on the effects of exogenous GB on relative physiological indices in *G. uralensi* seedling under salt stress

Fig. 9a shows the PCA of the effect of exogenous GB on physiological indices of *G. uralensis* seedling roots under NaCl stress, with the contribution value of the first and second principal components being PC1 = 53.9% and PC2 = 36.5%, respectively, and the two-dimensional plot reflecting 90.4% of the true information for each treatment. Triangles were formed by connecting the outermost treatment points (CK, NaCl, and NaCl+GB20). Three dashed lines perpendicular to the sides from the centre point divided the whole graph into three regions. The root biomass (RB), TRL, and TRSA are shown in the region, which means that the RB, TRL, and TRSA of the CK group were significantly higher than those in other treatments. The region with NaCl treatment as the vertex also included NaCl+GB80 treatment, and the indices in this region were MDA, H_2O_2, O_2^-, and O_2^- production rate, and all the angle between the index vectors were acute angles, indicating a positive correlation with each other. This result indicated that NaCl stress led to an increase in ROS content, enhanced membrane lipid peroxidation, and extravasation of intracellular substances in the root system of *G. uralensis*; therefore, the damage was

greater than that in other treatments. By contrast, the indicators of antioxidant enzymes, antioxidants, and osmoregulatory substances were distributed in the region with NaCl+GB20 treatment as the apex, and the region also included the NaCl+GB10 treatment and NaCl+GB40 treatment. This phenomenon showed that the addition of 20 mM GB treatment under NaCl stress had the highest antioxidant enzyme activity, antioxidant content, and osmoregulatory substances, which reduced the degree of peroxidation of membrane lipids and mitigated the negative effects of NaCl stress on the root system of *G. uralensis*, and that the positive correlation and the coordinated effect among the indices were able to better scavenge the excessive ROS produced by NaCl stress and stabilize the balance of cellular oxidative metabolism.

The PCA of leaf-related physiological indices is shown in Fig. 9b. The information loading of the first and second principal components were PC1 = 56.6% and PC2 = 29.4%, respectively, and the two-dimensional graph reflected 86.0% of the true information for each treatment. Connecting the vertices of each treatment in the outermost (CK, NaCl, and NaCl+GB20), vertical dashed lines were used for each side to divide the whole graph into three regions. Among them, MDA, H_2O_2, O_2^-, and O_2^- production rates were distributed in the region with NaCl treatment as the vertex. There was an acute angle between the index vectors, which suggested a positive correlation. Therefore, the MDA, H_2O_2, and O_2^-content of *G. uralensis* leaves was relatively high under NaCl treatment, and the degree of damage was greater than that of the other treatments. Indicators distributed within the region of CK were leaf biomass, which indicated that *G. uralensis* leaf biomass was the highest in the CK treatment. The NaCl+GB10 treatment and NaCl+GB40 treatment were also included in the region with NaCl+GB20 treatment as the apex. The indicators of antioxidant enzymes, antioxidants, osmoregulatory substances, and salt secretion rate were distributed in this region; moreover, the antioxidant enzyme activities were improved, osmoregulatory substances were increased. The secretion rate of leaves was promoted under the 20 mM GB treatment. Under NaCl stress, the ROS in the leaves of *G. uralensis* increased. This led to the increase in membrane lipid peroxidation and disrupted the oxidative metabolism equilibrium; meanwhile, the addition of GB was able to increase the activities of antioxidant enzymes and the contents of antioxidants and osmoregulators and promoted the secretion of salts by the leaves. This alleviated the stress damage and stabilised the oxidative metabolism level.

Discussion

Salt stress often inhibits plant growth through ionic toxicity, oxidative stress, and osmotic stress[21], and the strength of its inhibitory effects is directly reflected by growth indicators. We found that NaCl stress had a significant inhibitory effect on the accumulation of biomass in *G. uralensis* at the seedlings stage, and the seedlings may use more resources and energy to answer the high salt stress. Additionally, *G. uralensis* can self-regulate its biomass allocation; however, under salt stress conditions, it can allocate more biomass to the root system, adapting to the salinity environment. In the NaCl stress group, the RB percentage increased from 66.21% in CK to 72.22%. In addition to increasing the percentage of below-ground biomass to adapt to the adversity, exogenous application of different concentrations of GB alleviated the growth inhibition of *G. uralensis* seedlings by salt stress, especially in the treatment of applying 20 mM GB, the root, stem, and leaf biomass of *G. uralensis* increased significantly by 118.24%, 64.78%, and 128.22%, respectively. The inhibition of root growth is the most obvious characteristic of plants under salt stress at its early stage. TRL, root surface area, and root volume are indicators of root growth and development[22]. Shen (2022) found that NaCl stress inhibited the growth of the root system of *G. uralensis* and that the application of silicon was able to attenuate the detrimental effects of salt stress[23]; these results are similar to ours. We found that GB promoted the elongation of primary roots and the growth of lateral roots of *G. uralensis* under NaCl stress. Leading to a significant increase in the total length, total surface area, and volume of the root system. Thus, the exogenous GB expanded the range of water uptake and increased the efficiency of water and mineral absorption by the roots of *G. uralensis* under NaCl stress, which mitigated the adverse effects of salt stress. In contrast with other cash crops such as cotton (*Gossypium hirsutum* L.) and maize (*Zea mays* L.), widely grown in arid regions, the root system is the main economic organ of *G. uralensis*. Its growth status has a direct impact on the economic income of the farmers. The growth condition of the root system, especially the length and thickness of the main root, is a key indicator of the quality grade of liquorice herbs, and the thicker and longer the main root, the higher the grade of the herb. The results of this study showed that exogenous GB could significantly promote the elongation and thickening of the primary roots of *G. uralensis* seedlings under NaCl stress. This is conducive to the enhancement of the herb grade and plays a role in increasing the herb production and farmer's income.

The accumulation of endogenous plant betaine adversity stress is crucial for cellular osmoregulation[24]. Currently, there

are three main pathways for the accumulation of endogenous betaine in plants: Firstly, the synthesis and accumulation of betaine are induced in plants by drought[25], salt[5], and heavy metal stress[24]; Secondly, genes for key enzymes of betaine synthesis (e.g., BADH genes) are introduced into plants by using transgenic technology to equip plants with the ability to synthesize more endogenous betaine[26]; Thirdly, the synthesis and accumulation of endogenous betaine in plants is induced by chemical inducers such as inducing endogenous betaine synthesis and accumulation in plants through application of exogenous betaine[27]. Several studies have demonstrated that exogenous GB can promote the accumulation of endogenous GB in different crops under stress conditions. For example, Ji (2022) found that Pb stress induced the accumulation of endogenous GB in roots and shoots of pakchoi (*Brassica chinensis* L.), and the induced effect was more pronounced when 5 mM GB was applied under Pb stress[24]. In our study, we observed similar findings: NaCl stress significantly induced endogenous betaine accumulation in roots, stems, and leaves of *G. uralensis*, and root application of exogenous GB significantly increased the endogenous betaine content of *G. uralensis*. BADH is the rate-limiting enzyme for endogenous GB synthesis in plants[28]. In this study, exogenous application of GB was able to effectively increase the activity of BADH, and its variation pattern was similar to that of endogenous betaine content.

Under salt stress, the osmotic potential around the root system decreases, and osmoregulatory substances (e.g., soluble sugars, soluble proteins, free proline) are rapidly produced in the plant to avoid a large amount of water loss from the root cells[29]. This helps ensure a normal water supply to the plant to manage with the salt-stressed environment. Proline and soluble sugars are two important osmoregulatory substances that enable water transport across membranes in a direction that favours plant growth. Most of the soluble proteins in plants are enzymes involved in metabolism, and their content reflects the intensity of plant metabolism. Additionally, soluble proteins have strong hydrophilicity, which can improve cellular water retention performance and effectively prevent dehydration of the plant root system[30]. The results of this study showed that the content of soluble sugars, soluble proteins, and free proline in the roots and leaves of *G. uralensis* under NaCl stress were significantly higher than those of the CK. This indicated that the osmoregulatory system of *G. uralensis* produced a rapid response to NaCl stress. Moreover, the content of soluble sugars, soluble proteins, and free proline further significantly

increased after the application of GB under NaCl stress compared with NaCl-only treatment.This indicated that exogenous GB not only alleviated the damage cause by NaCl stress to *G. uralensis* through osmoregulation, but also enhanced the resistance of *G. uralensis* to salt stress by increasing its metabolic vigour under NaCl stress.

Under normal physiological conditions, the generation and removal of ROS in plants are a dynamic equilibrium, and ROS is maintained at a low level[31]. When subjected to severe salt stress, the structure and function of chloroplasts are damaged, and the reduction of CO_2 assimilation capacity causes a large amount of electron transfer to O_2^-, resulting in the excessive accumulation of ROS in the plant. Those ROS will attack the electron transport chain, destroy the structure of proteins and other biomolecules, and cause oxidative damage[32], which was also corroborated by our study. Plants have evolved specific scavenging systems to avoid the negative effects of ROS, and improving the antioxidant metabolic capacity to scavenge ROS is crucial for plant resistance to salt stress. The key factors in these processes are the enzymes that catalyse the metabolic reactions, among which SOD, POD, CAT, and APX are key antioxidant enzymes relevant to plant resistance[33]. For SOD, an important component, its main function is to disproportionate negative oxygen ions, produce H_2O_2 and O_2^-, and reduce the damage of ROS to the cells of a plant. POD and CAT are common oxidoreductase enzymes in plant and important endogenous ROS scavengers in a cell. This enables the plant cells to alleviate the oxidative damage of ROS to a certain extent. APX is an important enzyme involved in the ASA-GSH cycle, which catalyses the reaction between ASA and H_2O_2, scavenging excess H_2O_2 in plants[34]. In addition, ASA is an important non-enzymatic antioxidant, and a reactive oxygen scavenger produced during intracellular glucose metabolism. The degradation of H_2O_2 in plants is mainly accomplished by ASA, which also acts as a cofactor for some antioxidant enzymes, and feedback regulates antioxidant enzyme activities[34]. Therefore, maintaining a relatively stable level of ASA in plants under salt stress to maintain the normal physiological metabolism of cells and resist salt stress damage is critical.

Plants maintain the balance of ROS metabolism mainly by regulating the activities of the key aforementioned antioxidant enzymes antioxidants.This reduces the salt stress triggered is intracellular reactive oxygen radical accumulation and membrane lipid peroxidation injury triggered by saltstress. Improving the antioxidant capacity of plants is a key prerequisite

for plant growth in stressful environments[24]. Betaine plays an important role in antioxidant defence and ROS scavenging to protect plants from damage induced by oxidative stress, the finding was confirmed in studies on crops such as cotton (*Gossypium hirsutum* L.)[35] and lettuce (*Lactuca sativa*)[36]. Our results showed that NaCl stress significantly elevated MDA content and conductivity in roots and leaves, indicating that NaCl stress disrupted the integrity and stability of cell membranes. Application of 20 mM GB significantly enhanced SOD, POD, CAT, and PAX activities and ASA content in the antioxidant system of *G. uralensis.* This reduced the adverse effects of membrane lipid peroxidation, decreasing MDA and conductivity. Specifically, the application of 20 mM GB significantly increased SOD, POD, CAT, and PAX activities and ASA content in the root system by 59.55%, 413.07%, 225.91%, 300.00%, and 73.33%, respectively, compared with the NaCl-only treatment. MDA and conductivity were reduced by 27.62% and 60.23%, respectively ($P < 0.05$). The SOD, POD , CAT, and APX activities and ASA content of leaves were increased by 877.51%, 359.89%, 199.15%, 144.35%, and 108.11%, respectively, and their MDA content and conductivity were reduced by 45.20% and 59.30%, respectively, compared with the NaCl-treated group ($P < 0.05$). GB does not function as an antioxidant and cannot directly kerb ROS accumulation, but rather indirectly by activating the antioxidant enzyme system[37]. For example, Wang et al. (2016) found that exogenous GB produced more antioxidant enzymes in response to cold stress by promoting the transcription of POD, CAT, APX, GR (glutathione reductase), and LOX (lipoxygenase) in a cold-damaged treatment of chilli pepper (*Capsicum annuum* L. cv. Mutianqiushuo)[38]. Cisse, by contrast, demonstrated that exogenous GB increased the activities of SOD, POD, CAT, and APX in *Dalbergia odorifera* leaves under salt stress conditions. Nomura (1998) suggested that GB acts as a chaperone molecule that binds to enzyme proteins and enhances enzyme protein conformation in response to adversity stress in plants, further demonstrating that exogenous GB has a positive physiological regulatory effect on enhancing salt tolerance in plants[39].

Halophytes often have evolved a series of unique salt tolerance mechanisms to adapt to saline soil habitats. Majority of salt-secreting plants have salt-secreting structures, such as salt glands or salt vesicles, distributed on the stems and leaves, which are conducive to the organism's ability to excrete excess salts from the body. For example, *Limonium bicolor* L., a salt-

secreting saline plant of the Plumbaginaceae, showed a positive correlation between the rate of salt gland secretion and soil salt concentration. The rate of Na^+ secretion by salt glands in the leaves of *Limonium bicolor* seedlings reached a maximum under 400 mM NaCl stress[40]. In addition to its salt glands, the stomata of *G. uralensis* leaves also function as salt secretion[19]. These were also observed by scanning electron microscopy in this study. Using atomic absorption split-flame spectrophotometry to determine the content of ions on the surface of *G. uralensis* leaves, we found that the rate of leaf salt-secreting was significantly higher than that of the control group under NaCl stress, which implies that *G. uralensis* can excrete excessive salts from its body to alleviate the salinity-damaging effect under salt stress. The regulation the rate of salt secretion by exogenous substances in saline plants has become a key research focus. For example, Li (2020) used a Nikon fluorescence microscope and flame spectrophotometer to observe the morphology of salt glands of *Limonium bicolor* L. and measure the rate of Na^+ excretion and found that the addition of exogenous melatonin (MT) significantly increased the number of salt glands on its leaves and the radius of salt glands and promoted the rate of Na^+ excretion from salt glands, which effectively improved its salt tolerance[41].

In addition, Wei (2022) found that exogenous hydrogen sulfide (H_2S) treatment increased the rate of Na^+ excretion from the salt glands of the mangrove plant *Avicennia marina* (Forsk) under salt stress conditions and found that H_2S promoted Na^+ secretion from the salt glands through the upregulation of Na^+/H^+ antagonists and H^+-ATPase in the salt glands' plasma membranes and chromatophores as detected by qRT-PCR[42]. In our experiment, we observed the same phenomenon as aforementioned, with a significant increase in salt secretion around the salt glands and stomata of GB-treated *G. uralensis*. This may be attributed to the regulatory effect of exogenous GB on stomatal opening and closing[34]. When leaf leachate was examined using the atomic absorption flame spectrophotometer method, we found that the content of Na^+, K^+, and Ca^{2+} in the GB-treated group were significantly higher than that in the salt stress treatment, especially after the application of 20 mM GB. The rate of leaf secretion of Na^+ was 7.93 times higher than that of the NaCl-only treatment, which implies that the exogenous GB reduces the Na^+ accumulation in the plant by accelerating the rate of leaf secretion.

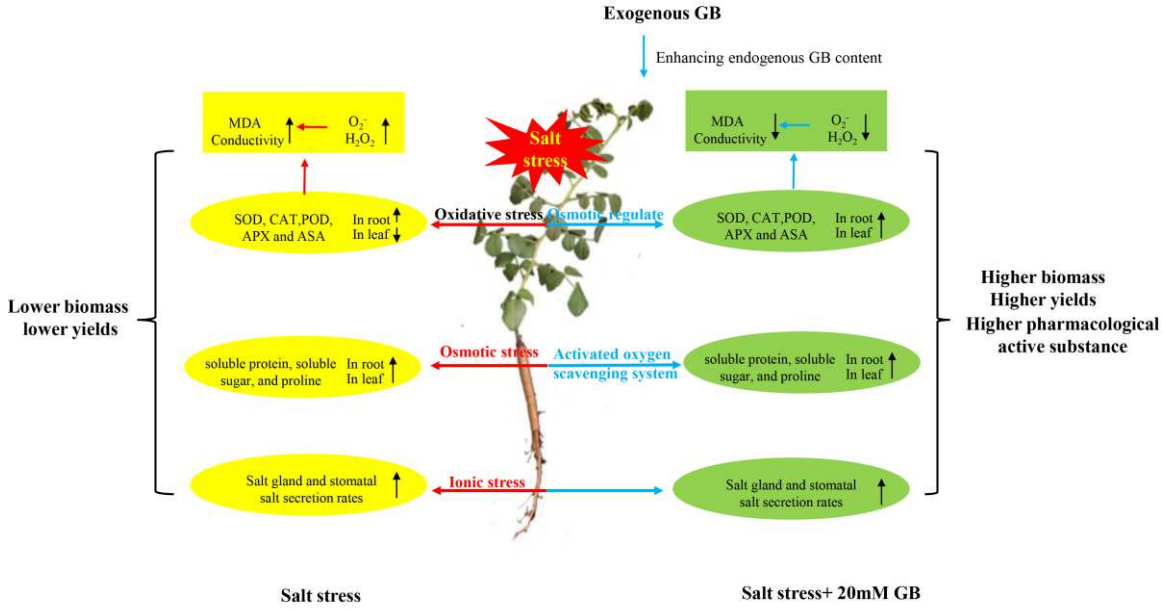


Fig.10 Summary of exogenous betaine on salt damage mitigation in *G. uralensis* under salt stress conditions

Conclusion

In summary, to determine how exogenous GB mitigates the adverse effects of salt stress on *G. uralensis* seedlings growths, we summarised the potential mechanisms of its action. In this study, NaCl stress reduced the biomass of *G. uralensis*. Exogenous GB increased the biomass accumulation of *G. uralensis* and enhanced the salt tolerance of *G. uralensis* seedlings by increasing the antioxidant enzyme activity, osmoregulatory capacity, and leaf salt secretion capacity (Fig. 10). Under this experimental conditions, the 20 mM GB treatment had the strongest positive effect on the growth of *G. uralensis* seedlings and the enhancement of herb yield and quality. This finding provides a theoretical basis for exogenous GB to alleviate NaCl stress in *G. uralensis* and a scientific basis for the development of high-yield and high-quality cultivation technology of liquorice herbs by using salinized soil.

Abbreviations

PH: plant height; RB: root biomass; SB: stem biomass; LB: leaf biomass; TRL: total root length; TRSA: total root surface area; TRV:total root volume; RGB: root GB content; SGB: stem GB content; LGB: leaf GB content; $RBADH_2$: root betaine aldehyde dehydrogenase; $SBADH_2$:stem betaine aldehyde dehydrogenase; $LBADH_2$: leaf betaine aldehyde dehydrogenase;

RSP: root soluble protein; LSP: leaf soluble protein; RSS:root soluble sugar; LSS: leaf soluble sugar; RP: root proline; LP: leaf proline; RH_2O_2: root hydrogen peroxide; LH_2O_2:leaf hydrogen peroxide; RO_2^-:root superoxide anion; LO_2^-: superoxide anion; RO_2^-PA:root superoxide anion production rate; LO_2^-PA:leaf superoxide anion production rate; RMDA:root malondialdehyde; LMDA:leaf malondialdehyde; RC:root conductivity; LC:leaf conductivity; RSOD:root superoxide dismutase; LSOD:leaf superoxide dismutase; RCAT:root catalase; LCAT:leaf catalase; RPOD:root peroxidase; LPOD:leaf peroxidase; RAPX:root ascorbate peroxidase; LAPX:leaf ascorbate peroxidase; RASA:root ascorbic acid; LASA:leaf ascorbic acid; K^+:potassium ion secretion; Na^+:sodium ion; Ca^{2+}:calcium ion.

Declarations

Ethics approval and consent to participate

The authors declare that the collection of plant material comply with relevant institutional, national, and international guidelines and legislation.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Author's Contributions

M M conceived and designed the research. XPD and XMM conducted all experiments, analyzed the data, interpreted the results, and wrote the manuscript. ZLZ provided assistance in data analysis.The authors have approved of publication, and there is no confict of interest. All the authors equally approve of publication.

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Consent for publication

Not applicable.

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