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RESEARCH PAPERS

Rhizospheric Bicarbonate Improves Glucose Metabolism and Stress Tolerance of *Broussonetia papyrifera* L. Seedlings under Simulated Drought Stress

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Abstract—Heavy concentrations of bicarbonates present throughout karst ecosystems necessitate identification of the role of HCO_3^- in plant growth, especially when under drought stress. We aim to elucidate effect of different concentrations of HCO_3^- on paper mulberry (*Broussonetia papyrifera* L.) seedlings growth under drought stress. Effects of four bicarbonate concentrations (0, 3, 6, and 9 mM) with simulated mild drought stress on growth, glucose metabolism, photosynthetic traits, self-protective abilities and extent of cell damage were examined. Compared with other treatments, plants under 3 mM bicarbonate with simulated mild drought stress showed the best osmotic adjustment and reactive oxygen species scavenging ability, the highest total activity of glucose catabolism enzymes and glucose-6-phosphate dehydrogenate activity, and the highest growth rate. Specific concentration of HCO_3^- reduce negative influence in *Broussonetia papyrifera* L. under drought stress. Due to both producing more metabolic substrate and activating pentose phosphate pathway, those plants showed stronger resistance, therefore might suffer less harm and retain a better growth status under mild drought stress. This suggests that when specific plants in a karst environment are suffering drought stress, specific concentration of HCO_3^- benefit to their growth.

Keywords: Broussonetia papyrifera, bicarbonate, drought, glucose metabolism, karst, stress tolerance

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INTRODUCTION

As open geochemical systems are located on and near the Earth's surface, karst systems are closely related to the atmosphere, hydrosphere, and biosphere, and their unique geological structure and soil composition affect plant growth. Due to the corrosion of limestone and dolomite in water, stable HCO_3^- sinks form in the soil and water of karst regions, and concentrations of HCO_3^- are significantly higher than in non-karst areas. Bicarbonate radical is usually available in concentrations several-fold higher than that of CO_2 at a pH above 7 [1] According to previous studies, bicarbonate concentrations are usually approximately 4.5 mM in karst rivers and lakes [2] and usually in the range between 1 and 5 mM in calcareous soils [3]. Simultaneously, the prevalence of bare rock, shallow and patchy soils, highly porous limestone bedrock, and complex underground drainage systems means that drought occurs frequently in karstic regions. With sufficient rainfall, the soil field capacity can maintain plant growth for as little as 7 to 14 days in karst regions [4].

Under stress conditions, plants alter their glucose metabolic pathway to adapt to environmental changes and maintain physiological vitality. These changes are closely related to the regulation of the pentose phosphate (PPP) and glycolytic pathways (EMP). The pentose phosphate and glycolytic pathways are central to the metabolic system of organisms. Previous studies have shown that in the presence of abiotic stress, glu-

Abbreviations: DIC—soluble inorganic carbon; DPPH—2,2-diphenyl-1-picrylhydrazyl; EMP—glycolytic pathways; G6PDH—glucose-6-phosphate dehydrogenate; g_s—stomatal conductance; PFK—phosphofructokinase; 6PGDH—6-phosphogluconate dehydrogenase; P_n—net photosynthesis rate; PPP—pentose phosphate pathway; RuBP—ribulose-1,5-disphosphate; RuMP—ribulose-5-phosphate; TBARS—thiobarbituric acid reactive substance.

cose metabolism is shifted from the glycolytic pathway to the pentose phosphate pathway [5]. Stimulation of the pentose phosphate pathway enhances the production of NADPH to support antioxidant enzyme activity and the regeneration of ribulose-5-phosphate (RuMP) to maintain the photosynthetic capacity of plants.

Unlike drought stress, bicarbonate generally inhibits plant growth in multiple ways: (1) plants are subject to salt stress and pH stress simultaneously with high levels of HCO_3^-. (2) HCO_3^- influences the absorption and utilisation of Fe, Zn, Mn, Ca, and K. (3) HCO_3^- plays an important role in the electron transmission process of the PSII complex [6]. (4) Low concentrations of HCO_3^- promote stomatal opening, but high concentrations of HCO_3^- lead to stomatal closure [7]. (5) More importantly, HCO_3^- is a source of carbon substrate for carbon assimilation by terrestrial plants [8]. In particular, upon stomatal closure, HCO_3^- often makes up a large proportion of the carbon assimilated by plants [9]. The proportion of CO_2 supplied from root-derived soluble inorganic carbon (DIC) accounted for approximately 20% of the total C gain observed when plants were under drought stress [10].

As mentioned above, extensive research has demonstrated that the regulation of the pentose phosphate pathway plays an important role in plant tolerance to drought stress, and furthermore, it is also known that rhizospheric bicarbonate can be used as a carbon source to alleviate the threat of carbon starvation in plants under drought stress. Although much is known about bicarbonate, which provides a substrate for photosynthesis and has positive effects on biomass, there is little known about the special role of HCO_3^- in plant growth when it coexists with drought stress in karst regions. Therefore, in this study, we aimed to elucidate the effect of different concentrations of HCO_3^- on plants under drought stress by determining growth parameters, photosynthetic capacity, antioxidant capacity and disproportionation of the glucose metabolic pathway in paper mulberry. We will answer the following two questions: 1) under drought stress, what role does rhizospheric bicarbonate play in the regulatory processes of sugar metabolism in plants, and 2) thus, what effect occurs on plant drought tolerance and growth. We will focus on the differences in the regulation of plant sugar metabolism, drought tolerance and growth resulting from different concentrations of bicarbonate treatments under drought stress.

Paper mulberry (*Broussonetia papyrifera* L.), a deciduous perennial woody species belonging to the family Moraceae, is widely distributed in eastern Asia. It is a type of calciphyte that is common in secondary karst forests growing at moderate elevations. Paper mulberry is highly tolerant to drought and tolerant to salt stress, can grow in acidic or neutral soils and be widely used in greening and rocky desertification control. The bark of the paper mulberry contains abundant fibre that can be used for making paper and cloth and its leaves are enriched with crude proteins, which makes it an ideal woody forage [11]. This paper is the first to explore the effects of bicarbonate concentrations on glucose metabolism and stress tolerance under drought stress in paper mulberry.

MATERIALS AND METHODS

Experimental design. Seeds of paper mulberry were obtained from the Guizhou Academy of Agricultural Sciences, China. Seedlings were sown directly in perlite and germinated at 25°C in a greenhouse. After germination, all seedlings were transferred to 1/2 Hoagland nutrient solution. The environmental conditions for germination were as follows: a 12-h photoperiod with an average day/night temperature of 25/20°C and a photosynthetic photon flux density of 300 μmol/(m^2 s) with 65% relative humidity. Plant height (H), basal diameter (D_b), number of leaves (N), and number of blades more than 70 mm wide (N_{70}) were selected as growth indices. Six-week-old plants were used for the following treatments: (i) addition of 50 g/L PEG 6000 in 1/2 Hoagland nutrient solution to simulate mild drought stress. (ii) Bicarbonate treatment was performed by adding one of four levels of bicarbonate [0 (T_1), 3 (T_2), 6 (T_3), or 9 (T_4) mM as $NaHCO_3$] to simulate mild drought stress, and a control solution was also used. To simulate the alkaline soil environment in the karst region, the solution pH was adjusted to 7.8 in all treatments by the addition of NaOH, and solutions were replaced every day after the imposition of stress treatments. Following treatment, the leaves approximately 70 mm wide were sampled once every three days (a total of four times), frozen in liquid nitrogen, and stored at −80°C until further analysis was performed, and plants were measured at the beginning and end of the10 d.

Radical-scavenging activity assay. The assay procedure was modified from that described by Kontogiorgis and Hadjipavlou-Litina [12]. One gram of plant material was ground with a mortar and pestle with 80% methanol. Samples were centrifuged for 10 min at 3500 *g* at 4°C, and the supernatant was kept at 4°C until analysis. The test tubes contained 0.1 mL of supernatant and 4.9 mL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) dissolved in 80% methanol. The mixture was incubated for 15 min in the dark at 20–22°C. Then, the OD of the solution was measured at 517 nm. DPPH radical-scavenging activity was calculated using the following formula:

$$\mathrm{DPPH\%} = \left(\mathrm{OD_{blank}} - \mathrm{OD_{sample}}\right)/\mathrm{OD_{blank}} \times 100, \quad (1)$$

where OD_{blank} and OD_{sample} are the absorbance values of the blank and sample, respectively.

Thiobarbituric acid reactive substance (TBARS) determination. Lipid peroxidation was determined by measuring the concentration of TBARS accumulation according to the method of Heath and Packer [13] with some modifications. Fresh plant material (0.1 g) was homogenised in 1 mL 0.1% (w/v) trichloroacetic acid and centrifuged at 10000 *g* for 20 min. Next, 0.5 mL of the supernatant was added to 1 mL of 0.5% (w/v) TBA in 20% TCA and heated in a water bath at 98°C for 30 min. The mixture was then quickly cooled by an ice bath for 10 min and centrifuged at 10000 *g* for 5 min again. To reduce interference, the absorbance of the supernatant was measured at 450, 532, and 600 nm. The lipid peroxide content was measured as nmol TBARS/g dry wt. The TBARS content was calculated using the following formula:

$$\text{Concentration of TBARS} = 6.45(A_{532} - A_{600}) - 0.56A_{450}. \quad (2)$$

Proline and soluble sugar content measurements. The proline content was assayed as described earlier [14]. Proline was extracted from 0.1 g of fresh leaves with 3 mL of methanol : chloroform : water (12 : 5 : 1, v/v). After centrifugation at 10000 *g* for 5 min, the supernatant was used for proline estimation. One millilitre of supernatant was transferred to a test tube and heated in a water bath until methanol evaporation, after which 0.33 mL ninhydrin solution, 0.33 mL of glacial acetic acid, and 0.33 mL of water was added to the sample. The tubes were then cooled to room temperature, and 2 mL of toluene was added. After 30 s of shaking, the two phases were separated, and the absorbance of the upper phase was measured at 520 nm. Soluble sugar was measured by the anthrone colorimetric method according to Zou [15]. Briefly, 0.1 g leaves were extracted with 5 mL distilled water two times (100°C, 30 min each). A volume of 0.5 mL of the extract was added to 1.5 mL distilled water and 1 mL 9% phenol and incubated for 20 min at 25°C. Then, 5 mL H_2SO_4 was added to the mixture and incubated for 30 min at 25°C. A 2 mL mixture was measured for its absorption at 485 nm.

Stomatal conductance (g_s), and net photosynthesis rate (P_n) measurements. The g_s and P_n values were measured using a Li-6400 portable photosynthesis measurement system (LI-COR, United States).

Plant extract preparation. Enzymes were extracted according to the method described by Liu et al. [5] with some modifications. Briefly, 0.1 g of leaf tissue was ground in liquid nitrogen and 1 mL of extraction buffer containing 50 mM Hepes-Tris (pH 7. 8), 3 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The homogenate was centrifuged at 12000 *g* for 20 min at 4°C.

Glucose-6-phosphate dehydrogenate (G6PDH, EC 1.1.1.49) activity assays. G6PDH activity was assayed as described by Tian et al. [16] with some modifications: 200 μL aliquots of extract were added to either 1.8 mL of the total dehydrogenase [G6PDH + 6-phosphogluconate dehydrogenase (6PGDH)] assay buffer containing 50 mM Hepes-Tris (pH 7.8), 3.3 mM $MgCl_2$, 0.5 mM D-glucose-6-phosphate disodium salt, 0.5 mM 6-phosphogluconate, and 0.5 mM NADP-Na_2, or 1.8 mL of the 6PGDH assay buffer, containing 50 mM Hepes-Tris (pH 7.8), 3.3 mM $MgCl_2$, 0.5 mM 6-phosphogluconate, and 0.5 mM NADP-Na_2. The reduction of $NADP^+$ to NADPH was measured as the rate of change in the absorbance at 340 nm during the initial 5 min at 25°C. The G6PDH activity was calculated as the total dehydrogenase activity with the 6PGDH activity subtracted.

Phosphofructokinase (PFK, EC 2.7.1.11) activity assays. ATP-PFK and PPi-PFK were assayed as described by Carnal and Black [17] with some modifications: 200 μL aliquot of the extract was added to 1.8 mL of assay buffer containing 50 mM Hepes-Tris (pH 7.8), 2.5 mM $MgCl_2$, 0.1 mM nicotinamide adenine dinucleotide, 5 mM F-6-P, 2 units/mL aldolase, 1 unit/mL triosephosphate isomerase, 2 units/mL α-glycerol-3-phosphate dehydrogenase and either 1 mM ATP or 1 mM PPi. The oxidation of NADH to NAD^+ was measured as the rate of change in the absorbance at 340 nm during the initial 5 min at 25°C. PFK activity was equal to ATP-PFK activity plus PPi-PFK activity.

Total activity of glucose catabolism and proportion of PPP. The glycolytic and pentose phosphate pathways are the two dominant processes of glucose catabolism. Approximately 75% of glucose is metabolised via the glycolytic pathway, and 15 to 30% of the hexose phosphate oxidised is processed by the pentose phosphate pathway in the plant cell under normal growth conditions [18, 19]. Therefore, the total activity of glucose catabolism may be simply represented by the PFK activity plus G6PDH activity, and the total activity of glucose catabolism was calculated as

$$G_c = A_{pfk} + A_{g6pdh}, \quad (3)$$

where G_c is the total activity of glucose catabolism, and A_{pfk} and A_{g6pdh} are the activities of PFK and G6PDH, respectively.

Furthermore, PFK activity and G6PDH activity are expressed by the rates of change in the contents of NADH and NADPH, respectively. The output and measurement of NADH and NADPH are highly consistent: (1) both measure the absorption change at 340 nm with a spectrophotometer, and both extinction coefficients are 6.22 mM^{-1} cm^{-1}. (2) One unit of glucose produces two units of NADH through the glycolytic pathway or produces two units of NADPH through the pentose phosphate pathway. Therefore, the ratio of PFK activity to G6PDH activity can represent the proportion of glucose metabolism through

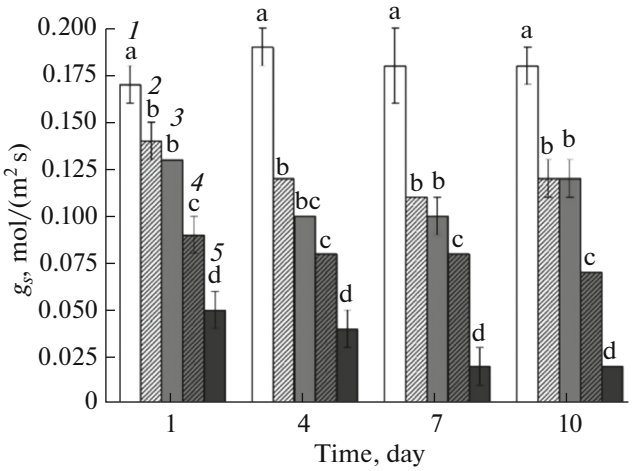

Fig. 1. The g_s of paper mulberry leaves under control, T_1, T_2, T_3 and T_4 for 1, 4, 7, and 10 d. (*1*) 1/2 Hoagland nutrient solution (Control); (*2*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 (T_1); (*3*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 +3 mM $NaHCO_3$ (T_2); (*4*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 6 mM $NaHCO_3$ (T_3); (*5*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 9 mM $NaHCO_3$ (T_4). The values are shown as mean ± SD, $n = 3$. The mean values marked with different letters significantly differ at $P < 0.05$.

the glycolytic and pentose phosphate pathways, and the proportion of PPP was calculated as

$$P_{g6pdh} = A_{g6pdh}/(A_{pfk} + A_{g6pdh}) \times 100\%, \quad (4)$$

where P_{g6pdh} is the proportion of glucose metabolism through PPP.

Rubisco (EC 4.1.1.39) activity assays. Rubisco activity was assayed as described by Sato et al. [20] with some modifications: 200 μL aliquots of the extract were added to 1.8 mL of assay buffer containing 100 mM Tris–bicine (pH 8.0), 10 mM $MgCl_2$, 0.2 mM EDTA, 5 mM dithiothreitol, 40 mM $NaHCO_3$, 4 mM ATP, 0.2 mM NADH, 0.2 mM ribulose-1,5-disphosphate (RuBP), and one enzyme unit of 3-phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase. The reaction was initiated by adding 0.2 mM RuBP, and all activity was assayed spectrophotometrically at 340 nm for 5 min at 25°C. Enzyme activities were expressed as μmol/g h.

Statistical analysis of the data. For each measurement, three replicates were used. Data are presented as the mean ± SD. Statistical analysis was performed using ORIGIN 9.0 software (OriginLab Inc., United States). Differences were considered significant when the *p* value was less than 0.05.

RESULTS

Stomatal Conductance

As shown in Fig. 1, the values of g_s under T_1, T_2, T_3, and T_4 treatments were all lower than the values under the control treatment in paper mulberry leaves. The value of g_s under T_2 treatment was almost equal to that under T_1 treatment but significantly higher than that under T_3 treatment and T_4 treatment.

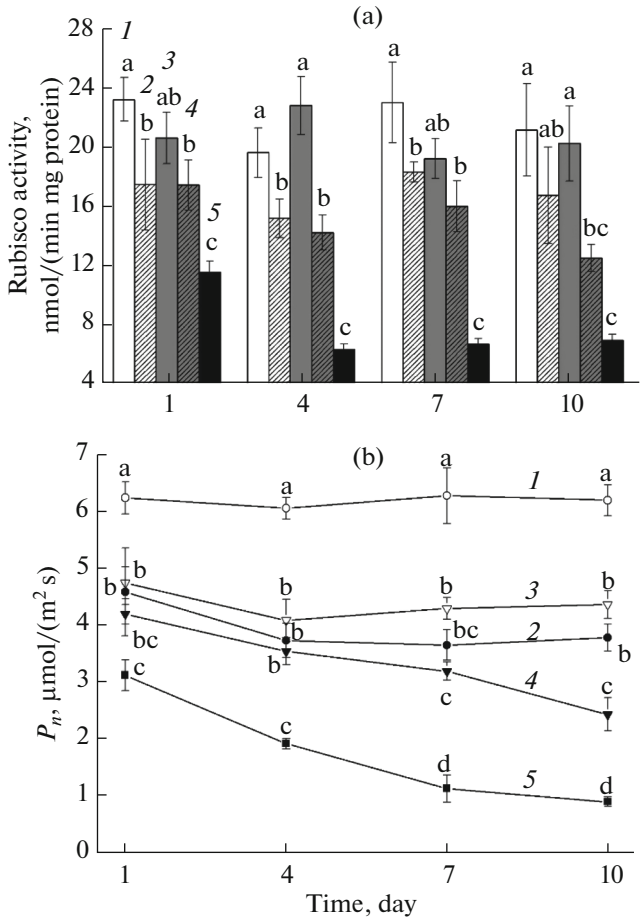

Fig. 2. The Rubisco activity (a) in paper mulberry leaves and P_n (b) of paper mulberry leaves under control, T_1, T_2, T_3 and T_4 for 1, 4, 7, and 10 d. (*1*) 1/2 Hoagland nutrient solution (Control); (*2*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 (T_1); (*3*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 +3 mM $NaHCO_3$ (T_2); (*4*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 6 mM $NaHCO_3$ (T_3); (*5*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 9 mM $NaHCO_3$ (T_4). The values are shown as mean ± SD, $n = 3$. The mean values marked with different letters significantly differ at $P < 0.05$.

Rubisco Activity and Net Photosynthesis Rate

As shown in Fig. 2a, the Rubisco activities under the T_1 treatment and T_2 treatments were stable. The Rubisco activities under the T_2 treatment were similar to those under the control treatment but were slightly higher than the Rubisco activity under the T_1 treatment. Under the T_3 treatment, the Rubisco activities were almost equal to those under the T_1 treatment at 1 d, and then, they decreased slightly to 59% of that under the

control treatment. The Rubisco activities under the T_4 treatment were at a minimum among all treatments assessed, at 50% of its activity under the control treatment at 1 d, then it decreased to 31% of its activity under control treatment and thereafter, was stable.

As shown in Fig. 2b, the values of net photosynthesis rate (P_n) under T_1, T_2, T_3, and T_4 treatments were all lower than the values under the control treatment in paper mulberry leaves. After 10 days of treatment, the P_n order of all treatments was $T_4 < T_3 < T_1 < T_2 <$ control.

The Status of Glucose Metabolism

As shown in Fig. 3a, G6PDH activities under the T_1, T_2, and T_3 treatments were all higher than the activity under the control treatment, and they were stable. The G6PDH activities under the T_1 and T_2 treatments were roughly equal but slightly higher than the G6PDH activity under the T_3 treatment. The G6PDH activity under the T_4 treatment was 271% of that under the control treatment at 1 d, and it then dropped rapidly over time; at 10 d, its activity was even lower than its activity under the control treatment. As shown in Fig. 3b, PFK activities under the T_1, T_2, T_3, and T_4 treatments were all significantly lower than its activity under the control treatment in paper mulberry leaves. Under the T_1, T_2 and T_3 treatments, the PFK activities were stable, and the PFK activities thereof were ranked as $T_3 < T_1 < T_2$. Under the T_4 treatment, the PFK activity continued to decrease until 10 d, reaching approximately 18% of its activity under the control treatment.

As shown in Figs. 3c, 3d, under the T_1 treatment, the total activity of glucose catabolism was lower than that of the control group and 88% of its activity under the control treatment at 10 d. Under the T_2 treatment, the total activity of glucose catabolism was increased and then became stable at approximately 127% of its activity under the control treatment. Under the T_3 treatment, the total activity of glucose catabolism was almost equal to its activity under the control treatment. Under the T_4 treatment, the total activity of glucose catabolism was first increased and then decreased sharply and was only 26% of that under the control treatment at 10 d. As shown in Figs. 3d, 3e, under the control, T_1, T_2 and T_3 treatments, the proportion of glucose metabolism through PPP was stable at approximately 33, 48, 57, and 63% of the total amount of glucose catabolism, respectively. Under the T_4 treatments, the proportion of glucose metabolism through PPP was increased to approximately 75% of the total amount of glucose catabolism in first 7 days, but after 7 days of treatment, the proportion of glucose metabolism through PPP decreased sharply and was only 44% of the total amount of glucose catabolism at 10 d.

Osmolytes Contents

Under the T_1 treatment, the proline content was approximately 160% of its content under control treatment and was shown to be stable after 4 d. Under T_2 treatment, the proline content increased gradually and reached approximately 190% of its content under control treatment at 10 d. Under the T_3 and T_4 treatments, the proline contents decreased after initially increasing, both of them reaching maxima at 4 d, 266 and 286% of those under the control treatment, respectively. The proline content under the T_4 treatment decreased significantly and was only 93% of that under the control treatment at 10 d (Fig. 4a).

As shown in Fig. 4b, under the T_1 treatment and T_2 treatments, the soluble sugar content was approximately 120% of its content under the control treatment, which was shown to be stable. Under the T_3 treatment, the soluble sugar content was shown at 1 d to reach its maximum and was 146% of its content under the control treatment, after which the content decreased gradually; at 10 d, its content was 83% of that under the control treatment. Under the T_4 treatment, at 1, 4, 7, and 10 d, the soluble sugar content was 161, 133, 95, and 47% of its content under the control treatment, respectively.

DPPH Radical-Scavenging Activity and TBARS Content

The scavenging ability of methanol extract from paper mulberry leaves was estimated using DPPH. As shown in Fig. 5a, under the T_1 and T_2 treatments, DPPH radical-scavenging activity was increased and then became stable at approximately 123 and 154% of its activity under the control treatment, respectively. Under the T_3 treatment, DPPH radical-scavenging activity peaked at 4 d, at 135% of its activity under the control treatment; thereafter, the activity decreased gradually over time and was at only 66% of its activity under the control treatment at 10 d. Under the T_4 treatment, DPPH radical-scavenging activity peaked at 1 d, reaching 152% of the activity under the control treatment. Thereafter, the DPPH radical-scavenging activity decreased sharply; at 10 d, its activity was even below that under the control treatment.

To determine the peroxidation of lipids, the concentration of TBARS accumulation was measured. As shown in Fig. 5b, under the T_1, T_2 and T_4 treatments, the TBARS contents were approximately 127, 117, and 215% of that under the control treatment, respectively, and these were shown to be stable after 4 d. Under the T_3 treatment, the TBARS content was approximately 187% of that under control treatment and was shown to be stable after 7 d.

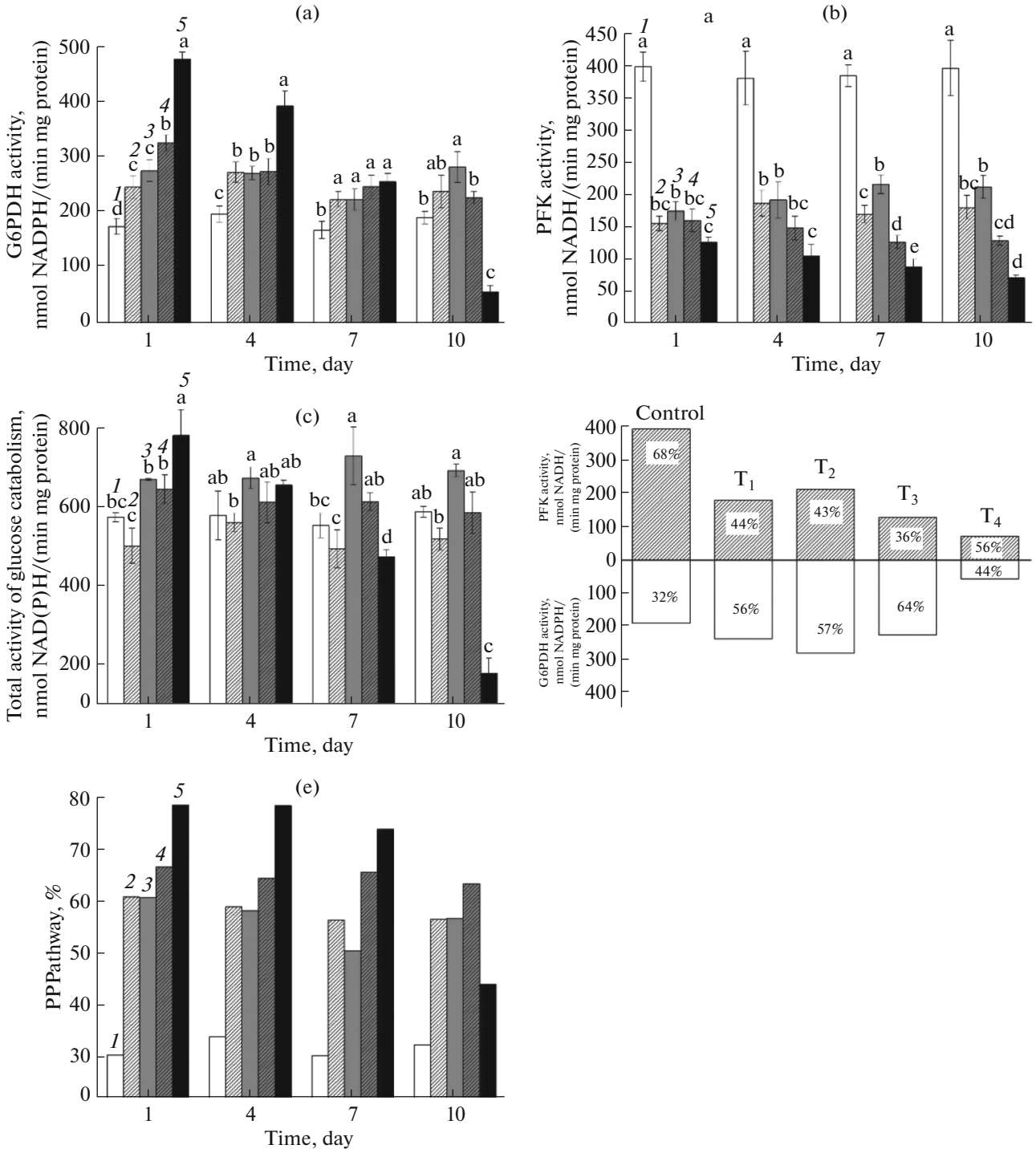

Fig. 3. The G6PDH activity (a), PFK activity (b), total activity of glucose catabolism (c), the activities of enzymes in glucose catabolism after 10 days treatments (d) and proportion of PPP in glucose catabolism (e) under control, T_1, T_2, T_3 and T_4 for 1, 4, 7, and 10 d in paper mulberry leaves. (*1*) 1/2 Hoagland nutrient solution (Control); (*2*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 (T_1); (*3*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 3 mM $NaHCO_3$ (T_2); (*4*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 6 mM $NaHCO_3$ (T_3); (*5*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 9 mM $NaHCO_3$ (T_4). The values are shown as mean ± SD, $n = 3$. The mean values marked with different letters significantly differ at $P < 0.05$.

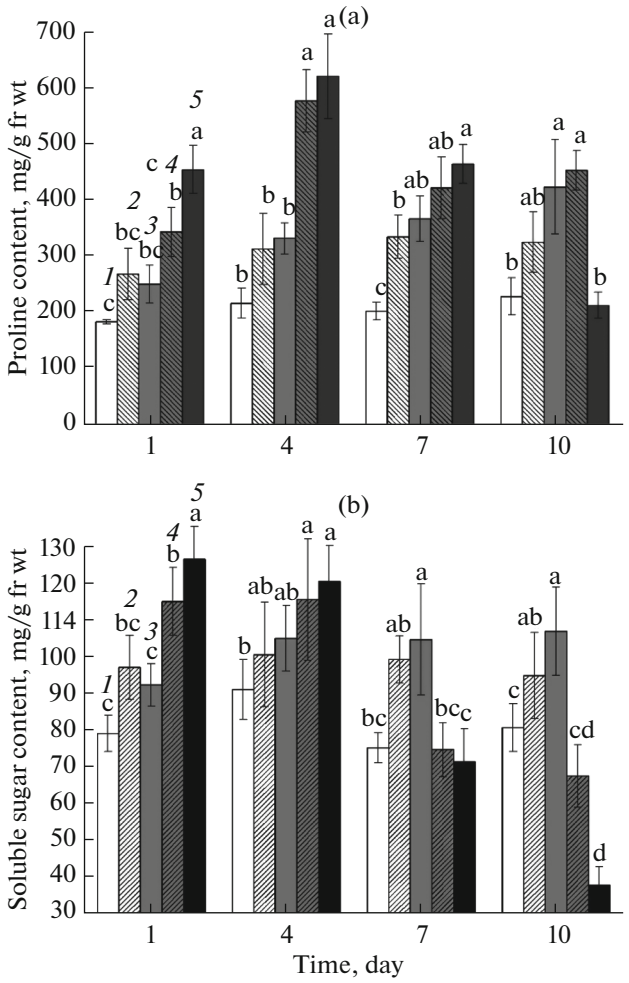

Fig. 4. The proline content (a) and soluble sugar content (b) in paper mulberry leaves under control, T_1, T_2, T_3 and T_4 for 1, 4, 7, and 10 d. (*1*) 1/2 Hoagland nutrient solution (Control); (*2*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 (T_1); (*3*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 3 mM $NaHCO_3$ (T_2); (*4*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 6 mM $NaHCO_3$ (T_3); (*5*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 9 mM $NaHCO_3$ (T_4). The values are shown as mean ± SD, $n = 3$. The mean values marked with different letters significantly differ at $P < 0.05$.

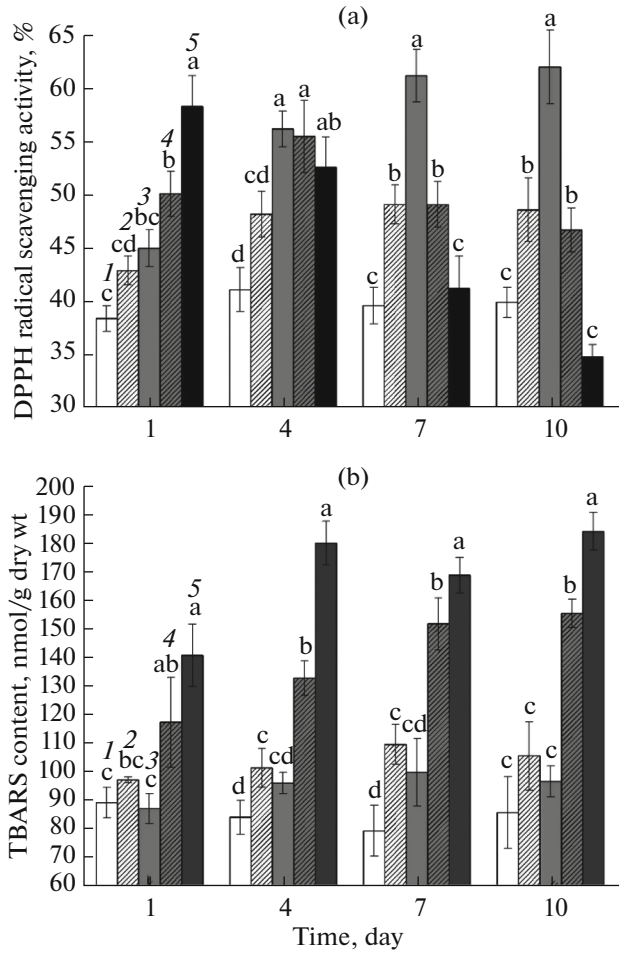

Fig. 5. DPPH radical-scavenging activity (a) and TBARS content (b) under control, T_1, T_2, T_3 and T_4 for 1, 4, 7, and 10 d in paper mulberry leaves. (*1*) 1/2 Hoagland nutrient solution (Control); (*2*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 (T_1); (*3*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 3 mM $NaHCO_3$ (T_2); (*4*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 6 mM $NaHCO_3$ (T_3); (*5*) 1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 9 mM $NaHCO_3$ (T_4). The values are shown as mean ± SD, $n = 3$. The mean values marked with different letters significantly differ at $P < 0.05$.

Growth Parameters

As shown in Table 1, after 10 days of treatment, the plants grew best under the T_2 treatment except when the plants grew under the control treatment. Plant growth under the T_3 treatment was inhibited compared with the plants grown under the T_1 treatment. The T_4 treatment significantly inhibited plant growth and nearly ceased plant growth.

DISCUSSION

In karst regions, plants often encounter drought, and higher concentrations of bicarbonate exert significant influence on the growth and development of plants. Because there are large amounts of limestone ($CaCO_3$) and dolostone [$CaMg(CO_3)_2$] [21], a stable HCO_3^- sink forms in the soils of karst regions.

Plants adapt to various stresses through metabolic regulation at the transcriptional and post-translational levels, and the pentose phosphate pathway is an integral part of a plant's defence system under stress conditions. G6PDH is the key regulatory enzyme of OPPP, which controls the flow of carbon and produces NADPH. All treatments exerted activating effects on G6PDH and had an inhibitory effect on PFK activity in paper mulberry leaves (Figs. 3a, 3b). These results reflect that carbon flux diverted from the catabolic glycolytic pathway to the anabolic pentose

Table 1. Growth indices of plants after 10 days of treatments

	Control	T_1	T_2	T_3	T_4
ΔH, cm	6.41 ± 0.59^{a}	4.75 ± 0.36^{b}	5.53 ± 0.81^{ab}	2.97 ± 0.49^{c}	1.20 ± 0.29^{d}
ΔD_b, mm	0.67 ± 0.09^{ab}	0.77 ± 0.04^{a}	0.71 ± 0.07^{a}	0.51 ± 0.08^{b}	0.33 ± 0.03^{c}
ΔN	3.67 ± 0.58^{a}	3.33 ± 0.58^{a}	3.67 ± 0.37^{a}	2.00 ± 0.00^{b}	1.00 ± 0.00^{b}
ΔN_{70}	2.00 ± 0.00^{ab}	1.33 ± 0.58^{ab}	2.33 ± 0.58^{a}	1.67 ± 0.23^{ab}	0.67 ± 0.57^{b}

*Plant height (H), basal diameter (D_b), number of leaves (N), and number of blades more than 70 mm wide (N_{70}) were selected as growth indices, and the Δ means the increase of the above indices from the beginning of treatments to the end of treatments. The mean values marked with different letters significantly differ at $P < 0.05$.

phosphate pathway in the leaves of this plant under stress. The stimulation of the pentose phosphate pathway enhances the production of NADPH to support antioxidant enzyme activity and meet the cellular needs of reductive biosynthesis (*e.g.*, proline) [22] and enhances the production of RuMP to improve the regeneration ability of RuBP in the Calvin-Benson cycle. Furthermore, the G6P shunt can relieve excess light energy at PSI to reduce the significant damage to the PSI reaction centre proteins cause by excess light energy arriving at reduced PSI [23]. More than 50% of glucose is metabolised via the pentose phosphate pathway when a plant is exposed to biotic and abiotic stresses [18, 19]. This shows that the pentose phosphate pathway is important to plants as they respond to various environmental stresses.

However, it is noteworthy that different concentrations of HCO_3^- in the plant rhizosphere have different effects on the regulation of the plant sugar metabolic pathway under drought stress. The addition of $NaHCO_3$ in the environment not only provides additional carbon sources for plants but also imposes salt stress on plants. Different concentrations of HCO_3^- in the growth medium stimulated or inhibited the growth of plants, and this effect depended on species and experimental conditions [24]. The utilisation of bicarbonate by terrestrial plants involves the action of carbonic anhydrase, which is continuously activated under stress [25]. Therefore, elucidation of the mechanism of bicarbonate effects on plant growth is important to understand plant adaptation to karst environments. The results showed that when a low concentration (3 mM) of HCO_3^- was added to the rhizosphere, the g_s (Fig. 1) did not decrease significantly in plant leaves compared with that under drought treatment. Previous studies have shown that low concentrations of bicarbonate can stimulate stomatal opening, while higher concentrations of bicarbonate force stomatal closure [26]. This may indicate that salt stress produced by 3 mM $NaHCO_3$ treatment had no significant negative impact on plants. Stomatal closure is the main factor inducing a decline in the plant photosynthetic rate in the early stage of drought stress, but non-stomatal limitation plays a major role in the middle and later stages. The insufficient regeneration of RuBP [27] and decline of Rubisco activity [28] both cause carboxylation efficiency to decrease in plant cells. Rubisco is the most important enzyme for carbon assimilation in C_3 plants and the catalysis of the reaction of RuBP with atmospheric CO_2. The activity of Rubisco reflects the photosynthetic capacity of C_3 plants to some extent. In this experiment, under the T_2 treatment and the control, plants had almost identical Rubisco activities in paper mulberry leaves and these Rubisco activities were significantly higher than those plant leaves under T_1 treatment (Fig. 2a). P_n under this treatment was also slightly higher than in the plant leaves under T_1 treatment (remarkably, owing to the measuring principle of Li6400, the P_n values here do not reflect the ability of plants to obtain CO_2 from roots for photosynthesis, so the actual net photosynthetic rate of plants should be higher than the P_n measured by Li-6400) (Fig. 2b). This indicates that the addition of carbon sources in the rhizosphere increased the photosynthetic capacity of plants and promoted the accumulation of metabolic substrates [soluble sugar (Fig. 4b)] and osmolytes [proline (Fig. 4a) and soluble sugar (Fig. 4b)] in plant. For the total sugar metabolism (Fig. 3c) of the plant increased, and PPP and EMP both strengthened. Enhanced PPP strengthens DPPH scavenging capacity (Fig. 5a) to increase the eliminated efficiency of antioxidant enzymes and restrain photosynthetic system damage by membrane-lipid peroxidation (Fig. 5b), increasing CO_2 receptor (RuBP) content to promote photosynthesis, thereby generating a virtuous cycle (Fig. 6). Enhanced glycolysis provided more ATP, NADH and other precursor metabolites for biomass production to meet the energy demands of metabolic activities. Therefore, the growth status of plants under T_2 treatment (Table 1) is better than that under simulated drought treatment alone, and the low concentration of $NaHCO_3$ in the rhizosphere of plants under drought stress mainly acts as a carbon source and has a positive effect on the plants.

On the other hand, when a high concentration (9 mM) of HCO_3^- was added to the rhizosphere, plants were subjected to strong salt stress. Higher osmotic

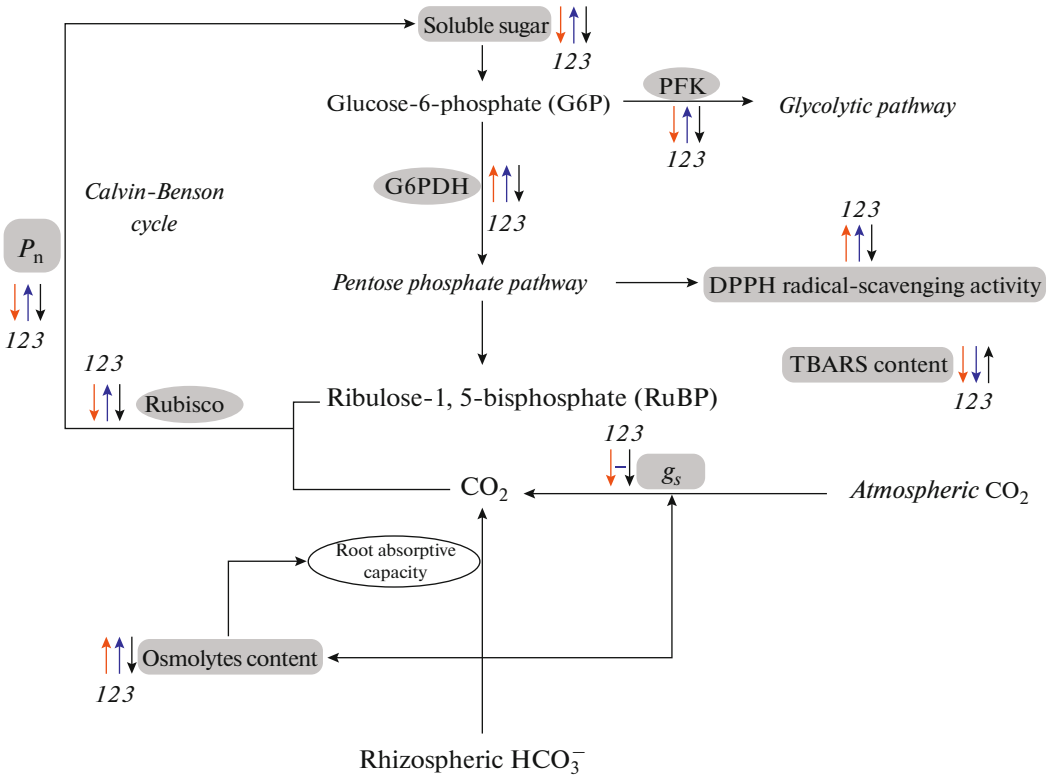

Fig. 6. Schematic representation of rhizospheric bicarbonate modulates glucose metabolism and stress tolerance of paper mulberry seedlings under simulated drought stress. Control—1/2 Hoagland nutrient solution; T_1—1/2 Hoagland nutrient solution + 50 g/L PEG 6000; T_2—1/2 Hoagland nutrient solution + 50 g/L PEG 6000 +3 mM $NaHCO_3$; T_3—1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 6 mM $NaHCO_3$; T_4 —1/2 Hoagland nutrient solution + 50 g/L PEG 6000 + 9 mM $NaHCO_3$. Arrows indicate, whether measurements have increased or decreased, and short dash indicate measurements have no significant changes. Brown arrows (*1*) indicate moderating effect of T_1 compared with control; Blue arrows and short dash (*2*) indicate moderating effect of T_2 compared with T_1; Black arrows (*3*) indicate moderating effect of T_4 compared with T_1.

stress not only caused further closure of stomata (Fig. 1) but also decreased the HCO_3^- absorbed from plant roots, combined with the toxicity of Na^+ enrichment on plants making the photosynthetic system and membrane system of plants more seriously damaged [29], causing the photosynthetic assimilation ability of plants to decrease seriously (Fig. 2b). Although the proportion of PPP in glucose metabolism further increased (Fig. 3e), due to the sharp decline in photosynthetic capacity (Fig. 2b) and total metabolic capacity of plants (Fig. 3c), the stress tolerance and growth of plants were worse than those under simulated drought treatment alone. Therefore, the high concentration of $NaHCO_3$ in the rhizosphere of plants under drought stress mainly causes salt stress and has a negative effect on the plants (Fig. 6). Previous research has also shown that low concentrations of DIC, when added to the rhizosphere, positively affect biomass production, while high concentrations inhibit both plant growth and metabolic processes [7].

It was concluded from this experiment that all stress treatments exerted activating effects on G6PDH in paper mulberry leaves. And the low concentration (3 mM) of $NaHCO_3$ in the rhizosphere of plants mainly acts as a carbon source that could enhance paper mulberry growth and metabolism and has a positive effect on the plants to a significant extent under simulated mild drought stress; high concentrations (9 mM) of $NaHCO_3$ in the rhizosphere of plants under drought stress mainly cause salt stress and cause the photosynthetic assimilation ability of plants to decrease seriously, which has a negative effect on the plants. This finding implies that a moderate amount of HCO_3^- in soil promotes paper mulberry growth in karst drought environments, especially when plants close their stomata under some stress conditions, although additional in situ experiments are needed.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflicts of interest.

Statement on the welfare of humans or animals. This article does not contain any studies involving animals performed by any of the authors.

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