



Gibberellic acid application on biomass, oxidative stress response, and photosynthesis in spinach (*Spinacia oleracea* L.) seedlings under copper stress

Qin Gong^{1,2} · Zhao-hua Li^{1,3} · Ling Wang¹ · Jing-yi Zhou¹ · Qun Kang¹ · Duan-dan Niu¹

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Abstract

The mechanism of Cu tolerance in plants and its control measures are of considerable significance for the remediation of Cu-contaminated soils. Gibberellic acid (GA₃) is involved in plant growth and development and in the response to heavy metal stress. In the present study, changes in the biomass, oxidative stress response responses, and photosynthesis of spinach seedlings were examined under Cu stress with exogenous GA₃ applied at concentrations of 0, 3, 5, 10, 20, 40, 60, or 80 mg L⁻¹. Under Cu stress, the plant Cu concentration and oxidative damage were greater, photosynthetic parameters and biomass declined, and antioxidant enzyme activities and the proline concentration increased. However, spinach growth did not terminate, indicating that spinach seedlings had strong Cu tolerance. When low concentrations of GA₃ (3–5 mg L⁻¹) were added to Cu-stressed spinach seedlings, the damage caused by Cu stress to spinach seedlings was reduced, and the Cu tolerance of spinach seedlings was enhanced, which mainly manifested as reduced oxidation damage, an increased proline concentration, elevated antioxidant enzyme activities, decreased Cu concentration in leaves, and increased Cu concentration in roots, increased photosynthetic parameters, and an increased in the total biomass. In contrast, additions of GA₃ at concentrations higher than 40 mg L⁻¹ intensified oxidative damage and decreased the activities of antioxidant enzymes, photosynthetic parameters, and biomass. Additionally, the Cu concentration increased in leaves and decreased Cu concentration in roots, indicating that high concentrations of GA₃ aggravated stress damage and severely influenced physiological functions in spinach seedlings. In summary, the application of 3–5 mg L⁻¹ GA₃ to spinach seedlings in Cu-contaminated soil can be used to reduce Cu toxicity to plants and increase Cu tolerance.

Keywords Gibberellic acid · Spinach seedlings · Copper stress · Plant growth · Antioxidant enzyme · Photosynthesis · Copper tolerance

Introduction

Heavy metal pollution in soil has become a global concern. In China, approximately 20 million ha of farmland are

contaminated by heavy metals (Li et al. 2011; Cai et al. 2019). According to the communique on the soil pollution survey conducted by the Ministry of Environmental Protection (MEP) of China and the Ministry of Land and Resources in 2014, the national total percentages of soil heavy metal pollution exceeded 16.1%. Cu was ranked fourth accounting for 2.1% of the total pollution by heavy metals (MEP 2014). The sources of excess Cu in soil were mainly mining and smelting waste (Acosta et al. 2011), garbage incineration, sewage application, and excessive application of Cu-containing pesticides and fertilizers (Możdżeń et al. 2016; Sun et al. 2018). Some reports showed that the level of soil Cu pollution reached 102.5 mg kg⁻¹ in 31 provincial capitals in China (Zhang et al. 2018), especially in the southwest, which is a major area for large-scale mining and smelting of non-ferrous metals such as Cu, Pb, Zn, and Cd. Mining activities

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✉ Zhao-hua Li
1298085286@qq.com

¹ Faculty of Resources and Environmental Science, Hubei University, Wuhan 430062, China

² Xinjiang Vocation College of Agriculture, Changji 831100, Xinjiang, China

³ Hubei Rural Safe Drinking Water Engineering Technology Research Center, Wuhan 430062, Hubei, China

contaminate the surrounding via the drainage of mine waste, spillage of tailings, and dust emission, among other pathways (Cheng et al. 2009). Excess Cu in the soil can then enter the human body through the food chain threatening human health (Cai et al. 2019).

In plants, excess Cu has many detrimental consequences, such as facilitating the production of reactive oxygen species (ROS), decreasing plant biomass, inactivating enzymes, and disturbing photosynthesis (Chandrasekhar and Ray 2017; Rombel-Bryzek et al. 2017). To counteract the adverse effects of Cu stress, plants have developed various defense mechanisms, including antioxidant defense and toxic ion chelation and detoxification (Yruela 2009; Song et al. 2014). In addition, signaling molecules such as gibberellic acid (GA₃), indole-3-acetic acid (IAA), and citric acid may also induce stress tolerance in plants (Agami 2016; Ben Massoud et al. 2017).

GA₃, as a signaling molecule in plants, is involved in eliciting specific responses to biotic and abiotic stresses. GA₃ provides protection against saline stress in tomato (Maggio et al. 2010) and wheat (Akman 2012), induces heat stress responses in winter rapeseed seedlings (Leul and Zhou 1999), and drought stress responses in tomato, pepper, and mint (Abdelkader 2015). Furthermore, GA₃ is involved in alleviating heavy metal stress (Piotrowska-Niczyporuk et al. 2012), reducing heavy metal accumulation (Abd El-Monem et al. 2009), and improving antioxidant capacities (Alonso-Ramirez et al. 2009) in plants. GA₃ application can reduce oxidative stress injury in Cu-stressed pea seeds (Ben Massoud et al. 2017). Foliar spray of GA₃ to Pb-stressed maize also significantly increased Pb uptake and improved plant growth (Hadi et al. 2010), and the application of 5 μmol L⁻¹ GA₃ improved root growth and reduced the Cd content and lipid peroxidation in *Arabidopsis thaliana* seedlings under Cd stress (Zhu et al. 2012).

Spinach (*Spinacia oleracea* L.; order Caryophyllales) contains large amounts of vitamins, carotenoids, organic acids, alkaline minerals, and antioxidants (Aehle et al. 2004; Lisiewska et al. 2011; Becker et al. 2014). It is characterized by having a large leaf area, high relative growth rate, and high heavy metal absorption rate (Alia et al. 2015). Irrigation of spinach with sewage water containing heavy metals resulted in higher heavy metal accumulation in the leaves of spinach seedling than in their roots (Naz et al. 2016). Similarly, spinach grown in sewage-irrigated soil (Cu²⁺ content of 7.93 mg L⁻¹) showed a Cu²⁺ content above the standard level, whereas spinach growth and yield were significantly increased (Naz et al. 2016). Spinach tolerance mechanisms mainly include an increase in antioxidant enzyme activities and an accumulation of secondary metabolites (Gong et al. 2019). However, little is known about the interaction between GA₃ and toxic heavy metals such as Cu in spinach. Therefore, we aimed to (i) compare the effects of different GA₃ concentrations on the

physiological mechanism of spinach seedlings under Cu stress and (ii) explore whether exogenous GA₃ can alleviate Cu toxicity in spinach, and if so, determine the optimal concentration of GA₃ and the possible mechanism of GA₃ mediated protection. Accordingly, plant growth, oxidative damage, antioxidant enzyme activities, and levels of photosynthetic pigments, gas exchange, and chlorophyll fluorescence were evaluated in spinach seedling leaves under Cu stress with and without GA₃ treatment.

Materials and methods

Culturing of test materials

The Japanese big-leaf spinach seeds used in the present study were provided by the seed breeding station of Wangzhendian, Qingxian County, Hebei Province, China. Healthy seeds were sterilized using 0.1% NaClO, rinsed with deionized water, and then evenly placed on filter paper in a Petri dish. Seeds were germinated in an incubator at 25 °C for 48 h, in the dark. Germinated seeds were selected and planted in peat soil until they had grown to approximately 10 cm. Ten seedlings showing similar growth characteristics were selected and transplanted into 53 × 23 × 18 cm (length × width × height) pots supported by a tray placed underneath. The potting matrix comprised of peat soil:perlite at a 7:3 ratio, of which 3 kg (2.1 kg dry weight) was used to fill each pot. Spinach plants were grown in an open greenhouse. Complete Hoagland nutrient solution was supplied to all pots for 20 days to establish a vigorous root system, after which the GA₃ treatment began. The modified complete Hoagland nutrient solution is comprised of Ca(NO₃)₂·4H₂O 5 mmol L⁻¹, KNO₃ 5 mmol L⁻¹, Fe₂SO₄·7H₂O 1 mmol L⁻¹, MgSO₄·7H₂O 2 mmol L⁻¹, H₃BO₃ 0.045 mmol L⁻¹, MnCl₄·4H₂O 0.01 mmol L⁻¹, ZnSO₄·7H₂O 0.8 mmol L⁻¹, CuSO₄·5H₂O 0.3 mmol L⁻¹, Na₂Mo₂O₄·2H₂O 0.4 mmol L⁻¹, and Na-EDTA·2H₂O 0.02 mmol L⁻¹ (Zhu et al. 2012; Ouzounidou and Ilias 2005).

GA₃ treatment

Plants were divided into nine groups. One group, to which only 400 mL Hoagland nutrient solution was added in a single dose, was used as the control (C1). The other eight groups were provided with a one-time irrigation of 400 mL Hoagland nutrient solution containing 700 mg kg⁻¹ Cu²⁺ in the form of CuSO₄ (analytical reagent, Wuxi Yatai United Chemical Co. LTD). The Hoagland nutrient solution, containing 4.56 mg kg⁻¹ Cu²⁺, was adjusted to pH 6.5 ± 0.3 using 0.1 mmol L⁻¹ NaOH or HCl.

To examine the effect of GA₃ on the Cu-stressed seedlings, GA₃ was sprayed on the leaves of the seedlings that received Cu²⁺ at concentrations of 0, 3, 5, 10, 20, 40, 60, and 80 mg L⁻¹

GA₃ (guarantee reagent, Fuzhou Feijing Biotechnology Co. LTD). These eight treatments are hereafter referred to as Cu + GA₃-0 (C2), Cu + GA₃-3 (T1), Cu + GA₃-5 (T2), Cu + GA₃-10 (T3), Cu + GA₃-20 (T4), Cu + GA₃-40 (T5), Cu + GA₃-60 (T6), and Cu + GA₃-80 (T7), respectively. The GA₃ solution was mixed with Tween-20 (C₅₈H₁₁₄O₂₆, a leaf surfactant) and a total of 20 mL of GA₃ was applied to each pot (one application per pot). The C2 plants were sprayed with an equal amount of water (20 mL/pot) mixed with Tween-20. Each treatment was replicated three times in a randomized block design.

Plants were watered with deionized water to maintain a weight of 3.4 ± 0.05 kg. Cultivation was conducted at a day/night temperature of 22/15 °C, respectively, under a relative humidity of 70–80%, with 14 h of illumination (light intensity of 8000 lux) and 10 h of darkness. Treatment concentrations were set according to the results of preliminary tests (Gong et al. 2019; Naz et al. 2016; Ji et al. 2015; Hadi et al., 2010; Falkowska et al. 2011). After 7 days of treatment, plant samples were collected to measure the plant stress indicators.

Plant stress indicator measurements

Determination of biomass

Seedlings were carefully removed from pots, rinsed with tap water, and then rinsed three times with deionized water. After drying with blotting paper, the leaves, stems, and roots were separated, and their fresh weight (FW) was immediately determined. Thereafter, samples were oven-dried at 105 °C for 10 min, and then at 80 °C until a constant weight was obtained, which was recorded as the dry weight (DW) (Gong et al. 2019).

Determination of Cu concentration

Seedling roots were soaked in 20 mmol L⁻¹ Na₂-EDTA solution for 3 h to remove the Cu²⁺ adsorbed on their surfaces and then washed repeatedly with deionized water. Samples were then oven-dried at 105 °C for 20 min and then at 80 °C for 72 h. The dried plant samples were ground and collected in a porcelain crucible, and then digested with HNO₃ and HClO₄ (4/1, v/v) on a hot plate. Dense yellow fumes appeared from the flask and H₂O₂ was added continuously until dense yellow fumes disappeared. When the samples became colorless, the porcelain crucibles were removed from a hot plate, and volume was made up to 25 mL using distilled water. Cu concentrations were determined in the School of Chemistry, Hubei University, using inductively coupled plasma atomic emission spectroscopy (Fisons ARL Accuris, Ecublens, Switzerland) (Zhu et al. 2012).

Determination of malondialdehyde and proline contents

Malondialdehyde (MDA) was determined by the thiobarbituric acid (TBA) method (Tewari et al. 2002). Fresh leaf samples (0.3 g) were collected and ground with phosphate buffer to form a slurry, which was then centrifuged at 3000 ×g for 15 min. The resulting supernatant was isolated, and after the addition of 5 mL 0.5% thiobarbituric acid (TBA), the solution was mixed by shaking while being heated at 100 °C for 10 min. The mixture was subjected to rapid cooling centrifugation, the absorbance was determined at 532 nm and 600 nm using TBA as a blank sample. The measurements were performed using a UV-1750 spectrophotometer (Shimadzu, Kyoto, Japan).

Proline content was determined by acidic ninhydrin colorimetry (Tewari et al. 2002). Fresh leaf samples (0.5 g) were weighed and mixed with 5 mL sulfosalicylic acid for extraction in a boiling water bath for 10 min, after which the mixture was allowed to cool. The resulting supernatant was isolated by suction, followed by addition of 2 mL glacial acetic acid and 4 mL acidic ninhydrin in a boiling water bath. The mixture was then allowed to cool to room temperature, after which 4 mL toluene was added. The mixture was thoroughly shaken and allowed to stand until stratified; after isolation, the upper layer absorbance was determined at 520 nm using a UV-1750 spectrophotometer (Shimadzu, Kyoto, Japan).

Determination of antioxidant enzyme activities

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was determined according to Beauchamp and Fridovich's method (Beauchamp and Fridovich 1971). The reaction mixture contained the enzyme extract, 20 μmol L⁻¹ riboflavin, 750 μmol L⁻¹ nitroblue tetrazolium (NBT), 13.37 mM methionine, 0.1 mM EDTA, and 50 mmol L⁻¹ phosphate buffer (pH 7.8). One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the initial NBT reduction under light. The measurements were performed using a UV-1750 spectrophotometer (Shimadzu, Kyoto, Japan).

Peroxidase (POD, EC 1.11.1.7) activity was assayed in a 2.9 mL reaction mixture containing 50 μL 0.02 mol L⁻¹ guaiacol, 0.1 mL enzyme extract, and 10 μL 0.04 mol L⁻¹ H₂O₂. The optical density at 470 nm for 1 min at 25 °C was recorded using a UV-1750 spectrophotometer (Shimadzu, Kyoto, Japan) (Batish et al. 2006).

Catalase (CAT, EC 1.11.1.6) activity was assayed according to the method of Lu et al. (2018). The 3.3-mL reaction mixture contained 100 mmol L⁻¹ phosphate buffer (pH 7.4), 0.1 mL enzyme extract, 0.5 mL 65 μmol L⁻¹ H₂O₂, and 0.5 mL 32.7 mol L⁻¹ ammonium molybdate. The absorbance of

the sample was determined at 405 nm using a UV-1750 spectrophotometer (Shimadzu, Kyoto, Japan).

Total ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined by monitoring the ascorbate oxidation rate at 290 nm (Nakano and Asada 1981). The protein extract was added to a 1.7 mL reaction solution, containing 50 mmol L⁻¹ phosphate buffer (pH 7.8), 0.1 mmol L⁻¹ EDTA, and 5 mM ascorbic acid. The reaction was initiated by the addition of 100 μL of 20 mM H₂O₂. The decrease in absorbance was monitored using a UV-1750 spectrophotometer (Shimadzu, Kyoto, Japan).

Determination of photosynthetic pigments, photosynthetic parameters, and chlorophyll fluorescence parameters

Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid contents were determined according to Hiscox and Israeltem’s methods (Hiscox and Israeltem 1979). The leaf content (per gram) was calculated according to Arnon’s formula (Arnon 1949).

$$C_a = 13.95A_{665} - 6.88A_{649}; C_b = 24.96A_{649} - 7.32A_{665}; C_T = C_a + C_b$$

$$C_{x-c} = (1000A_{470} - 2.05 C_a - 114.8 C_b) / 245$$

The net photosynthetic rate (*P_n*), intercellular CO₂ concentration (*C_i*), stomatal conductance (*g_s*), and transpiration rate (*T_r*) of functional leaves were determined using a Li-6800 portable photosynthesis meter (LI-COR, Lincoln, NE, USA). Chlorophyll fluorescence parameters were determined using a PAM-2100 chlorophyll fluorometer (Walz, Wurzburg, Germany) and included minimum fluorescence (*F₀*), maximum fluorescence after dark

adaptation (*F_m*), photochemical quenching (*qP*), non-photochemical quenching (*NPQ*), and electron transport rate (*ETR*). The ratio of *F_v*/*F_m* was calculated according to the formula (*F_m* - *F₀*) / *F_m* (Gong et al. 2019).

Data analyses

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). One-factor ANOVA was performed to identify statistically significant differences among treatments, followed by Duncan’s multiple comparisons (*P* < 0.05).

Results

Effects of Cu stress and GA₃ on spinach seedlings biomass

As shown in Fig. 1, Cu stress (treatment C2) significantly inhibited spinach seedling growths, as the total FW and total DW in C2 decreased by 31.9% and 41.0%, respectively (*P* < 0.05), compared with that of C1. When 3–5 mg L⁻¹ GA₃ was applied (treatments T1–T2), the inhibitory effects of Cu stress on spinach biomass were reversed. In treatments T1 and T2, total FW increased by 25.5% and 24.3%, respectively, while total DW increased by 27.2% and 26.8%, respectively, compared with that of C2. When high concentrations of GA₃ were used (treatments T3–T7), the increment of biomass increase was reduced. In treatments T6 and T7, the total FW only increased by 10.8% and 4.4%, respectively, while the total DW only increased by 11.5% and 8.6%, respectively. The changes in the FW and DW of

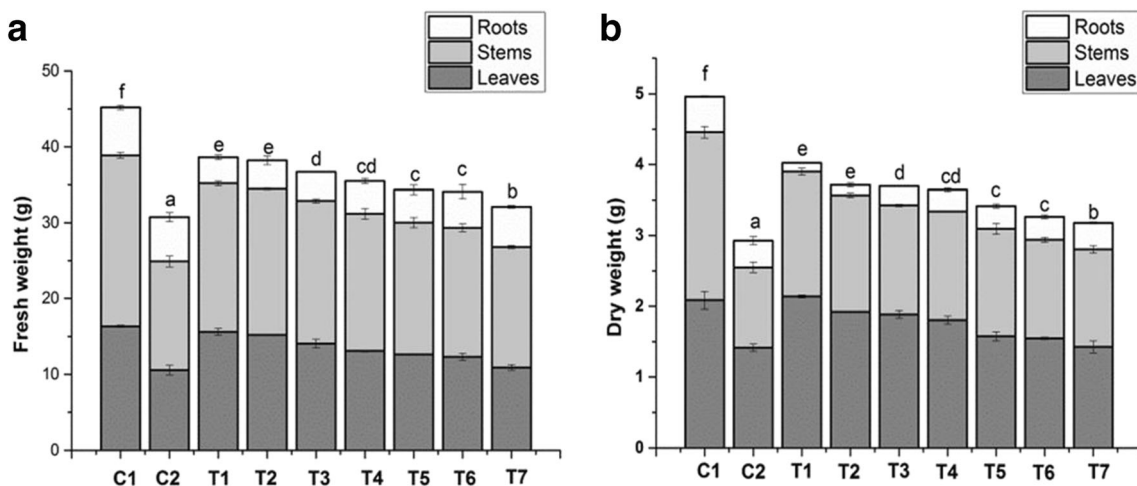


Fig. 1 Effects of GA₃ on the fresh weight (a) and dry weight (b) of spinach seedlings under Cu stress. Values and error bars indicate means ± standard deviations (*n* = 3). Different letters indicate a significant difference between treatments at *P* < 0.05

Table 1 Effects of GA₃ on Cu accumulation in spinach seedlings under Cu stress

Treatment	Cu accumulation in leaves (μg g ⁻¹)	Cu accumulation in roots (μg g ⁻¹)
C1	1.79 ± 0.01a	0.72 ± 0.006a
C2	544.51 ± 0.41h	32.36 ± 1.18b
T1	340.19 ± 3.04d	111.20 ± 3.76g
T2	210.05 ± 1.19b	178.31 ± 4.13h
T3	332.95 ± 2.20c	104.29 ± 0.36f
T4	354.64 ± 0.58e	112.97 ± 1.24g
T5	465.76 ± 4.51g	57.94 ± 1.28d
T6	466.88 ± 1.50g	73.38 ± 0.67e
T7	453.36 ± 3.77f	51.26 ± 0.06d

leaves, stems, and roots exhibited essentially the same trend as that of total biomass.

Effects of Cu stress and GA₃ on Cu accumulation in spinach seedlings

Compared to seedlings of C1, Cu concentration significantly increased in leaves and roots under Cu stress (treatment C2) (Table 1), with leaves having higher concentration than roots. When GA₃ was applied to Cu-stressed seedlings (treatments T1–T7), the Cu concentration decreased in leaves and increased in roots. Notably, the lowest leaf Cu concentration and the highest root Cu concentration were observed in treatment T2. In addition, compared with C2, both the decrement of Cu concentration in the leaves and the increment of Cu concentration in the roots decreased in treatments T1–T7. Similarly, the decrement of leaf Cu concentration and the increment of the root Cu concentration reached the maximum values in treatment T2.

Data are mean ± standard error ($n = 3$). Values within a row followed by the same letter are not significantly different ($p < 0.05$)

The influence of Cu stress and GA₃ on lipid peroxidation and proline contents of spinach leaves

As shown in Fig. 2, Cu stress (treatment C2) significantly increased the MDA content ($P < 0.05$) compared with C1. When exogenous GA₃ was applied (treatments T1–T7), the MDA contents were higher than that in C2, except for treatment T1 in which the MDA content was 18.2% lower than that in C2. In T6 and T7, the MDA contents increased by 75.9% and 62.6% of that in C2, respectively.

Proline content in C2 was higher than in C1 (Fig. 2b). When GA₃ was added (treatments T1–T7), the proline contents showed an initial increase followed by a decrease. Proline decreased by 74.4% and 45.4% in T1 and T2, respectively, compared with that in C2. Proline content peaked in T5, increasing by 18.1% of that in C2.

Effects of Cu stress and GA₃ on antioxidant enzyme activities of spinach leaves

Exposure of spinach seedlings to Cu stress (treatment C2) significantly increased the activities of antioxidant enzymes (SOD, POD, CAT, and APX), as shown in Fig. 3. However, the maximum increase occurred in treatments T1 and T2 for all four enzyme activities, with average increase of 19.3%, 127.3%, 3.7%, and 2.7%, respectively, compared with treatment C2. In treatments T5–T7, SOD, CAT, and APX activities declined to their minimum values, decreasing by 27.9%, 15.2%, 15.8%, and 35.2%, respectively, on average, compared to C2.

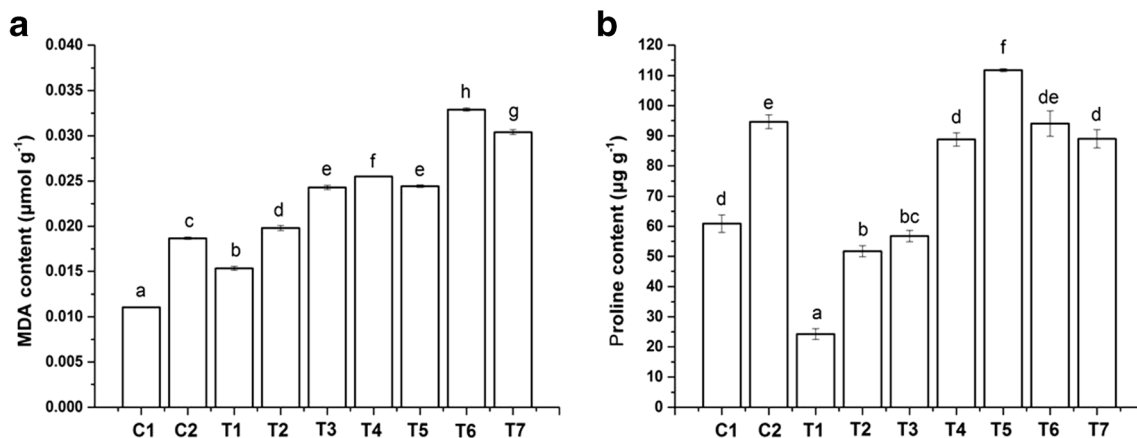


Fig. 2 Effects of GA₃ on MDA (a) and proline (b) contents of spinach seedlings under Cu stress. Values and error bars indicate means ± standard deviation ($n = 3$). Different letters indicate a significant difference between treatments at $P < 0.05$

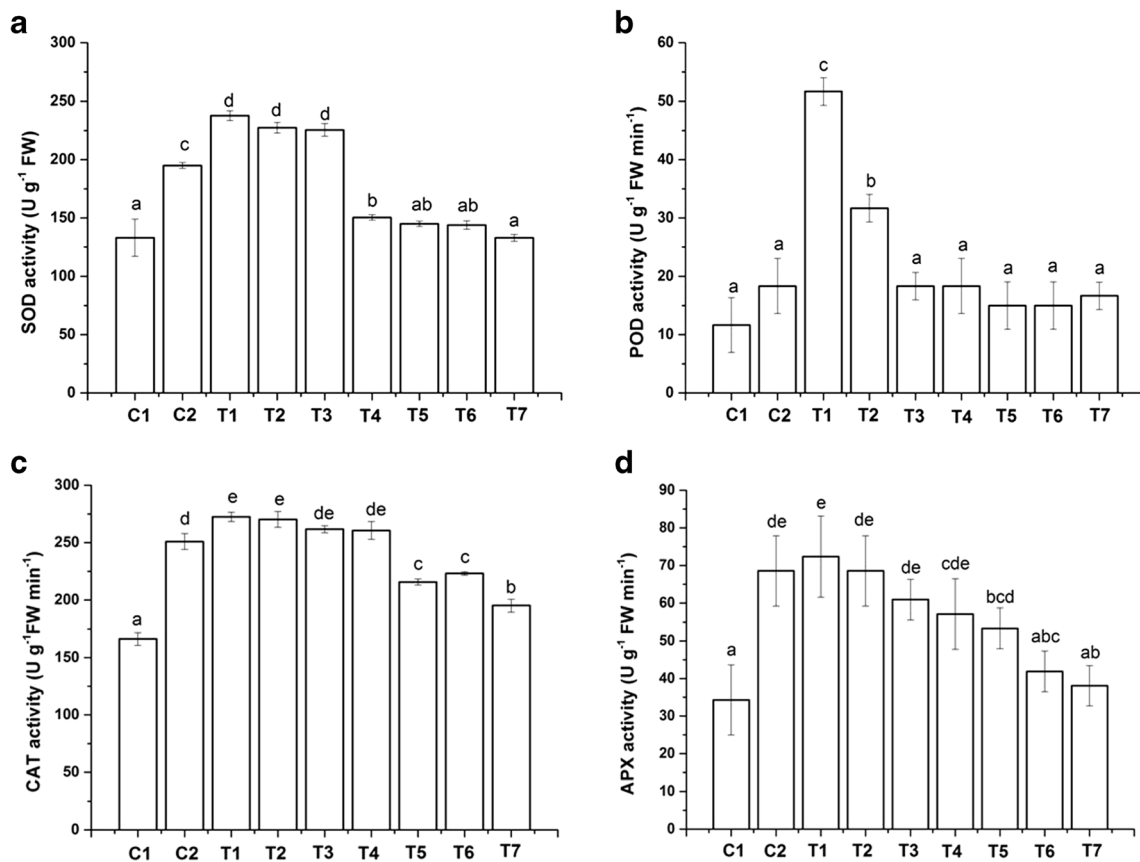


Fig. 3 Effects of GA₃ on SOD (a), POD (b), CAT (c), and APX (d) activities of spinach seedlings under Cu stress. Values and error bars indicate means ± standard deviation (*n* = 3). Different letters indicate a significant difference between treatments at *P* < 0.05

Effects of Cu stress and GA₃ treatment on chlorophyll contents and photosynthetic characteristics of spinach leaves

The Chl *a*, Chl *b*, and carotenoid content was significantly lower under Cu stress (treatment C2) than that in C1 (*P* < 0.05) (Table 2). When GA₃ was sprayed on Cu-stress seedlings (treatments T1–T7), Chl *a* contents showed a decreasing trend; however, it was higher than that under Cu stress without application of GA₃ (treatment C2). However, Chl *b* contents exhibited a decreasing trend from T4 to T7. The highest Chl *b* content was observed in T1 (0.44-fold higher than in C2), and the lowest in T7, and there was no significant difference from that in C2 (*P* > 0.05).

When compared to C1, the *P_n*, *g_s*, and *T_r* values decreased in treatment C2, while *C_i* values increased (Table 2). When GA₃ was added (treatments T1–T7), the *P_n* value showed a trend of gradual decline, with a minimum value in T7, which was lower than that in C2. However, the *g_s*, *T_r*, and *C_i* values first increased and then decreased, reaching maximum values in T3, in which they increased by 83.1%, 20.3%, and 87.5%, respectively, compared to C2.

Data are mean ± standard error (*n* = 3). Values within a row followed by the same letter are not significantly different (*p* < 0.05)

Effects of Cu stress and GA₃ on chlorophyll fluorescence parameters of spinach leaves

Cu stress (treatment C2) significantly reduced chlorophyll fluorescence parameters of spinach leaves, and the Fv/Fm, NPQ, qP, and ETR values in C2 were 11.8%, 21.2%, 60.8%, and 77.0% lower than those in C1, respectively (Fig. 4). However, exogenous GA₃ reversed the reduction of chlorophyll fluorescence parameters. When compared with values in C2, the Fv/Fm, qP, and ETR values in T1 and T2 showed the largest increments, increasing by averages of 0.14, 0.84, and 1.09-fold, respectively. The Fv/Fm values in T6 and T7 only increased by averages of 0.03, 0.24, and 0.17-fold, respectively, in comparison to in C2. The NPQ values increased significantly in T1–T5 (0.75, 1.01, 0.72, 0.91, and 0.77-fold, respectively) and decreased significantly in T6 and T7 (0.18 and 0.27-fold, respectively) compared to C2.

Discussion

High Cu concentration inhibited the physiological activity of spinach seedlings

Excess Cu affects the physiological activities of plants, leading to decreased plant growth, inhibited photosynthesis, and

Table 2 Effects of GA₃ on chlorophyll contents and photosynthetic characteristics of spinach seedlings under Cu stress

Treatment	Chl a content (mg g ⁻¹)	Chl b content (mg g ⁻¹)	Carotenoid content (mg g ⁻¹)	P_n (μmol m ⁻² s ⁻¹)	g_s (mmol m ⁻² s ⁻¹)	C_i (μmol m ⁻¹)	T_r (mmol m ⁻² s ⁻¹)
C1	1145.69 ± 4.35i	520.58 ± 3.06e	235.77 ± 0.76ef	14.43 ± 0.008i	267.17 ± 8.51h	270.78 ± 5.93a	2.55 ± 0.008h
C2	695.94 ± 5.24a	328.69 ± 4.87a	164.65 ± 9.09ab	6.38 ± 0.002b	154.18 ± 2.03c	314.40 ± 5.75cd	0.80 ± 0.009a
T1	1088.39 ± 7.44h	474.14 ± 25.76d	242.70 ± 4.33e	8.11 ± 0.009h	196.28 ± 8.85f	319.44 ± 1.19d	1.04 ± 0.002c
T2	987.81 ± 16.67g	456.64 ± 12.55d	207.19 ± 11.66cd	7.70 ± 0.009g	210.05 ± 9.09g	320.65 ± 7.04d	1.32 ± 0.003e
T3	913.30 ± 7.13f	405.89 ± 2.00c	182.44 ± 14.75bc	7.68 ± 0.005f	282.36 ± 5.64h	320.77 ± 8.47d	1.50 ± 0.01g
T4	865.91 ± 5.16e	359.60 ± 10.66ab	212.52 ± 2.37d	7.36 ± 0.001e	181.57 ± 0.91e	308.74 ± 5.65bcd	1.44 ± 0.006f
T5	820.69 ± 9.07d	476.73 ± 37.49d	150.71 ± 18.41a	6.85 ± 0.006d	130.69 ± 2.31b	302.91 ± 4.54bc	1.04 ± 0.005c
T6	783.88 ± 7.98c	386.63 ± 16.88bc	152.42 ± 19.50a	6.55 ± 0.007c	168.91 ± 3.85d	299.52 ± 7.11b	1.18 ± 0.007d
T7	728.61 ± 7.48b	338.24 ± 15.33a	170.62 ± 13.87ab	5.37 ± 0.009a	102.38 ± 4.13a	295.14 ± 8.02b	0.94 ± 0.005b

changes to physiological and metabolic processes (Dai et al. 2016; Chandrasekhar and Ray 2017). Our present data showed that, compared with the control treatment (C1), the addition of 700 mg kg⁻¹ Cu (treatment C2) significantly increased the Cu, MDA, and proline content, as well as the activities of four antioxidant enzymes (SOD, POD, CAT, and APX), and decreased the chlorophyll contents, gas exchange parameters (P_n , g_s , and T_r), chlorophyll fluorescence

parameters (Fv/Fm, qP, NPQ, and ETR), and total biomass. Because seedlings survived, high Cu concentration led to Cu²⁺ accumulation and an increase in the oxidative stress response in spinach seedlings. However, seedlings resisted oxidative stress damage by increasing antioxidant enzyme activities to maintain vital functions. In addition, the content of proline, which is an osmotic regulatory compound with antioxidant effects, increased in spinach seedlings improving the

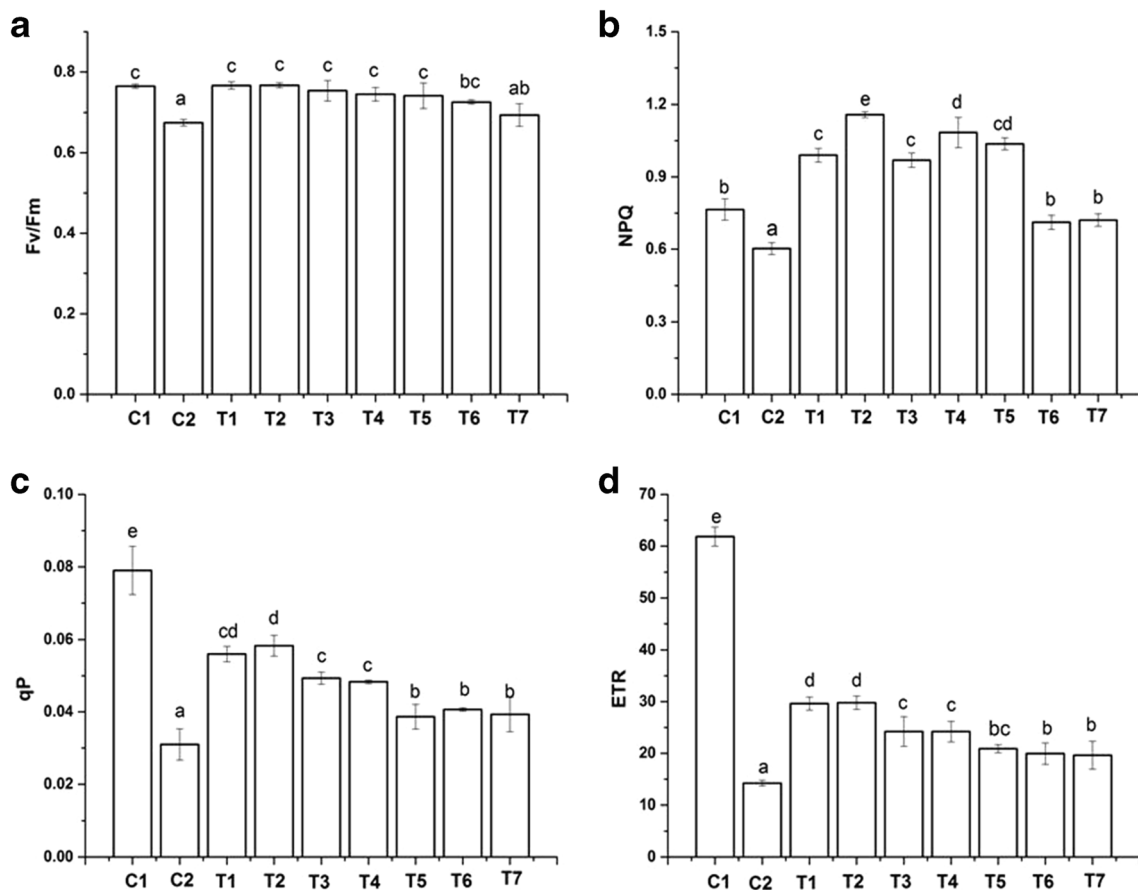


Fig. 4 Effects of Cu stress and GA₃ on Fv/Fm (a), NPQ (b), qP (c), and ETR (d) of spinach seedlings. Values and error bars indicate means ± standard deviation ($n = 3$). Different letters indicate a significant difference between treatments at $P < 0.05$

antioxidant capacity of plants and chelating Cu^{2+} to reduce its toxic accumulation (Sharma and Dietz 2006; Dresler et al. 2014) thereby improving the Cu tolerance of spinach seedlings.

However, different plants have varying Cu tolerances. *Elsholtzia haichowensis*, a plant with strong Cu tolerance, can survive under soil with Cu concentrations up to 600 mg kg^{-1} (Chen et al. 2015) and *Sedum sediforme* were able to survive extreme phytotoxicity conditions in soil containing 5000 mg kg^{-1} Cu (Poschenrieder et al. 2001). However, the biomass and photosynthesis rate of cucumber treated with 10 $\mu\text{g g}^{-1}$ Cu for 5 days (Vinit-Dunand et al. 2002) and the biomass of tomato treated with 5 $\mu\text{mol L}^{-1}$ Cu decreased (Zhang et al. 2017). In the present study, although the Cu concentrations were not as high as those applied to *E. haichowensis* and *S. sediforme*, spinach seedlings showed strong Cu tolerance compared to that of tomato and cucumber observed in previous studies.

Low-concentration GA_3 alleviated the damage of Cu stress to spinach seedlings

As an active member of the signal cascade involved in the induction of plant stress responses (Tuna et al. 2008), GA_3 can reduce heavy metal accumulation in plants and alleviate oxidative damage when applied to plants under heavy metal stress (Zhu et al. 2012; Ben Massoud et al. 2017). The results of the present study showed that low GA_3 concentrations (3–5 mg L^{-1}) applied to Cu-stressed seedlings could significantly decrease the Cu contents in the leaves, but that in the roots significantly increased. These results indicated that GA_3 applied at low concentrations reduced Cu^{2+} accumulation in the leaves and reduced the toxic influence of Cu (Falkowska et al. 2011), but promoted Cu^{2+} accumulation in the roots (Zhu et al. 2012). It is also possible that GA_3 inhibited Cu^{2+} migration from roots to leaves (Fujita et al. 2006). The root is the main organ of plants exposed to Cu-contaminated soil, and Cu^{2+} enters the root through the plant's physiological activities such as transpirational pull and active and passive water absorption, which increased Cu^{2+} accumulation in the roots. However, roots prevent the extensive migration of Cu^{2+} to the leaves through ion chelation, ion regionalization, and other routes (Yruela 2009; Song et al. 2014). Thus, when GA_3 was applied, the damage caused by Cu stress in leaves was alleviated and Cu tolerance in roots was enhanced, removing excess Cu^{2+} from the soil. Similar results have been obtained in previous studies: with the application of 10 mg L^{-1} GA_3 , Cd concentrations increased in the stems of potato (*Solanum tuberosum* L.) under Cd stress, while Cd concentrations in the leaves decreased (Ji et al. 2015); when GA_3 was applied to maize under Pb stress, Pb uptake by roots was significantly increased (Hadi et al. 2010). In addition, our results

also indicated that the total Cu content in leaves and roots was the lowest at the low GA_3 concentrations (3–5 mg L^{-1}). It may be that low GA_3 concentrations aggravated root cell wall embolization and that lignification, and the cuticle of the cell wall formed protective substances; the barrier function of root apoplasts was enhanced, and thus a large number of Cu^{2+} were prevented from entering the plant resulting in damage caused by excess Cu^{2+} to be alleviated (Krishnamurthy et al. 2009; Lu et al. 2019; Yuankun et al. 2020). This is also a strong evidence that low GA_3 concentrations can alleviate the damage to plants caused by Cu^{2+} stress.

Exogenous addition of plant hormones can improve heavy metals tolerance by reducing ROS accumulation in the membrane peroxidation reaction and enhancing the antioxidant defense ability of plants (Lu et al. 2010; Agami 2016). The MDA content is an important indicator of the degree of membrane lipid peroxidation and damage in plants (Rombel-Bryzek et al. 2017). In the present study, when low GA_3 concentrations (3–5 mg L^{-1}) were applied, the MDA contents remarkably decreased while the activities of antioxidant enzymes (SOD, POD, CAT, and APX) and biomass significantly increased. Possible reasons for this phenomenon are as follows: (i) low GA_3 concentration can reduce the oxidative damage to plants caused by high Cu concentration and thus, alleviate the damage to plants caused by Cu and (ii) a low GA_3 concentration may induce upregulation of antioxidant enzyme genes in plants, thus enhancing their ability to resist oxidative stress (Kaur Kohli et al. 2018). Hence, plant growth and Cu tolerance were enhanced in spinach seedlings after the addition low GA_3 concentrations. Similar observations have been reported in previous studies. Ji et al. (2015) found that addition of 1000 mg L^{-1} GA_3 to black nightshade (*Solanum nigrum* L.) could increase plant biomass under Cd stress and promote its phytoremediation ability in Cd-contaminated soil. Zhu et al. (2012) showed that GA_3 could reduce the Cd damage to lipid peroxidation in *A. thaliana* plants and enhance their Cd tolerance. In the present study, when low GA_3 concentrations were added, the chlorophyll contents increased, as well as P_n , C_i , Fv/Fm, NPQ, qP, and ETR values. The reasons for this may be that (i) GA_3 , as a signaling molecule, can induce the PSII reaction center to be in an open state, allowing the use of more excitation energy for electron transmission thereby improving the electron flow efficiency of PSII (Zhang et al. 2015; Możdżeń et al. 2016); (ii) GA_3 may trigger some protective mechanisms of the photosynthetic apparatus resulting in the stability of PSII (Ouzounidou and Ilias 2005); and (iii) GA_3 enhances photosynthetic CO_2 uptake, probably by increasing the coupling between electron transport and phosphorylation (Tamas et al. 1974; Ouzounidou and Ilias 2005), thereby promoting photosynthesis in spinach seedlings.

High concentration of GA₃ inhibits physiological activities of spinach seedlings under Cu stress

The timing, concentration, and utilization techniques of exogenous plant growth regulators should be considered. Among them, concentration is an important factor affecting plant growth (Saeid and Aboalfazl 2019). In the present study, the effects of GA₃ on plant growth physiology differed depending on the applied concentration (Muniandi et al. 2018). When GA₃ was applied at concentrations higher than 40 mg L⁻¹ (treatments T5–T7), the Cu contents were higher in leaves and lower in roots than that following application at when lower GA₃ concentrations (treatments T1 and T2), indicating that the application of high GA₃ concentrations promoted Cu²⁺ accumulation in leaves while reducing Cu²⁺ accumulation in roots. The reason for this may be that (i) GA₃ can alter the concentrations of heavy metals accumulated in plants (Falkowska et al. 2011; Hadi et al. 2010); (ii) spinach leaves are large and have a high water content, which means that plants need to transport a large amount of water from roots to leaves, resulting in Cu²⁺ accumulation in leaves. Similarly, Khan et al. (2011) found that application of a high concentration of plant growth regulation hormones reduced growth of *Salix tetrasperma* Roxb. In addition, when GA₃ concentrations were higher than 40 mg L⁻¹ (treatments T5–T7), the MDA contents significantly increased, and the activities of antioxidative enzymes (SOD, POD, CAT, and APX) decreased, and the total biomass of spinach seedlings decreased, indicating that the high GA₃ concentration aggravated the damage of membrane lipid peroxidation to seedlings under Cu stress, leading to the decline of plant antioxidant defense functions and plant biomass (Muniandi et al. 2018). Similar results have been reported in previous studies. For example, CAT and APX activities decreased when 1 μmol L⁻¹ GA₃ was applied to pea (*Pisum sativum* L.) seedlings under Cu stress (Ben Massoud et al. 2017). Addition of 24-epibrassinolide and salicylic acid to Indian mustard (*Brassica juncea* L.) under Pb stress decreased SOD activity (Kaur Kohli et al. 2018).

Photosynthesis is one of the most sensitive physiological activities of plants to environmental stress, and chlorophyll fluorescence is the most affected (Diao et al. 2014; Zhang et al. 2015). In the present study, the application of GA₃ at concentrations higher than 40 mg L⁻¹ (treatments T5–T7) led to declines in Chl *a* and carotenoid contents compared with low GA₃ concentration (treatments T1 and T2); gas exchange parameters (P_n , C_i , g_s , and T_r) and chlorophyll fluorescence parameters (Fv/Fm, NPQ, qP, and ETR) were also considerably reduced. These results suggested that (i) high GA₃ concentrations interfered with electron donation to photochemical reactions in PSII, leading to a reduction of the energy distributed to heat dissipation, decreasing the prevention exerted upon damaging free radicals formation (Muniandi et al. 2018) and (ii) the high GA₃ concentration inhibited the growth of the leaf primordia within the terminal bud, resulting in decreases in the number and shape of leaves,

the photosynthetic area, photosynthetic products, and the photosynthetic electron transfer rate (Gou et al. 2010; Wang et al. 2015). Eventually, the photosynthetic rate reduced, and physiological functions were affected.

Conclusion

Soil containing 700 mg kg⁻¹ Cu significantly increased the Cu contents and decreased the biomass of spinach seedlings; however, the antioxidant defense ability of plants was enhanced, which enabled the seedlings to maintain their survival and demonstrate strong Cu tolerance. While the addition of 3–5 mg L⁻¹ GA₃ could alleviate the damage from Cu stress and enhance the Cu tolerance of spinach seedlings, application of GA₃ at concentrations higher than 40 mg L⁻¹ aggravated the damage caused by Cu stress, as the biomass, antioxidant enzyme activities, and photosynthesis of spinach seedlings decreased. Therefore, our results suggest that application of 3–5 mg L⁻¹ GA₃ to spinach seedlings can alleviate the toxicity of Cu-contaminated soil to plants.

Abbreviations FW, Fresh weight; DW, Dry weight; MDA, Malondialdehyde; TCA, Trichloroacetic acid; SOD, Superoxide dismutase; POD, Peroxidase; CAT, Catalase; APX, Ascorbate peroxidase; ROS, Reactive oxygen species; Chl *a/b*, Chlorophyll *a/b*; PSII, Photosystem II; P_n , Net photosynthetic rate; g_s , Stomatal conductance; C_i , Internal CO₂ concentration; T_r , Transpiration rate; Fv/Fm, Maximum quantum yield of PSII photochemistry; qP, Photochemical quenching; NPQ, Nonphotochemical quenching; ETR, Electron transport rate; NBT, Nitroblue tetrazolium

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Authors' contribution Qong Gin, Ling Wang, and Jing-yi Zhou conducted the experiments, collected and analyzed the samples, and drafted the manuscript. Qun Kang and Duan-dan Niu analyzed the data and revised the manuscript. Zhao-hua Li and Qong Gin conceived and designed this work. All authors read and approved the manuscript.

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Declarations

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