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1 **Physiological responses of *Elaeocarpus glabripetalus***
2 **seedlings exposed to simulated acid rain and cadmium**

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Abstract

Combined effects of cadmium (Cd) and acid rain on physiological and biochemical characteristics in *Eleocarpus glabripetalus* seedlings were investigated under controlled conditions. The single Cd treatment and the combined Cd and acid rain treatment increased growth at low Cd concentrations, while decreased growth and photosynthesis at high Cd concentrations. A low Cd concentration ($50\text{mg}\cdot\text{kg}^{-1}$) combined with different acid rain treatments increased the seedling biomass, while there was no significant difference in chlorophyll fluorescence and antioxidative enzyme activities compared with the control plants. A high Cd concentration ($100\text{mg}\cdot\text{kg}^{-1}$) under different acid rain treatments significantly decreased the biomass, the Fe and Mg content, chlorophyll fluorescence, photosynthetic parameters (the maximum net photosynthetic rate, A_{max} , and the light saturation point, L_{SP}). Relative electric conductivity and peroxidase (POD) activity were significantly higher while the reduced glutathione (GSH) content and catalase (CAT) activity were significantly lower at high Cd concentration under acid rain when compared to the control plants. The results indicated that the combination of a high concentration of Cd and acid rain aggravated the toxic effect of Cd or acid rain alone on the growth and physiological parameters of *E. glabripetalus* due to serious damage to the chloroplast structure. However, *E. glabripetalus* showed tolerance to a low concentration of Cd and low pH induced by acid rain.

Additional Key words: Interactive effects; Cadmium; Acid rain; Physiology;

Elaeocarpus glabripetalus.

32 ***Abbreviation***

33 *AQY* – apparent quantum; *A_{max}* – maximum net photosynthetic rate; CAT – catalase;
34 Chl – chlorophyll; GSH – glutathione; *F_m* – maximal fluorescence in dark-adapted
35 state; *F_m'* – maximal fluorescence in the light-adapted state; *F_s* – steady state value of
36 fluorescence; *F_v* – variable fluorescence; *F_v/F_m* – maximum quantum yield; *F_v/F₀* –
37 potential activity of PSII; *F₀'* – basal fluorescence after farred illumination; *L_{CP}* – light
38 compensation point; *L_{SP}* – light saturation point; *NPQ* – non-photochemical
39 quenching of fluorescence; PAR – photosynthetically active radiation; *P_n* – net
40 photosynthetic rate; POD – peroxidase; PSII – photosystem II; PPFD – photosynthetic
41 photon flux density; *qP* – photochemical quenching; ROS – reactive oxygen species

1 Introduction

Cadmium (Cd) is one of the most toxic elements that can be found as a contaminant in the soil. According to Zhou et al. (2004) and Jin et al. (2010), the concentration of Cd in some severe polluted soils was higher than 100 mg·kg⁻¹. Cadmium contents in soil have dramatically increased during recent years and its toxicity limits plant growth and development. Cadmium can be easily taken up by roots and it tends to accumulate within plant organs (Gill et al., 2012). Cd inhibits plant growth and biomass production, affects mineral uptake, photosynthesis and the protective system, and negatively interferes with essential physiological processes (Januškaitienė, 2012, Parmar et al., 2013, Li et al., 2015, Liu et al., 2017,).

In the nature, plants are rarely exposed to a single hazardous stress. Different contaminants may show additive, antagonistic or synergistic interactions. Acid rain is one of the well-known environmental problems, which cause negative impacts on plant growth and health. It has been found that acid rain destroys leaves, causes deleterious effects on photosynthesis and inhibits growth (Sun et al., 2016, Debnath et al., 2018). Most agroforestry areas with acid rain pollution have also other contaminants, such as Cd, leading to combined pollutions effects, which are major problems for sustainable forestry and environmental safety.

There are many previous investigations on the effects of acid rain or Cd alone on plants (Chmielowska-Bąk et al., 2014, Li et al., 2015, Zheng et al., 2017, Ren et al., 2018), while only a few studies have reported results on their combined adverse effects on plant growth (Liao et al., 2003, Liu et al., 2011, Sun et al., 2012). Such

combined pollution could have harmful effects on plant growth through the inhibition of photosynthesis, because the photosynthetic apparatus appears especially sensitive to Cd (Gill et al., 2012). Previous studies have found that Cd combined with acid rain may damage the chloroplast structure, thus reducing photosynthesis (Sun et al., 2012), and decrease the activities of antioxidative enzymes (Liu et al., 2011). The combined effect of acid rain and heavy metal exposure would inhibit plant growth through reducing biomass and mineral uptake (Zhang et al., 2016). Many of the previous studies on the combined effect of heavy metals and acid rain have focused on crops and only few on woody plants. Compared with herbaceous plants, woody plants usually grow slowly with a deep root system and long generation time, and they are more easily affected adversely by pollution.

Eleocarpus glabripetalus is a heliophilous and dominant species in forest ecosystems of China. It is widely distributed in regions affected by severe acid rain but also by Cd pollution. Our previous studies showed that *E. glabripetalus* seedlings have tolerance to acid rain (Liu et al., 2015). However, we don't know how it would grow under the combined pollution of acid rain and Cd. Therefore, the objectives of our present study are: (1) to investigate the responses of chlorophyll fluorescence and photosynthetic parameters to the combined pollution of Cd and acid rain; (2) to reveal the activities of antioxidative enzymes and the content of reduced glutathione (GSH) under the combined pollution of Cd and acid rain; (3) to show the combined effect on biomass allocation, and on the content of Cd, Mg and Fe in plants. The results obtained from this study will enhance understanding of the mechanisms of tolerance to the combined

pollution of Cd and acid rain in *E. glabripetalus*.

2 Materials and methods

2.1. Preparation of simulated acid rain and Cd solution

According to the average pH value of acid rain in southern China (Hangzhou Municipal Environmental Protection, 2013), two pH values (pH 3.0 and 5.6) were selected for the experiment. Correspondingly, according to the average concentration of Cd in polluted soil (Zhou et al., 2004, Jin et al., 2010), the final concentrations of 50 and 100 mg·kg⁻¹ were selected. Two solutions were prepared: (1) According to the general anion composition of rainfall in southern China, the SO₄²⁻/NO₃⁻ ratio is 2.87 (Xu et al., 2011). Considering that the proportion of NO₃⁻ has been rising during recent years, a simulated acid rain solution stock of pH 3.0 was prepared with a H₂SO₄ and HNO₃ ratio of 1.0:1.0 (v/v), and then used as such or diluted to pH 5.6 to be utilized as the spraying solution to simulate acid rain. (2) The Cd solutions (0.15 mM and 0.30 mM, calculated for 10 kg soil in the pot and 100-day stress according to 50 and 100 mg·kg⁻¹) were prepared by dissolving appropriate quantities of CdCl₂·5H₂O (Chemical Co. Ltd., China) in distilled water (pH 7.0).

2.1 Plant material and stress treatments

The soil, a mixture of reddish brown forest soil, was sampled from the depth of 0-20 cm. Then, the soil sample was air-dried and ground to pass through a 2 mm sieve, and 10 kg was divided into each pot. The initial soil pH was 6.88 and available Cd was 0.082 mg·kg⁻¹. Healthy one-year old seedlings of *E. glabripetalus* with height of 40-

60 cm and a basal diameter of approximately 0.8-1.0 cm were planted into pots with an inner diameter of 30 cm and a depth of 40 cm, one seedling per pot. A PVC plate was placed under each pot. The seedlings were kept under a canopy in the Botanical Garden of the Zhejiang Agriculture and Forestry University (119°44' E, 30°16' N), East China. After growing for two months, seedlings were subjected to 9 treatments of Cd and simulated acid rain, (1) The control group (CK): *E. glabripetalus* seedlings were watered daily by distilled water (pH 7.0) without Cd. (2) Single treatment with Cd: soil was sprayed daily with Cd solutions (0.15 mM or 0.30 mM). (3) Single treatment with simulated acid rain: *E. glabripetalus* seedlings were sprayed daily with acid rain solutions (pH 3.0 or 5.6) on leaves. (4) Combined treatment with Cd and simulated acid rain: *E. glabripetalus* seedlings were sprayed with acid rain solutions (pH 3.0 or 5.6) on leaves and Cd solutions (0.15 mM or 0.30 mM) into soil every day. For these 9 treatments, the amount of simulated acid rain or distilled water or Cd solutions was 300 mL per pot every day, as calculated according to precipitation and evaporation in southeastern China. All treatments were replicated in 12 pots. During the 100-day experiment, the places of pots were often randomly switched to avoid differences in microclimate.

2.2 Measurements of chlorophyll fluorescence and photosynthetic parameters

After the 100-day experiment, chlorophyll fluorescence was measured from 09:00-11:30 a.m. using a portable chlorophyll fluorometer (PAM-2000 WALZ, Germany). Three seedlings were selected from each treatment, with measurements performed for

a leaf from the upper, middle and lower parts of the plant. The sampled leaves were
 dark-adapted for 20 minutes using a dark leaf clip before each chlorophyll
 fluorescence measurement. The initial fluorescence (F_0), which was measured using
 an analytical light of $0.05 \mu\text{mol m}^{-2} \text{s}^{-1}$ PFD, was recorded after dark adaptation and,
 subsequently, a saturating pulse ($6,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PFD, 2s) was given to measure
 the maximal fluorescence (F_m). The steady state value of fluorescence (F_s), the
 maximal fluorescence in the light-adapted state (F_m') and the basal fluorescence after
 farred illumination (F_0') were determined by a procedure described by Guo (2005),
 with six measurements conducted per replication. The maximum quantum yield ($F_v /$
 F_m) was determined by $F_v / F_m = (F_m - F_0) / F_m$. F_v is variable fluorescence calculated
 as $F_v = F_m' - F_0'$. Non-photochemical quenching of fluorescence (NPQ) was calculated
 as $NPQ = (F_m - F_m') / F_m'$. Potential activity of photosystem II (PSII) (F_v / F_0) was
 calculated by $F_v / F_0 = (F_m - F_0) / F_0$. Photochemical quenching (qP) was calculated by
 $qP = (F_m' - F_s) / (F_m' - F_0')$.
 Photosynthetic parameters were measured for the same leaves at the same time as for
 chlorophyll fluorescence, using a portable photosynthesis system, Li-6400 (Li-Cor
 Inc. Lincoln, Nebraska, USA). Leaf temperature was 25°C with a relative humidity of
 80% inside the leaf chamber during measuring. Photosynthetic photon flux density
 (PPFD) was controlled at $500 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Ambient CO_2 concentration was 400 ± 5
 $\mu\text{mol} \cdot \text{mol}^{-1}$. Responses to photosynthetically active radiation (PAR) were measured at
 0, 10, 20, 50, 80, 100, 200, 300, 500 and $800 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Net photosynthetic rate
 (P_n) - PAR response curves were generated by plotting P_n as a function of PAR. These

curves were modeled by fitting data to a nonrectangular hyperbola (Lambers et al., 1998) by means of the nonlinear least squares curve-fitting procedure of *Photosyn Assistant software* (Dundee Scientific, Scotland, UK). This model is expressed as follows:

$$P_n = [AQY \times PAR + A_{max} - \sqrt{(AQY \times PAR + A_{max})^2 - 4 \times AQY \times A_{max} \times PAR \times K} / 2 \times K] - R_d$$

where *AQY* is apparent quantum yield [$\mu\text{mol (CO}_2\text{)} \mu\text{mol}^{-1}$ (photon)], *PAR* is the photosynthetic active radiation, *A_{max}* is the maximum net photosynthetic rate, *K* is the curvature factor, and *R_d* is leaf-level “dark” respiration [$\mu\text{mol (CO}_2\text{)} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$]. Light saturation point (*L_{SP}*) and light compensation point (*L_{CP}*) were estimated.

2.3 Measurements of antioxidant system

1 g leaves from control and treated plants were ground in liquid nitrogen, then extracted with 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 1% (w/v) PVP, 0.1 mM PMSF, and 0.2% (v/v) Triton X100 for the measurements of peroxidase (POD) and catalase (CAT) activities and the content of reduced glutathione (GSH). The homogenate was centrifuged at 14 000 ×g at 4°C for 15 min. The supernatants were used for the enzyme assays. The means ± SE were calculated from the data of at least 3 independent measurements.

POD activity was determined by monitoring the increase in absorbance at 470 nm during the oxidation of guaiacol (Upadhyaya et al., 1985). The amount of enzyme producing 1 $\mu\text{mol/min}$ of oxidized guaiacol was defined as 1 U. CAT activity was determined by UV spectrophotometry by a direct measurement of H₂O₂

decomposition at 240 nm, following the procedure of Aebi (1984); 1 U equals the amount of H₂O₂ (in μmol) decomposed in 1 min.

Reduced (GSH) contents were measured according to Griffith (Griffith, 1980). The contents were determined in a reaction mixture containing leaf extract, 100 mM sodium phosphate buffer (pH 7.7) and 100 mM sodium phosphate buffer (pH 6.8) containing 4 mM 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction mixture was maintained at 25°C for 10 min and the absorbance was read at 412 nm. A standard graph was drawn using different concentrations of a GSH solution. With the help of the standard graph, reduced GSH contents in the leaves were calculated.

Relative electric conductivity was determined using the conductivity instrument. First, 1 g fresh leaves were cut and completely immersed in 20 ml deionized water, and then degassed for 15min. The initial conductivity (E₁) was measured with a conductivity instrument (LC116, Mettler Toledo Instruments Co., Ltd, Shanghai, China) after 30 mins. The solution was then incubated at 100 ° C for 15 min and cooled to room temperature, and the final conductivity (E₂) was determined. The conductivity of deionized water was also determined as blank conductivity (E₀). The relative conductivity was calculated according to the following formula: relative electric conductivity (%) = (E₁ - E₀) / (E₂ - E₀) × 100%.

2.4 Measurements of biomass, and element and Cd contents of plants

Three replicated plants from each treatment were harvested at 100 days. Plant parts, such as stems, leaves and roots (carefully washed and blotted), were separated and dried in an oven at 80 °C for 48h to constant weight and weighed. Dry mass

accumulation was then calculated. The contents of elements (Mg, Fe and Cd) in the leaves, stems and roots of *E. glabripetalus* were measured by ICP-AES (PerkinElmer Inc., Shelton, CT, USA) with three replicates per treatment.

2.5 Data analysis

The significance of differences between the treatments was analyzed by one-way analysis of variance (ANOVA) using SPSS17.0. The interactions between Cd and acid rain were analyzed by the two-way analysis of ANOVA. Prior to analysis, the data were checked for normality and homogeneity of variances. Differences between treatments were detected and mean values were compared by the Tukey's test ($p = 0.05$). All data were expressed as means $\pm$ SE ($n \geq 3$).

3 Results

3.1 Effects of Cd and acid rain on chlorophyll fluorescence in *E. glabripetalus* seedlings

Fig.1 shows the changes in chlorophyll fluorescence parameters when *E. glabripetalus* seedlings were treated with Cd and acid rain. When treated with acid rain only, the F_v/F_m , NPQ , qP and F_v/F_0 values of *E. glabripetalus* seedlings increased compared with control plants. F_v/F_m decreased by 1.20% at pH 5.6 and increased by 0.41% at pH 3.0. NPQ , qP and F_v/F_0 increased by 8.99%, 2.66% and 0.46%, respectively, at pH 5.6, and increased by 10.99%, 1.75% and 0.37%, respectively at pH 3.0. When *E. glabripetalus* seedlings were treated with Cd alone, F_v/F_m decreased by 1.22% and 2.98% at 50 and 100 mg·kg⁻¹ Cd, respectively, in comparison with the control plants. NPQ , qP and F_v/F_0 increased by 16.63%, 0.63 %

and 1.06%, respectively, at 50 mg·kg⁻¹ Cd, while they decreased by 8.36%, 2.11% and 9.88%, respectively, at 100 mg·kg⁻¹ Cd.

When *E. glabripetalus* seedlings were treated with acid rain and 50 mg·kg⁻¹ Cd, F_v/F_m increased by 0.64% at pH 5.6 and 0.08% at pH 3.0 compared with the control plants, by 1.86% and 1.88% compared with the single treatment with pH 5.6 acid rain and the single treatment of 50 mg·kg⁻¹ Cd, respectively (Fig.1). However, when *E. glabripetalus* seedlings were treated with acid rain and 100 mg·kg⁻¹ Cd, F_v/F_m decreased compared with control or acid rain-treated plants. NPQ , qP and F_v/F_0 decreased by 7.05%, 2.51% and 5.80%, compared with control plants. Decreases in NPQ , qP and F_v/F_0 were found in some combined treatments with Cd and acid rain. For example, when the seedlings were treated with acid rain at pH 3.0 and 100 mg·kg⁻¹ Cd, NPQ decreased by 22.40%, 30.08% and 15.32%, respectively, compared with control plants and those treated with 100 mg·kg⁻¹ Cd alone and acid rain at pH 3.0 alone, respectively. The two-way ANOVA analysis indicated that there was no interaction between Cd and acid rain on chlorophyll fluorescence parameters (F_v/F_m , NPQ , qP and F_v/F_0). The values of NPQ , qP and F_v/F_0 of *E. glabripetalus* seedlings kept under the combined treatment with a high concentration of Cd (100 mg·kg⁻¹) and low pH acid rain (pH 3.0) were lower than those in seedlings exposed to a single treatment with Cd or acid rain, indicating that 100 mg·kg⁻¹ Cd and pH 3.0 acid rain have a synergistic effect on most chlorophyll fluorescence parameters.

3.2 Effects of Cd and acid rain on photosynthetic parameters in E. glabripetalus seedlings

When the seedlings were treated with acid rain only, A_{max} increased at pH 3.0 and pH 5.6 compared with control seedlings ($p > 0.05$) (Table 1). A_{max} increased by 30.36% at pH 5.6 and 11.04% at pH 3.0. When the seedlings were treated with Cd alone, A_{max} increased by 52.65% at 50 mg·kg⁻¹ Cd, while it decreased slightly at 100 mg·kg⁻¹ Cd compared with control seedlings. However, when *E. glabripetalus* seedlings were treated with acid rain and 50 mg·kg⁻¹ Cd, A_{max} increased by 1.49% at pH 5.6 and 15.07% at pH 3.0 ($p > 0.05$), while there was a decrease by 57.96% at pH 5.6 and 39.07% at pH 3.0 under 100 mg·kg⁻¹ Cd treatment ($p > 0.05$). AQY increased when the seedlings were treated with acid rain or Cd treatment only. The combined treatment with pH 3.0 acid rain increased AQY by 80% at 50 mg·kg⁻¹ Cd and by 56% at 100 mg·kg⁻¹ Cd ($p < 0.05$). Compared with control plants, L_{CP} decreased in seedlings treated with acid rain or Cd only ($p < 0.05$). When *E. glabripetalus* seedlings were treated with pH 3.0 acid rain and Cd, L_{CP} decreased by 57.89% at 50 mg·kg⁻¹ Cd and by 21.05% at 100 mg·kg⁻¹ Cd. L_{SP} increased at 50 mg·kg⁻¹ Cd and decreased at 100 mg·kg⁻¹ Cd ($p < 0.05$), and acid rain treatment alone decreased L_{SP} ($p < 0.05$). Under a combined treatment with acid rain and 100 mg·kg⁻¹ Cd, L_{SP} decreased by 65.18% at pH 5.6 and by 54.31% at pH 3.0 ($p < 0.05$) (Table 1). The two-way ANOVA analysis indicated that there was no interaction effect between Cd and acid rain on photosynthetic parameters.

3.3 Effects of Cd and acid rain on POD and CAT activities, relative electric conductivity and GSH content in *E. glabripetalus* seedlings

When *E. glabripetalus* seedlings were treated with acid rain alone, POD and CAT

262 activities increased along with decreasing pH values but no significant difference
 263 were detected compared with the control plants ($p > 0.05$) (Fig.2). The POD activity
 264 increased with an increasing Cd concentration. At 100 mg·kg⁻¹ Cd, the POD activity
 265 was significantly higher than that of the control plants ($p < 0.05$), with an increase of
 266 63.80%. When the seedlings were treated by both acid rain and Cd, the POD activity
 267 was significantly higher than that of control plants ($p < 0.05$), increases equaling
 268 176.45% at pH 5.6 and 226.05% at pH 3.0 under 100 mg·kg⁻¹ Cd. The CAT activity
 269 increased at 50 mg·kg⁻¹ Cd ($p > 0.05$) while decreased at 100 mg·kg⁻¹ Cd ($p < 0.05$).
 270 The CAT activity was higher than that of control plants under a combination of pH 3.0
 271 acid rain and 50mg·kg⁻¹ Cd, while the CAT activity was lower under a combination of
 272 100 mg·kg⁻¹ Cd and pH 3.0 / 5.6 acid rain than that of control plants ($p < 0.05$).
 273 When *E. glabripetalus* seedlings were treated with single acid rain, relative electric
 274 conductivity decreased but showed no significant difference compared with the
 275 control plants ($p > 0.05$), (Fig.2). When treated with Cd alone, relative electric
 276 conductivity increased significantly at 100 mg·kg⁻¹ Cd ($P < 0.05$). Under the
 277 combined treatment with acid rain and 100 mg·kg⁻¹ Cd, relative electric conductivity
 278 increased by 73.93% at pH 5.6 and 165.64% at pH 3.0 ($p < 0.05$).
 279 Compared with the control seedlings, the GSH content decreased under acid rain
 280 (Fig.2). At 50 mg·kg⁻¹ Cd ($p < 0.05$), the GSH content increased but decreased at 100
 281 mg·kg⁻¹ Cd ($p < 0.05$). Under the combined treatment with acid rain and 100mg·kg⁻¹
 282 Cd, the GSH content decreased by 21.30% at pH 5.6 and 25.16% at pH 3.0 ($p < 0.05$).
 283 The ANOVA analysis showed that there was a significant interaction effect between

Cd and acid rain on POD and CAT activities, and on the GSH content. Under the combined treatment with a high concentration of Cd and low pH acid rain, the POD and CAT activities of *E. glabripetalus* seedlings were lower than those under Cd or acid rain alone, thus indicating that 100 mg·kg⁻¹ Cd and pH 3.0 acid rain have a synergistic effect on antioxidant enzymes.

3.4 Effects of Cd and acid rain on biomass in *E. glabripetalus* seedlings

Table 2 shows the biomass accumulation of *E. glabripetalus* exposed to simulated acid rain and Cd. A acid rain treatment alone increased the total biomass by 15.92% at pH 5.6 and 20.66% at pH3.0 compared with the control plants. The biomass allocation of plants kept in control conditions was 2.99% in leaves, 46.70% in stem and 25.31% in roots. The root biomass increased by 34.98% at pH 5.6, while there was more biomass in leaves at pH 3.0 (37.30%). When the seedlings were treated by Cd alone, the stem biomass reduced by 38.03% and 41.47% at 50 and 100 mg·kg⁻¹ Cd, respectively, in comparison with control plants. Also, 50 mg·kg⁻¹ Cd increased the total biomass by 20.37% while 100 mg·kg⁻¹ Cd decreased the total biomass by 11.00%. The combined acid rain and Cd stress decreased biomass allocation into the stem. For example, the proportion of biomass in stem was 37.70% and 37.93% at pH 3.0 and 5.6 under 100 mg·kg⁻¹ Cd treatment, respectively. The total biomass increased under the combined 50 mg·kg⁻¹ Cd and acid rain treatment ($p > 0.05$), while it decreased under 100 mg·kg⁻¹ Cd and acid rain ($p < 0.05$).

3.5 Effects of Cd and acid rain on Mg, Fe and Cd contents in *E. glabripetalus* seedlings

306 Acid rain treatment alone decreased significantly the Mg content of stems and leaves
 307 compared with the control plants, while the slight decrease in roots was not significant
 308 (Table 3). When seedlings were treated by Cd alone, the Mg content of roots and
 309 stems decreased but not significantly ($p > 0.05$). However, 50 mg·kg⁻¹ Cd increased
 310 the Mg content of leaves by 6.82%, while 100 mg·kg⁻¹ Cd decreased it by 30.68% ($p <$
 311 0.05) in comparison with the control plants. When the seedlings were treated with the
 312 combined stress of pH 5.6 acid rain and Cd, the Mg content of roots decreased
 313 significantly ($p < 0.05$), by 16.20% and 12.55% at 50 and 100 mg·kg⁻¹ Cd,
 314 respectively. Similarly, the Mg content of leaves decreased significantly compared
 315 with control plants ($p < 0.05$) under the combined stress of acid rain and Cd. The total
 316 Mg content of plants decreased under acid rain or Cd stress alone and under the
 317 combined stress.

318 The acid rain treatment increased the Fe content in roots compared with the control
 319 plants ($p < 0.05$) (Table 4). When the seedlings were treated by Cd alone, the Fe
 320 content of roots decreased significantly at 100 mg·kg⁻¹ Cd ($p < 0.05$). However, 50
 321 mg·kg⁻¹ Cd increased the Fe content of stems ($p < 0.05$). When the seedlings were
 322 exposed to pH 5.6 acid rain and 100 mg·kg⁻¹ Cd, the Fe content of roots decreased
 323 significantly compared with control plants ($p < 0.05$). Mostly, there were no
 324 significant differences in the Fe contents of stems and leaves between the treatments
 325 and controls. The total Fe content of plants increased under acid rain stress alone
 326 (pH3.0, $p < 0.05$) and under the combination of 50mg·kg⁻¹ Cd and acid rain, while it
 327 decreased under the combined stress of 100mg·kg⁻¹ Cd and acid rain ($p < 0.05$).

The Cd content of plants was higher at 100 mg·kg⁻¹ Cd compared with 50 mg·kg⁻¹ Cd (Table 5). Most Cd distributed into roots under exposure to Cd alone, with the proportions equaling 91.27% and 91.45% at 50 and 100 mg·kg⁻¹ Cd, respectively. Acid rain was found to decrease the total Cd content of plants compared with the single Cd treatment. The total Cd content of plants decreased by 24.82% under the combined treatment with 50 mg·kg⁻¹ Cd and pH 3.0. However, in most cases, there were no significant differences between the effects of the single Cd treatment and the combined treatment on roots, stems or leaves.

4 Discussion

It has been reported that single treatments with Cd or acid rain decrease photosynthesis (Parmar et al., 2013, Li et al., 2015, Sun et al., 2016). Chlorophyll fluorescence and photosynthetic parameters are easily affected by environmental stress and they are commonly used as sensitive indicators of a plant's photosynthetic performance. In our study on *E. glabripetalus* seedlings, the treatment by acid rain only increased F_v/F_m , NPQ , qP , F_v/F_0 , A_{max} and AQY , which showed that the absorption of light energy, electron transport, conversion of light energy and net photosynthesis rate increased (Fig. 1 and Table 1). The results were in agreement with previous reports on the effects of acid rain on the photosynthesis in *E. glabripetalus* (Liu et al., 2015). Both Cd treatments decreased F_v/F_m and the 100 mg·kg⁻¹ Cd treatment decreased NPQ , qP , F_v/F_0 , A_{max} and AQY . These results showed that high Cd concentrations decrease the light reaction of photosynthesis, conversion of light energy and net photosynthesis rate, resulting in the inhibition of photosynthesis in *E.*

glabripetalus seedlings. The effects of Cd on *E. glabripetalus* seedling are similar to most reports on other plant species (Santos et al., 2012, Xue et al., 2013). An acid rain treatment alone or a high Cd concentration decreased L_{CP} and L_{SP} of *E. glabripetalus* seedlings, which indicated enhanced and reduced light use efficacy at low and high light levels, respectively. Interestingly, the combined treatment of Cd and acid rain did not alleviate the effect induced by the Cd treatment alone; instead, the combination decreased the chlorophyll fluorescence parameters induced by the Cd treatment. Thus, the combined stress led to a more serious decrease in the photosynthesis of *E. glabripetalus* seedlings. In addition, the degree of change in chlorophyll fluorescence and photosynthetic parameters in seedlings treated with Cd and acid rain depended on the concentration of Cd and the pH value used to simulate acid rain. In most cases, the combined treatment with Cd and acid rain decreased NPQ , qP and F_v/F_0 , and there was a greater inhibition in the absorption of light energy, electron transport and light reaction than under the Cd treatment alone (Fig.1).

Using two-way ANOVA analyses, we found that there are no significant interactive effects between Cd and acid rain on chlorophyll fluorescence and photosynthetic parameters mentioned above. However, the values of NPQ , qP , F_v/F_0 , A_{max} and L_{SP} in *E. glabripetalus* seedlings exposed to the combined treatment with a high concentration of Cd ($100 \text{ mg}\cdot\text{kg}^{-1}$) and low pH acid rain (pH 3.0) decreased more than those in seedlings exposed to other treatments, indicating that a high concentration Cd and low pH acid rain show a synergistic effect on most chlorophyll fluorescence parameters of *E. glabripetalus*. Our result is different from a previous report that

373 showed that there is an interactive effect of Cd and acid rain on chlorophyll
374 fluorescence parameters in soybean seedlings (Sun et al., 2012). It has been
375 previously shown that a high amount of Cd in soil can inhibit the chlorophyll
376 biosynthesis, lead to the reduction in chlorophyll contents and photoactivation of PSII
377 (Sun et al., 2012, Dias et al., 2013, Li et al., 2015). We conclude that in *E.*
378 *glabripetalus* seedlings, the combination of low pH acid rain and a high Cd
379 concentration would damage the chloroplast structure and lead to a greater decrease in
380 the chlorophyll fluorescence parameters, net photosynthesis rate and light use
381 efficacy.

382 In general, antioxidant metabolism is regarded as an important mechanism that helps
383 to reduce the excessive accumulation of reactive oxygen species (ROS) induced by
384 environment stress (Chaoui et al., 1997, Zhang et al., 2015). Acid rain or Cd stress
385 can induce membrane damage, increase membrane permeability and the accumulation
386 of free radicals in plants (Chaoui et al., 1997, Mobin and Khan, 2007, Debnath et al.,
387 2018). However, active oxygen-scavenging systems can protect membranes from the
388 deleterious effects (Jaleel et al., 2009). Previous studies have indicated that activities
389 of antioxidant enzymes and relative electric conductivity are correlated with plant
390 tolerance to abiotic stresses. Cd-induced damage has been shown to be negatively
391 correlated with the capacity to increase POD and CAT activities and relative electric
392 conductivity in many plants (Liu et al., 2017). According to our research, single acid
393 rain increased the POD and CAT activities in leaves. Generally, Cd toxicity can
394 increase (Srivastava et al., 2014) or decrease (Hassan et al., 2005) POD or CAT

activities depending on the stress intensity and duration or on the plant's developmental stage (Liu et al., 2017). However, a low Cd concentration (50 mg·kg⁻¹) increased CAT activity, while a high Cd concentration (100 mg·kg⁻¹) decreased the CAT activity and relative electrolyte leakage in leaves. This is in agreement with a report on soybean published by Liu (2011). Similarly to single Cd or acid rain treatments, the combined treatment with acid rain and Cd increased the POD activity significantly ($p < 0.05$). The combination of a high Cd concentration (100 mg·kg⁻¹) and acid rain (pH 3.0 and 5.6) inhibited the CAT activity ($p < 0.05$), while the POD activity and relative electrolyte leakage increased (Fig.2). Thus, a moderate Cd concentration and acid rain induced lipid peroxidation and increased POD and CAT activities, while a high Cd concentration (100 mg·kg⁻¹) inhibited the CAT activity and increased relative electrolyte leakage. The same effects have been observed in wheat treated with La³⁺ (d'Aquino et al., 2009) and soybean treated with La³⁺ and acid rain (Zhang et al., 2015). The increased POD and the decreased CAT could not alleviate the peroxidation damage to the cell membrane. The deleterious effects on the cell membrane aggravated the electrolyte leakage from the cytoplasm (the membrane permeability), as indicated by the increased relative electrolyte leakage. In addition, we analyzed the interaction between Cd and acid rain on the antioxidant enzyme system of *E. glabripetalus* seedlings and found that there was a synergistic effect of Cd and acid rain on the antioxidant enzyme system of *E. glabripetalus* seedlings.

GSH is a major low molecular-weight free thiol tripeptide in plant and animal cells. It

417 is involved in the cellular defense against the toxic action of oxyradicals, salinity,
418 acidity and heavy metal (David et al., 2005). GSH serves as an important defense line
419 against ROS to protect cells from oxidative stress damage and to modify metal
420 toxicity . It has been reported that the GSH content of a cell could either decrease or
421 increase under metal exposure (Maity et al., 2018, Kim et al., 2018). When *E.*
422 *glabripetalus* seedlings were treated with either acid rain or a high concentration of
423 Cd (100 mg·kg⁻¹), the GSH content decreased. Also the combination of acid rain and
424 100mg·kg⁻¹ Cd decreased the GSH content ($p < 0.05$). This result indicated that GSH
425 is involved in the cellular defense against acid rain and Cd stress and alleviates
426 oxidative stress. It may be an antioxidant intervention strategy to prevent oxidative
427 stress under Cd and acid rain stress.

428 The total biomass increased when *E. glabripetalus* seedlings were treated by acid rain,
429 as also reported in our previous study (Liu et al., 2015). The reason may be that
430 nitrogen (N) is added into the soil in the acid rain, which then accelerates the biomass
431 accumulation. We found that a high pH value enhances biomass accumulation in
432 leaves, while a low pH value facilitates root growth. However, a low Cd concentration
433 (50 mg·kg⁻¹ Cd) (with or without acid rain) increases the total biomass, while a high
434 Cd concentration (100 mg·kg⁻¹) (with or without acid rain) decreases the total
435 biomass. It has been found also in soybean that a low Cd concentration shows a
436 positive effect on biomass accumulation (Liu et al., 2011). Yet, despite the different
437 effects of Cd and acid rain the, two-way ANOVA analysis indicated that there was no
438 significant interaction between Cd and acid rain on the total biomass of *E.*

439 *glabripetalus* seedlings.

440 Cd, which is a common heavy metal pollutant in soil, could accumulate within plant

441 organs and negatively affect essential physiological processes, thus limiting plants'

442 productivity (Gill et al., 2012). The exposure of *E. glabripetalus* to Cd stress leads to

443 an accumulation of Cd mainly in roots (> 90%). These results are consistent with

444 other reports showing that Cd is mainly retained in roots (Gill et al., 2012, Dias et al.,

445 2013). Partitioning of heavy metals in different plant organs is a strategy to avoid

446 toxicity for a plant (López-Climent et al., 2011, Dias et al., 2013).

447 Acid rain was found to decrease the total Cd content of plants compared with the

448 single treatment of Cd. The total Cd content in plants decreased evenly by 24.8% at

449 50 mg·kg⁻¹ Cd and pH 3.0 acid rain. There is a competition between H⁺ and Cd²⁺

450 uptake (Li et al., 2009). At low pH, Cd is likely to form insoluble precipitates with thiol

451 base synthesized by sulphur ions under acid rain, which may inhibit the absorption of

452 Cd and affect the Cd transportation in plants. However, in most cases, there were no

453 significant differences between the effects of single Cd treatments and combined

454 treatments on roots, stems or leaves. We suggest that an acid rain treatment inhibits *E.*

455 *glabripetalus* seedlings from absorbing more Cd, leading to a higher accumulation of

456 Cd in roots when compared to a single Cd treatment.

457 It is important to notice that Cd influences the uptake of other mineral nutrients in

458 plant (Dias et al., 2013). In this study, we detected a significant reduction of Fe and

459 Mg only at a high Cd concentration, which suggests that Cd interferes with the

460 translocation of micronutrients to leaves and roots. Fe plays an important role in

electron transfer, because it is a cofactor in the photosynthetic processes, such as PSII and PSI. Although acid rain increases the total amount of Fe in plants, Cd-induced Fe deficiency affects the photosynthetic electron transport. In agreement with this, the reduction in F_v/F_m may be associated to the decreased amount of Fe. These results are similar to those reported in other plant species (López-Millán et al., 2009, Dias et al., 2013). Fe and Cd have similar binding sites at the cell; therefore, a high concentration of Cd leads to a decrease in Fe absorption by the cell. Mg is an essential component of the chlorophyll (Chl) molecule with a very important role in photosynthesis, such as Chl biosynthesis and photochemical reactions (Peng et al., 2015). In addition, Mg acts as a cofactor for many enzymes and functions in many other physiological processes, such as the formation of reactive oxygen species (ROS) and related photooxidative damage (Verbruggen and Hermans, 2013, Peng et al., 2015). The total Mg content in plants decreased under acid rain, Cd stress and the combined acid rain and Cd stress. The decrease in Mg might cause an increase in the production of ROS and antioxidant enzymes and, furthermore, impaired photosynthesis (Peng et al., 2015). Our results clearly showed that under a low Cd concentration and acid rain, decreases in Mg in the roots and leaves of *E. glabripetalus* caused decreases in A_{max} and L_{CP} , and increases in the POD and CAT activities, GSH content and relative electric conductivity.

5 Conclusion

(1) Combined treatment of a high concentration of Cd ($100 \text{ mg}\cdot\text{kg}^{-1}$) and low pH acid rain (pH 3.0) decreased NPQ , qP , F_v/F_0 , A_{max} and L_{SP} in *E. glabripetalus* seedlings, indicating that Cd and acid rain have a synergistic effect on most chlorophyll fluorescence and photosynthesis parameters. (2) Combined treatment of a moderate concentration of Cd ($50 \text{ mg}\cdot\text{kg}^{-1}$) and acid rain increased POD and CAT activities, GSH content and relative electric conductivity, while the combined stress with a high concentration of Cd and low pH acid rain decreased the GSH content and CAT activity. (3) Combined treatment of high concentration of Cd ($100 \text{ mg}\cdot\text{kg}^{-1}$) and acid rain decreased the total biomass and changed biomass allocation into different plant organs. (4) Cd mainly accumulated in roots, and acid rain decreased the total Cd content. (5) Combined treatment of a high concentration of Cd ($100 \text{ mg}\cdot\text{kg}^{-1}$) and acid rain decreased Fe and Mg contents, further resulting in the inhibition of photosynthesis, increases or decreases in the antioxidant enzyme activities, and reduction in biomass.

According to our results, a high concentration of Cd ($100 \text{ mg}\cdot\text{kg}^{-1}$) and low pH acid rain (pH 3.0) have toxic effects on the physiological processes of plants. Our study provides novel insights into the combined effects of Cd and acid rain on woody plants and might also serve as a guide to evaluate forest restoration and biological safety in areas with Cd pollution in acid rain.

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Table 1 Effects of Cd and simulated acid rain on photosynthetic parameters, including the maximum net photosynthetic rate (A_{max}), apparent quantum yield (AQY), light saturation point (L_{SP}) and light compensation point (L_{CP}), in *Elaeocarpus glabripetalus* seedlings.

Cd (mg·kg ⁻¹)	Acid rain pH	A_{max} ($\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	AQY ($\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	L_{CP} ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	L_{SP} ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)
0	7.0	4.71±0.02ab	0.025±0.001c	30.4±0.2ab	500.8±34.3a
	5.6	6.14±0.04a	0.053±0.001ab	24.0±0.3b	260.8±20.6bc
	3.0	5.23±0.18ab	0.025±0.001c	25.6±0.2ab	364.8±29.7b
50	7.0	7.19±0.04a	0.281±0.001a	14.4±0.9c	526.4±32.9a
	5.6	4.78±0.03ab	0.063±0.001ab	17.6±1.0bc	468.8±27.3ab
	3.0	5.42±0.19ab	0.045±0.001b	12.8±1.0c	569.6±47.3a
100	7.0	4.69±0.03b	0.149±0.001a	16.0±0.6bc	96.0±14.5c
	5.6	1.98±0.01c	0.019±0.002c	38.4±0.9a	174.4±13.3c
	3.0	2.87±0.01c	0.039±0.001bc	24.0±0.5b	228.8±28.6bc

Data are expressed as mean values ± SE of three replicates. Significant differences at $p < 0.05$ are showed with different letters in each column.

Table 2 Effects of Cd and simulated acid rain on the biomass in *Elaeocarpus glabripetalus* seedlings

Cd (mg·kg ⁻¹)	Acid rain pH	Leaf biomass (g)	Stem biomass (g)	Root biomass (g)	Total biomass (g)
0	7.0	6.66±0.42b	11.12±0.99a	6.02±0.52b	23.80±1.76b
	5.6	7.64±0.84ab	10.33±0.87ab	9.65±0.82a	27.62±2.39a
	3.0	10.71±0.98a	10.25±0.97ab	7.75±0.85ab	28.71±0.14a
50	7.0	9.02±0.38ab	10.90±0.36a	8.74±0.67ab	28.65±1.10a
	5.6	7.92±0.78ab	9.34±0.56ab	7.34±0.51ab	24.60±0.23ab
	3.0	7.37±0.85ab	10.01±0.95ab	6.98±0.43ab	24.36±2.24ab
100	7.0	6.55±0.89b	8.78±0.42ab	5.84±0.67b	21.17±2.21bc
	5.6	6.61±0.88b	7.64±0.81b	6.37±0.78ab	20.62±2.88bc
	3.0	5.98±0.96b	7.06±0.67b	5.58±0.45b	18.62±2.76c

Data are expressed as mean values ± SE of three replicates. Significant differences at $p < 0.05$ are showed with different letters in each column.

Table 3 Effects of Cd and simulated acid rain on the Mg content in *Elaeocarpus glabripetalus* seedlings

Cd (mg·kg ⁻¹)	Acid rain pH	Leaf Mg (mg·kg ⁻¹)	Stem Mg (mg·kg ⁻¹)	Root Mg (mg·kg ⁻¹)	Total Mg (mg·kg ⁻¹)
0	7.0	0.88±0.07a	0.69±0.05ab	8.21±0.12a	9.78±0.23a
	5.6	0.71±0.08c	0.65±0.06b	8.16±0.036ab	9.52±0.32a
	3.0	0.86±0.05b	0.56±0.04c	7.29±0.14ab	8.71±0.24b
50	7.0	0.94±0.01a	0.66±0.04b	7.23±0.41ab	8.83±0.13ab
	5.6	0.61±0.08d	0.61±0.03b	6.88±0.63b	8.10±0.12c
	3.0	0.63±0.05d	0.71±0.02ab	7.48±0.46ab	8.82±0.15ab
100	7.0	0.61±0.08d	0.67±0.07b	7.42±0.53ab	8.70±0.35b
	5.6	0.65±0.03cd	0.75±0.05a	7.18±0.55b	8.58±0.53bc
	3.0	0.64±0.08cd	0.64±0.04b	7.54±0.46ab	8.82±0.14ab

Data are expressed as mean values ± SE of three replicates. Significant differences at $p < 0.05$ are showed with different letters in each column.

Table 4 Effects of Cd and simulated acid rain on the Fe content in *Elaeocarpus glabripetalus* seedlings

Cd (mg·kg ⁻¹)	Acid rain pH	Leaf Fe (mg·kg ⁻¹)	Stem Fe (mg·kg ⁻¹)	Root Fe (mg·kg ⁻¹)	Total Fe (mg·kg ⁻¹)
0	7.0	3.08±0.16b	3.21±0.23bc	6.67±0.62c	12.96±0.56b
	5.6	2.88±0.19b	3.35±0.22bc	9.50±0.31b	15.70±0.67ab
	3.0	3.09±0.21b	3.28±0.06bc	14.50±0.95a	20.87±0.87a
50	7.0	3.33±0.01ab	4.33±0.08a	6.83±0.22c	14.40±0.34ab
	5.6	3.35±0.34ab	3.59±0.15b	7.81±0.35c	14.70±0.44ab
	3.0	3.81±0.38a	4.49±0.02a	6.46±0.21c	14.76±0.49ab
100	7.0	2.79±0.11b	3.09±0.23c	4.82±0.41d	10.70±0.31c
	5.6	2.59±0.26b	3.02±0.11c	3.58±0.32d	9.19±0.24c
	3.0	2.65±0.02b	3.43±0.16bc	5.47±0.03c	12.55±0.35c

Data are expressed as mean values ± SE of three replicates. Significant differences at $p < 0.05$ are showed with different letters in each column.

Table 5 Effects of Cd and simulated acid rain on Cd content in *Elaeocarpus glabripetalus* seedlings

Cd (mg·kg ⁻¹)	Acid rain pH	Leaf Cd (mg·kg ⁻¹)	Stem Cd (mg·kg ⁻¹)	Root Cd (mg·kg ⁻¹)
50	7.0	0.010±0.006ab	0.29±0.04a	3.25±0.24ab
	5.6	0.008±0.0006b	0.23±0.05ab	2.77±0.28ab
	3.0	0.007±0.0002b	0.12±0.01b	2.55±0.16b
100	7.0	0.027±0.005a	0.33±0.03a	3.82±0.32a
	5.6	0.017±0.006ab	0.35±0.05a	3.17±0.42ab
	3.0	0.014±0.003ab	0.32±0.03a	3.49±0.25ab

Data are expressed as mean values ± SE of three replicates. Significant differences at $p < 0.05$ are showed with different letters in each column.

Fig.1 Effects of Cd and simulated acid rain on the maximum quantum yield (F_v/F_m), non-photochemical quenching of fluorescence (NPQ), potential activity of PSII (F_v/F_0) and photochemical quenching (qP) in *Eleocarpus glabripetalus* seedlings. Significant differences at $P < 0.05$ are shown with different letters.

Fig.2 POD and CAT activities, GSH contents and relative electric conductivity in *Elaeocarpus glabripetalus* seedlings under acid rain and Cd treatments. Different lowercase letters denote significant differences between treatments at the 5% level.