



Research article

Growth and physiological response of an endangered tree, *Horsfieldia hainanensis* merr., to simulated sulfuric and nitric acid rain in southern China

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ABSTRACT

As nitrogen deposition increases, acid rain is gradually shifting from sulfuric acid rain (SAR) to nitric acid rain (NAR). Acid rain can severely affect plant growth, damage ecosystems, and reduce biodiversity. Thus, a shift in acid rain type presents another challenge to the conservation of endangered plant species. We investigated the effect of three acid rain types (SAR, mixed acid rain [MAR], and NAR) and pH on the growth of an endangered Chinese endemic tree, *Horsfieldia hainanensis* Merr., using simulated rain in a greenhouse environment. Over nine months, growth indices, chlorophyll content, antioxidant enzyme activity, malondialdehyde content, and chlorophyll fluorescence parameters were investigated for treated and control saplings. The results indicated that at a pH of 5.6, *H. hainanensis* could adapt to SAR and MAR, but NAR inhibited below-ground growth. At a pH of 2.5 and 4.0, SAR inhibited stem and leaf biomass accumulation, whereas NAR inhibited root biomass accumulation and altered root morphology. MAR had intermediary effects between those of SAR and NAR. Adverse effects on leaf physiology were reduced as the rain type shifted from SAR to NAR; however, roots were increasingly adversely affected. Our results suggest that conservation efforts for *H. hainanensis* should shift from an above-ground to a below-ground focus as acid rain transitions toward NAR.

1. Introduction

Horsfieldia hainanensis Merr. (Myristicaceae) is an endemic species to China (Jiang et al., 2017). It contains fatty acids such as myristic and lauric acid, which are vital raw materials for the medical (Tada et al., 2016), cosmetic (Dumitrascu et al., 2018; Garg et al., 2017), spice (Hwang et al., 2011; Srinivasan, 2013), and textile industry (Biçer et al., 2015). Recently, *H. hainanensis* was listed as an endangered tree species and a second-grade protected plant due to illegal logging and habitat destruction (Jiang et al., 2017).

Acid rain is a global pollutant, especially in northern Europe, North America, and China (Ren et al., 2018). In China, rapid development has led to the increased production of the acid precursors sulfur dioxide (SO₂) and nitrogen dioxide (NO_x) (Mao et al., 2014), which react with water to form acids (Zhang et al., 2007). Due to coal combustion, SO₄²⁻ is the main anion in acid rain in China (Liang et al., 2016; Zhang et al.,

2017), and high levels of acid rain fall throughout the country, particularly in the south (Sun et al., 2016).

Successful efforts to significantly reduce SO₂ emissions in China have included closing high-emission factories, using cleaner production technologies, and implementing industrial emission control measures (Chan and Yao, 2008). Yet, NO_x emissions have increased due to a rapid increase in the number of motor vehicles (Liu et al., 2018a), and acid rain is gradually shifting from sulfuric acid rain (SAR) to nitric acid rain (NAR) (Lv et al., 2014). Research has shown that the ratio of SO₄²⁻ to NO₃⁻ in acid rain influences soil microorganisms, soil enzyme activity, litter decomposition, fine-root element contents, and antioxidant enzyme activities (Lv et al., 2014; Liu et al., 2018a). Liu et al. (2018b) reported that pH and SO₄²⁻:NO₃⁻ ratios impacted Chinese fir saplings, and acid rain with a high concentration of NO₃⁻ could change root and leaf characteristics, and inhibit growth in this species. Chen et al. (2013) showed that *Liquidambar formosana* was more affected by SAR

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than *Schima superba*, and SAR caused more harm to plants than mixed acid rain (MAR) or NAR. Lee et al. (2006) found that acid rain containing SO_4^{2-} did more harm to stems and roots than acid rain without SO_4^{2-} . Most research on the effects of acid rain on plants has focused on a single acid rain type, typically SAR (Wang et al., 2014; Hu et al., 2016). Furthermore, there is a lack research on the effects of different acid rain types on *H. hainanensis*. The response of this species to various acid rain types is unknown, as are conservation measures to mitigate any potential negative effects caused by the shift from SAR to NAR in China.

We conducted a series of pot experiments to investigate the effect of acid rain on the growth of *H. hainanensis* saplings. Specifically, we evaluated the effects of acid rain type and pH on biomass, root index, antioxidant enzyme activity, chlorophyll (Chl) content, malondialdehyde (MDA) content, and Chl fluorescence parameters. These indices provide insight into the response of *H. hainanensis* saplings to changes in acid rain type. Understanding the response of this valuable species aids in protecting remaining populations and provides a theoretical basis for biodiversity protection in southern China in response to the threat of global environmental change.

2. Materials and methods

2.1. Plant materials and experimental treatments

Sapling experiments were conducted in a greenhouse at the Forestry College of Guangxi University (22°85'N, 108°29'E), Guangxi Zhuang Autonomous Region, China, from March to November 2018. The average relative humidity was 79% and greenhouse temperatures ranged from 25 to 35 °C. *Horsfieldia hainanensis* Merr. saplings (50 ± 1.50 cm in height and 10 ± 0.5 mm in diameter at the base) were provided by the Guangxi Academy of Forestry Sciences. In March 2018, saplings with good growth were planted individually in plastic nutrient pots (30 cm × 24 cm). The cultivation medium was prepared based on the soil properties of natural stands of *H. hainanensis* in Guangxi Zhuang Autonomous Region, China. Our medium was composed of lateritic red soil and sandy soil at a dry-mass ratio of 3:1. The soil texture was sticky and contained quartz sand, with a pH of 6.83 ± 0.01. After transplanting, saplings were allowed to recover for two months and were watered with deionized water for the duration of the recovery period.

In early May 2018, 80 *H. hainanensis* saplings with similar growth were selected for the simulated acid rain trials, which used a randomized grouping design. To mimic the real world change in acid rain, three rain types were prepared by mixing H_2SO_4 (percent concentration 98%, concentration 1.84 g mL⁻¹) and HNO_3 (percent concentration 68%, concentration 1.4 g mL⁻¹) at a volume ratio of 8:1 (SAR), 1:1 (MAR), and 1:8 (NAR), respectively. Deionized water was then used to adjust the solution pH to 2.5, 4.0, and 5.6 for each acid rain type, respectively, yielding nine acid treatments and an additional control treatment (deionized water with no acid). Eight replicates were used per treatment.

The volume of acid rain sprayed on treated plants was determined according to the average annual rainfall of Nanning, Guangxi Zhuang Autonomous Region (1304 mm). Over the course of the experiment, rainfall was approximately three quarters (978 mm) of the annual average, and average monthly rainfall during the experiment was 140 mm. Given the average monthly rainfall and the area of the plastic nutrient pot (452 cm²), the simulated rainfall amount was set to 6330 mL month⁻¹. Plants were sprayed three times a week at a volume of 528 mL per application. During spraying, care was taken to cover the whole seedling and the soil surface.

2.2. Growth indices

On November 24th, 2018, three plants were selected from each of

the ten treatments. Plant roots were washed and dried, and an Epson root scanner (WinRhizo PRO, America) was used to scan the root system of all saplings. A root analysis system (EPSON Expression 10000XL 1.8V1.0 2.00) was used to analyze the scanned root pictures, from which estimates of total root length, total root surface area, average root diameter, and total root volume were obtained.

On November 25th, 2018, three plants were again selected from each treatment. The leaves and roots of each plants were washed and cleaned saplings were put in paper bags in an oven. Saplings were first dried at 100 °C for 30 min, then dried to constant weight at 75 °C, and the dry weight of each part of the plant (roots, stems, and leaves) was obtained (Yi et al., 2018).

2.3. Chlorophyll content

Chl content was measured from the second fully expanded leaves of selected plants in the early stage (May 3rd), mid-term (August 3rd), and late stage (November 3rd) of acid rain stress, respectively. According to the method described by Yi et al., (2018), 0.1 g of fresh leaf tissue was added to 15 mL of 96% ethanol and placed in darkness with intermittent oscillations until the leaf tissue was completely white. The sample volume was then adjusted to 25 mL by adding 96% ethanol. Absorption values of samples were recorded at 663 and 645 nm using an ultraviolet spectrophotometer (UV-2450, Shimadzu, Japan). Three duplicate samples were taken from each treatment.

2.4. Antioxidant enzyme activity

We determined antioxidant enzyme activity according to Liu et al., (2018c). Briefly, 0.1 g of fresh leaf or root tissue was ground into a homogenate in cold buffer (50 mM phosphate buffer [pH 7.0], 1 mM ethylene-diamine-tetraacetic acid, 2% (w/v) polyvinylpyrrolidone, and 1 mM phenylmethylsulfonyl). The homogenate was then centrifuged at 11,000 rpm for 15 min at 4 °C, and the supernatant was taken as the enzyme extract. Superoxide dismutase (SOD) activity was measured by photochemical reduction of a nitrotetrazolium blue (NBT) solution (50 mM phosphate buffer [pH 7.8], 53 mM NBT, 10 mM methionine, 5.3 mM riboflavin, and sample enzyme extract). This solution was reacted for 20 min under 4000 lux light intensity. Absorption values were recorded at 560 nm, and SOD activity was calculated according to Beyer and Fridovich (1987). Peroxidase (POD) activity was measured by guaiacol oxidation. The 2-min increase in absorbance of the reaction solution (50 mM phosphate buffer [pH 7.0], 20 mM guaiacol, 10 mM H_2O_2 , and supernatant) was recorded at 470 nm and POD activity was calculated according to Yordanova (2004). Catalase (CAT) activity was determined by the reaction of a solution (50 mM phosphate buffer [pH 7.0], 0.1 mM H_2O_2 , and the supernatant) at 240 nm for 2 min and calculated according to Muradian et al. (2002). Three duplicate samples from each treatment were measured from the early stage, mid-term, and late stage of acid rain stress, respectively.

2.5. Malondialdehyde content

MDA content was calculated according to Tai'bi (Tai'bi et al., 2016). We ground 0.25 g of fresh leaf and root tissue, respectively, into a homogenate in 5% (w/v) trichloroacetic acid (TBA), then centrifuged the homogenate at 10,000 rpm for 10 min at 4 °C. We then added 4 mL of 0.5% (w/v) thiobarbituric acid and 20% (w/v) TBA to 1 mL of supernatant, and heated the solution for 30 min at 95 °C. The solution was then rapidly cooled. Absorbance of the supernatant was read at 532 and 600 nm, respectively, to calculate MDA. Three duplicate samples from each treatment were measured from the early stage, mid-term, and late stage of acid rain stress, respectively.

2.6. Chlorophyll fluorescence parameters

Chl fluorescence parameters were measured with a mini blue version of the Imaging-PAM Chl fluorometer (IMAGMIN/B, Walz, Effeltrich, Germany) on November 5th. The minimum fluorescence (F_0) of leaves was measured after dark adaptation for 30 min, and maximum fluorescence (F_m) of the same leaves was measured with a saturating blue pulse. Then, we used actinic illumination and applied a saturated pulse at 20-s intervals for 5 min to obtain maximum fluorescence yield (F_m'), Chl fluorescence yield (F_s), and minimum fluorescence yield (F_0'). The maximum variable fluorescence ($F_v = F_m - F_0$) and the photochemical efficiencies of PSII (F_v/F_m) were calculated based on Maxwell and Johnson (2000). Photochemical efficiency of photosystem II (Φ_{PSII}), photochemical quenching coefficient (qP), and non-photochemical quenching coefficient (NPQ) were calculated according to Hazrati et al. (2016). Three replicates were measured from each treatment.

2.7. Statistical analyses

All analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance was used to analyze the significance of difference among different acid rain treatments. We used generalized linear models to determine the main factors influencing these same parameters and to quantify the effect of an interaction between acid rain type and pH.

3. Results

3.1. Phenotypic variation

We observed wide variation in the morphological characteristics of *H. hainanensis* under different treatments at the late stage of acid rain stress (Table 1). At pH 2.5, there were obvious holes and significant corrosion in sapling leaves. Saplings were yellowed and lost a large number of leaves under the SAR treatment, yet under MAR and NAR, saplings remained green and no defoliation was observed. At pH 4.0, seedling leaves had a limited number of holes and did not yellow under the SAR treatment. No obvious differences were observed between MAR- and NAR-treated plants and the controls. Saplings raised at pH 5.6 appeared more vigorous than those of the control.

3.2. Biomass

Different acid rain types promoted or inhibited biomass accumulation in treated saplings (Table 2). The biomass of all sapling parts (stems, roots, and leaves) decreased with decreasing pH. At pH 2.5, the total biomass of saplings treated with SAR, NAR, and MAR was 29.03, 36.78, and 32.81 g, respectively, which was 66.7, 84.5, and 75.4% of the biomass obtained from the controls (43.53 g), respectively. At pH 4.0, the above-ground growth of saplings was slightly inhibited, and below-ground growth was slightly promoted, in SAR-treated plants, but those treated with MAR and NAR showed the opposite trend. At pH 5.6, the above-ground growth of saplings was promoted under all three acid rain treatments, and the total biomass of saplings treated with SAR, NAR, and MAR was 53.43, 60.32, and 54.78 g, respectively, an increase of 22.8, 38.6, and 25.9% relative to the control plants. The root biomass of saplings treated with NAR was 13.39 g, which was significantly lower than that of the controls (17.29 g) ($P < 0.05$). At the same pH, the above-ground biomass (stems and leaves) of NAR-treated saplings was higher than that of SAR- and MAR-treated saplings, but the below-ground biomass (roots) of SAR-treated saplings was higher than those treated with MAR or NAR (Table 2).

3.3. Root morphology

At the same pH, root morphology indices of SAR-treated saplings

were greater than those of MAR-treated saplings, which were in turn greater than those of NAR-treated saplings (Table 3). Root morphology indices decreased with decreasing pH under the same acid rain type. Root indices of NAR-treated saplings were significantly lower than those of the control plants ($P < 0.05$).

Generalized linear models indicated that the effect of acid rain type on root length, average root diameter, and total root volume was greater than that of pH and the interaction between acid rain type and pH ($P_{\text{Type}} < P_{\text{pH}} < P_{\text{Type} \times \text{pH}}$). In contrast, the effect of pH on root surface area was greater than that of acid rain type and the interaction between acid rain type and pH ($P_{\text{pH}} < P_{\text{Type}} < P_{\text{Type} \times \text{pH}}$). The interaction between acid rain type and pH had no obvious effect on root morphological indices, excluding total root surface area and total root length ($P > 0.05$).

3.4. Chlorophyll content

The chlorophyll A (Chla) content, total Chl content, and the ratio of Chla to chlorophyll B (Chlb) decreased over time under the strong acid rain treatment (pH = 2.5). The Chla content of saplings treated with SAR, NAR, and MAR was 0.60, 0.93, and 0.80 mg g⁻¹, respectively, which was 40.8, 63.3, and 54.4% of the content found in control plants (1.47 mg g⁻¹). The total Chl content of saplings treated with SAR, NAR, and MAR was 1.06, 1.42, and 1.26 mg g⁻¹, respectively, which was 53.8, 72.3, and 64.3% of that of the control plants (1.97 mg g⁻¹). The ratio of Chla:Chlb in saplings treated with SAR, NAR, and MAR was 1.30, 1.90, and 1.69, respectively, which was 44, 64.6, and 57.5% of the ratios measured in control plants (2.94). However, we observed no significant change in the Chlb content among treatments (Fig. 1B). In addition, under pH 5.6, the content of Chla, Chlb, and total Chl in saplings treated with all three acid rain types increased gradually with treatment time. At the end of the study, the Chla, Chlb, and total Chl contents were all greater among treated saplings than those of the control ($P < 0.05$). Chl content decreased with decreasing pH. We found that acid rain type, pH, and the interaction of acid rain type and pH had a significant effect on the ratio of Chla:Chlb ($P < 0.01$).

3.5. Antioxidant enzyme activity

All acid rain types significantly enhanced the activity of antioxidant enzymes in leaves of treated plants relative to controls by the end of the study ($P < 0.05$) (Fig. 2A, C, E). Under strong acid rain condition (pH = 2.5), SOD and POD activity in leaves first increased, then decreased over time (Fig. 2A and C). The activity of SOD and POD in leaves of saplings treated with SAR was significantly lower than that of saplings treated with NAR at the end of the study (late-stage acid rain stress) ($P < 0.05$). In contrast, mid-term results indicated the opposite trend, SOD and POD in saplings treated with SAR was significantly higher than that of saplings treated with NAR. At pH 4.0, the activity of SOD and POD in leaves increased with time. Activity in saplings treated with SAR was significantly higher than in saplings treated with NAR at the end of the study ($P < 0.05$).

CAT activity in leaves increased over treatment time (Fig. 2E). At pH 2.5 and 4.0, CAT activity in the leaves of SAR-treated saplings was significantly higher than that of NAR-treated saplings by the end of the study ($P < 0.05$). Antioxidant enzyme activity in roots increased with time (Fig. 2B,D, F). At pH 2.5, the activity of SOD, POD, and CAT in the roots of saplings treated with NAR (SOD = 457.301 U·g⁻¹·Pro, POD = 38.345 U·g⁻¹·Pro·min⁻¹, CAT = 23.68 7U·g⁻¹·Pro·min⁻¹) was highest at the end of the study. Generalized linear models suggested that pH and the interaction of acid rain type and pH significantly affected antioxidant enzyme activity in leaves ($P < 0.01$), and pH and acid rain type significantly affected antioxidant enzyme activity in roots ($P < 0.01$).

Table 1
Effects of different types of acid rain on phenotypic variation of *H. hainanensis* saplings.

pH	Acid rain type		
	Sulfuric acid rain	Nitric acid rain	Mixed acid rain
2.5			
4.0			
5.6			
Control			

3.6. Malondialdehyde content

Under strong acid rain conditions (pH = 2.5), the MDA content in leaves increased with treatment time (Fig. 3A). Saplings treated with SAR, NAR, and MAR (23.67, 17.08, and 18.37 mmol g⁻¹·FM, respectively) had significantly higher MDA content than control plants (4.16 mmol g⁻¹·FM) at the end of the study (P < 0.05). At pH 2.5 and 4.0, the MDA content was significantly higher in SAR-treated saplings than those treated with NAR and MAR at the end of the study (P < 0.05). At pH 5.6, the MDA content in saplings across all treatments was not significantly different than that of control saplings (P > 0.05).

Root MDA content trends were contrary to those found in leaves. At pH 2.5, the MDA content in the roots of NAR-treated saplings (13.867 mmol g⁻¹·FM) was significantly higher than that found in the other treatments (P < 0.05). Generalized linear models indicated that acid rain type, pH, and the interaction of acid rain type and pH significantly affected the MDA content of both leaves (P < 0.01) and roots (P < 0.01) (Fig. 3).

3.7. Chlorophyll fluorescence parameters

Chl fluorescence parameters varied with pH and acid rain type

(Table 4). The ratios of F_v:F_m and F_v:F_o, Φ_{PSII}, and qP increased with increasing pH; however, NPQ increased with decreasing pH. At pH 2.5, the NPQ of SAR-, NAR-, and MAR-treated saplings was 1.759, 1.658, and 1.728, respectively, which was significantly higher than the NPQ values observed in control plants (1.167, P < 0.05). Generalized linear models indicated that acid rain pH had a highly significant effect on Chl fluorescence parameters (P < 0.01). Acid rain type had a significant effect on the F_v:F_m ratio (P < 0.01), as well as the F_v:F_o ratio, Φ_{PSII}, and qP (P < 0.05 for all), but no effect on NPQ. The interaction of pH and acid rain type had a significant effect on the F_v:F_o ratio, but not on the F_v:F_m ratio, Φ_{PSII}, qP, or NPQ.

4. Discussion

Total biomass in plants reflects differences in resource capture and biomass production rates (Dovrat et al., 2019). Plants have a remarkable ability to coordinate organ growth; therefore, a balance in stem and root biomass is typically observed (Poorter and Nagel, 2000). Here, we found wide variation in biomass accumulation among plant parts (stems, roots, and leaves) in *H. hainanensis* saplings exposed to different acid rain types, indicating that acid rain disrupts the balance of biomass distribution. As acid rain type shifted from SAR to NAR, the accumulation of above-ground biomass was first inhibited, then promoted,

Table 2
Effects of different types of acid rain on biomass accumulation of *H. hainanensis* saplings.

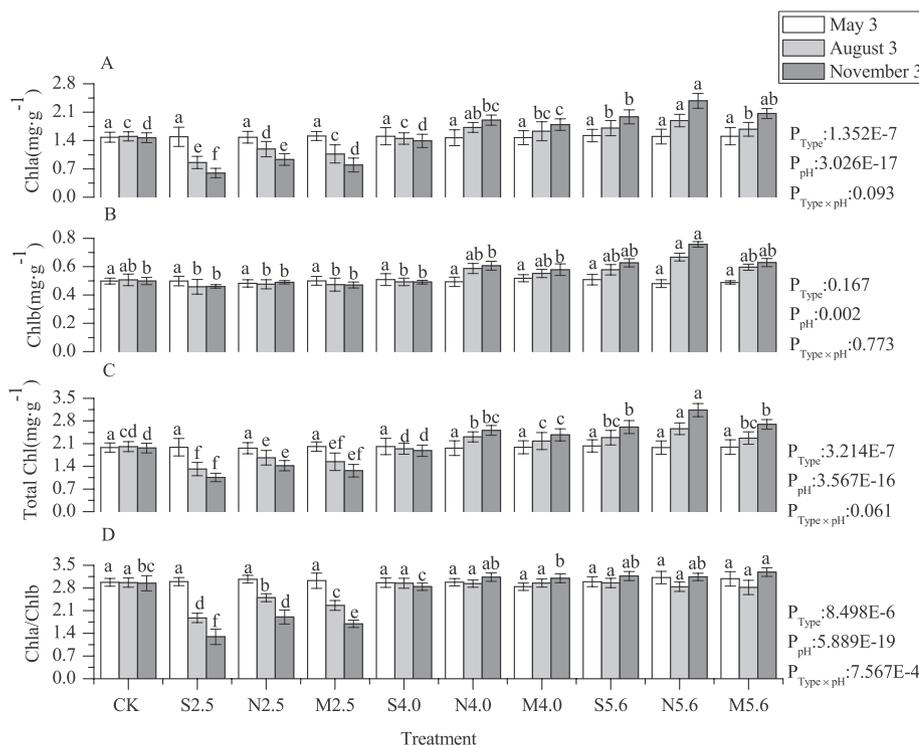
Types of acid rain	pH	Root biomass/g	Stem biomass/g	Leaf biomass/g	Total biomass/g
CK	6.5	17.29 ± 2.86a	12.56 ± 0.98bc	13.67 ± 2.16bc	43.53 ± 2.05bc
SAR	2.5	13.02 ± 1.51b	9.01 ± 0.20c	7.00 ± 0.92c	29.03 ± 1.31c
	4.0	20.34 ± 0.84a	11.27 ± 0.52c	11.79 ± 0.78bc	43.39 ± 2.11bc
	5.6	20.19 ± 1.12a	16.84 ± 0.94b	16.39 ± 2.59b	53.43 ± 4.43 ab
NAR	2.5	8.62 ± 0.76c	13.47 ± 0.99bc	15.03 ± 0.72b	36.78 ± 2.34bc
	4.0	11.31 ± 1.41bc	18.69 ± 2.14 ab	22.07 ± 2.84 ab	52.07 ± 7.90 ab
	5.6	13.39 ± 0.05b	22.64 ± 2.58a	24.29 ± 2.22a	60.32 ± 3.27a
MAR	2.5	12.24 ± 0.65bc	10.17 ± 1.18c	10.39 ± 1.66bc	32.81 ± 3.25c
	4.0	13.13 ± 1.03b	16.20 ± 3.27bc	17.23 ± 3.64 ab	46.56 ± 6.61b
	5.6	16.99 ± 0.37 ab	19.01 ± 1.92 ab	19.13 ± 0.71 ab	54.78 ± 1.17 ab
P _{Type}		4.485E-7	0.003	0.003	0.100
P _{pH}		5.396E-6	6.709E-5	0.002	9.939E-6
P _{Type × pH}		0.045	0.855	0.984	0.998

Note: The experimental treatments are: CK = control check; SAR = sulfuric acid rain; NAR = nitric acid rain; MAR = mixed acid rain. Different letters indicate significant difference (p < 0.05) among different acid rain treatments on one-way ANOVA, followed by a Duncan test. Type, type of acid rain; pH, acid rain pH. The general linear model analysis was applied to indicate significant difference among variances.

Table 3
Effects of different types of acid rain on root morphology of *H. hainanensis* saplings.

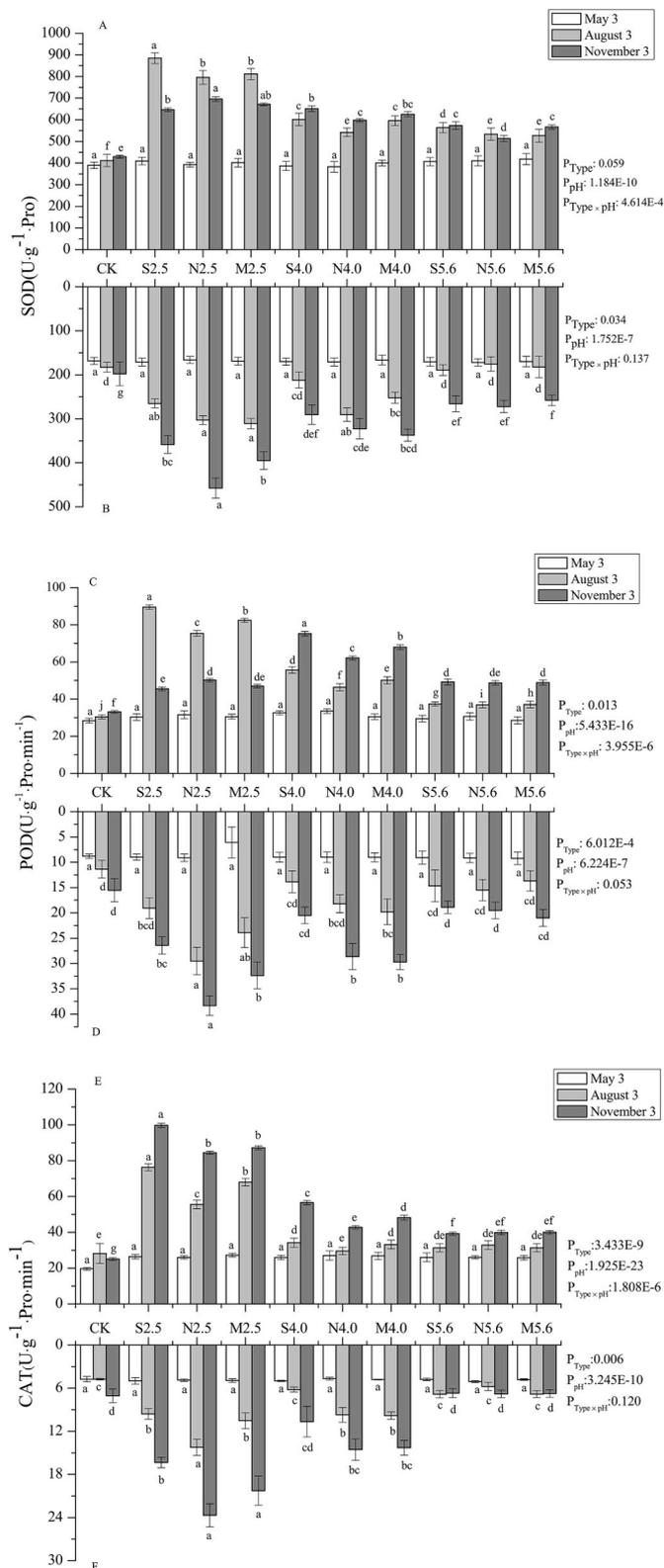
Types of acid rain	pH	Length/cm	SurfArea/cm ²	AvgDiam/mm	Root Volume/cm ³
CK	6.5	2413.51 ± 42.54b	1154.91 ± 28.64d	4.15 ± 0.31b	50.33 ± 2.39c
SAR	2.5	2447.51 ± 101.53b	901.09 ± 16.45f	3.42 ± 0.11bc	45.78 ± 0.98cd
	4.0	3874.15 ± 48.23a	1616.81 ± 19.60b	5.42 ± 0.02a	62.65 ± 1.67b
	5.6	3974.92 ± 107.92a	1847.13 ± 39.66a	5.52 ± 0.39a	71.69 ± 2.18a
NAR	2.5	1028.65 ± 11.79f	497.13 ± 28.47h	2.37 ± 0.31d	18.59 ± 1.50f
	4.0	1683.90 ± 48.76e	933.45 ± 11.42f	3.37 ± 0.25c	34.64 ± 2.79e
	5.6	2216.34 ± 86.11c	1054.82 ± 14.26e	3.21 ± 0.39c	41.90 ± 1.39d
MAR	2.5	1745.18 ± 57.51e	723.82 ± 11.78g	2.75 ± 0.17cd	29.66 ± 2.94e
	4.0	2021.93 ± 20.44d	1143.49 ± 14.38d	3.53 ± 0.09bc	46.13 ± 3.15cd
	5.6	2497.90 ± 25.69b	1245.36 ± 15.03c	4.17 ± 0.29b	49.74 ± 0.84c
P _{Type}		6.973E-19	7.333E-19	1.701E-7	2.288E-12
P _{pH}		5.091E-15	9.261E-20	2.099E-6	7.238E-11
P _{Type × pH}		3.431E-7	2.762E-8	0.087	0.667

Note: The experimental treatments are: CK = control check; SAR = sulfuric acid rain; NAR = nitric acid rain; MAR = mixed acid rain. Different letters indicate significant difference (p < 0.05) among different acid rain treatments on one-way ANOVA, followed by a Duncan test. Type, type of acid rain; pH, acid rain pH. The general linear model analysis was applied to indicate significant difference among variances.



while below-ground biomass accumulation was first promoted, then inhibited. This may be the result of acid rain causing changes to the physical and chemical properties of the soil, thereby indirectly affecting plant growth (Tamm et al., 1977). Nitric acid acts as a nitrogen fertilizer to promote above-ground biomass accumulation in *H. hainanensis* (Magill et al., 1997; Knops and Reinhart, 2000; Mofunanya and Soonen,

Fig. 2. Changes of antioxidant enzymes activity in leaves (A, C, E) and roots (B, D, F) of *H. hainanensis* saplings under different acid rain treatments. A and B, superoxide dismutase; C and D, peroxidase; E and F, catalase. The experimental treatments are: CK = control check; S2.5 = pH2.5 sulfuric acid rain; S4.0 = pH4.0 sulfuric acid rain; S5.6 = pH5.6 sulfuric acid rain; N2.5 = pH2.5 nitric acid rain; N4.0 = pH4.0 nitric acid rain; N5.6 = pH5.6 nitric acid rain; M2.5 = pH2.5 mixed acid rain; M4.0 = pH4.0 mixed acid rain; M5.6 = pH5.6 mixed acid rain. Different letters indicate significant difference ($P < 0.05$) among different acid rain acidity with the same phase based on one-way ANOVA, followed by a Duncan test. Type, type of acid rain; pH, acid rain pH. The general linear model analysis was applied to indicate significant difference among variances.



2017). However, the capacity for exchange with hydroxyl groups (OH^-) is lower for NO_3^- than for SO_4^{2-} , meaning that over time soil acidification will accelerate under NAR (Lindberg et al., 1990). Therefore, the adverse effects of NAR on soil pH and soil microbial activity are greater than those of SAR (Lv et al., 2014; Liu et al., 2017), and providing exogenous sulfur can improve soil conditions and increase the number of soil microorganisms under SAR (Zhao et al., 2008). In this study, we demonstrated that root morphology indices responded in a similar way to root biomass under various types of acid rain. This indicates that the restrained part of *H. hainanensis* transfers from the above-ground to below-ground as acid rain type shifts from SAR to NAR.

Chla and Chlb can be interchanged through 7-hydroxymethyl chlorophyll (Anderson, 1986; Tanaka et al., 1998). Plants can potentially use chlorophyll transformation to adjust the ratio of Chla:Chlb to meet their physiological and environmental needs (Rüdiger, 2002). It has been suggested that a Chlb deficiency can accelerate rice senescence (Yang et al., 2015). Kusaba et al. (2007) proposed that Chl degradation was faster in mutant rice that was deficient in Chlb. We did not observe any significant change in Chlb content in *H. hainanensis* treated with acid rain at pH 2.5 relative to control plants. However, Chla decreased significantly at this pH. This was likely a result of a transformation of Chla into Chlb to reduce leaf senescence and Chl decomposition in acid-stressed plants. At pH 4.0 and 5.6, the total Chl and Chla contents of NAR- and MAR-treated saplings were significantly higher than that of control plants. This is attributed to the nitrogen in NAR and MAR causing an increase in Chl content in leaves, given that nitrogen is the most important element in Chl biosynthesis (Cechin and de Fátima Fumis, 2004; Yadav and Garg, 2015).

SOD, POD and CAT are the main protective enzymes for scavenging reactive oxygen species (ROS) in cells, the ability to scavenge ROS is the key factor determining the resistance of cells to stress, the defensive ability of protective enzymes depends on the synthetical results of co-ordination of these enzymes (Jaspers and Kangasjärvi, 2010; Vaahera and Brosché, 2011), and MDA content can be used as an index to analyze lipid peroxidation and membrane damage (Yamauchi et al., 2008). We found that SOD and POD activity in leaves first increased, then decreased, over treatment time at pH 2.5. Simultaneously, MDA content accumulated in leaves over time, which may be due to long term acid-stress-induced destruction of SOD and POD, consistent with the findings of Liu and Liu (2010). We observed differences in CAT activity relative to SOD and POD in leaves over treatment time; this is likely because different enzymes have different sensitivities to the environment (Chen et al., 2013). At pH 2.5 and 4.0, the degree of change in antioxidant enzyme activities and MDA content in the leaves of SAR-treated saplings were higher than saplings treated with MAR and NAR. However, enzyme activity and MDA content in roots showed a contrary pattern. This indicates that the inhibitory effect of SAR on leaf physiology is higher than that of MAR and NAR, and the inhibitory effect of NAR on root physiology is higher than that of MAR and SAR.

Chl fluorescence parameters are ideal methods for exploring the effects of stress on plants (Guo et al., 2016). Parameters such as $F_v:F_o$ and $F_v:F_m$ can be used to determine if plants are inhibited by

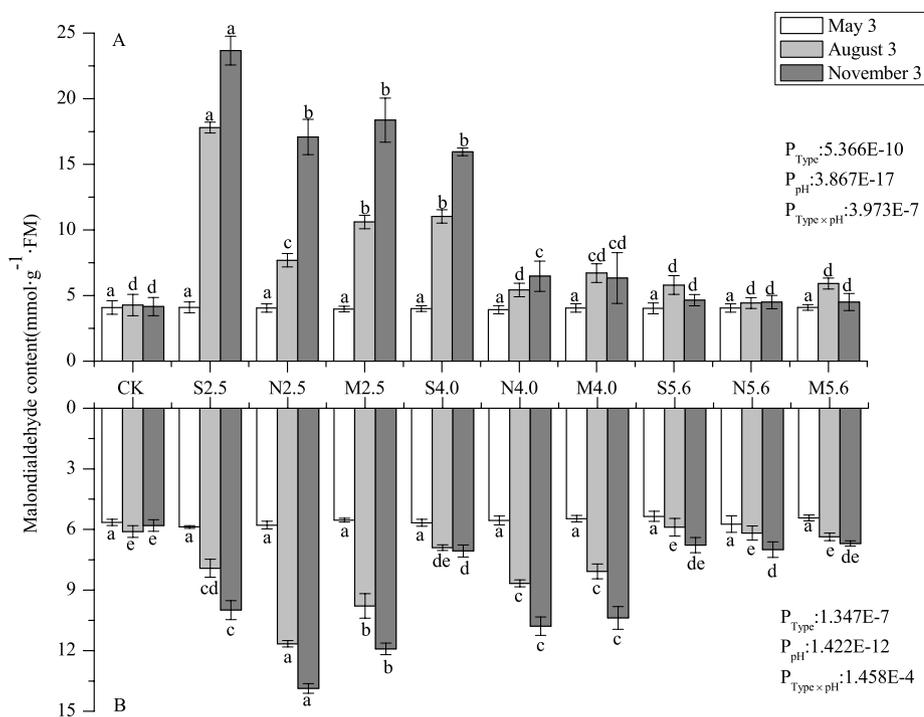


Fig. 3. Changes of malondialdehyde content of *H. hainanensis* saplings under different acid rain treatments. A, MDA content in leaves; B, MDA content in roots. The experimental treatments are: CK = control check; S2.5 = pH2.5 sulfuric acid rain; S4.0 = pH4.0 sulfuric acid rain; S5.6 = pH5.6 sulfuric acid rain; N2.5 = pH2.5 nitric acid rain; N4.0 = pH4.0 nitric acid rain; N5.6 = pH5.6 nitric acid rain; M2.5 = pH2.5 mixed acid rain; M4.0 = pH4.0 mixed acid rain; M5.6 = pH5.6 mixed acid rain. Different letters indicate significant difference ($P < 0.05$) among different acid rain acidity with the same phase based on one-way ANOVA, followed by a Duncan test. Type, type of acid rain; pH, acid rain pH. The general linear model analysis was applied to indicate significant difference among variances.

photoinhibition (Queiroz et al., 2016), qP represents the energy consumed by photosynthesis, NPQ represents the amount of excessive radiation converted into heat (Pinnola et al., 2013), and Φ_{PSII} can be used to estimate efficiency (Baker, 2004). At pH 5.6, $F_v:F_o$, $F_v:F_m$, qP, and NPQ in treated saplings were not significantly different from those of control plants, but Φ_{PSII} was significantly higher in treated saplings. This indicates that *H. hainanensis* has a greater adaptability to acid rain at pH 5.6. There is a negative correlation between qP and NPQ, and between Φ_{PSII} and NPQ (Fu et al., 2012; Hazrati et al., 2016), plants could reduce excessive accumulation of excitation energy through heat dissipation (Gill et al., 2013). We found that NPQ increased significantly in acid rain treated saplings at pH 2.5 and 4.0 relative to control plants. This suggests that under acid-stress conditions, the light absorbed by *H. hainanensis* is released more in the form of heat, thus reducing the utilization efficiency of light energy. This is supported by the findings of Wang et al. (2017).

Plant growth and physiological responses are dependent on their environment. In this study, growth, physiological, and Chl fluorescence parameters were comprehensively analyzed under various acid rain

treatments. We found that as acid rain shifted from SAR to NAR, the negative effect of acid rain on above-ground growth, physiology, and Chl parameters in *H. hainanensis* weakened, consistent with the findings of Lee et al. (2006). However, our results indicate that the negative effects of acid rain on below-ground growth and physiology are exacerbated with a shift from SAR to NAR. This suggests that conservation measures for *H. hainanensis* under NAR must shift to a below-ground focus.

5. Conclusion

H. hainanensis is an endangered species endemic to China that is affected by acid rain throughout its geographic range. Using a simulated acid rain experiment, we demonstrated that three acid rain types at three pH levels had differing effects on the growth and physiology of *H. hainanensis*. The results showed that the adverse effects of acid rain on the physiology of Leaves, $F_v:F_m$, $F_v:F_o$, Φ_{PSII} , qP and NPQ of *H. hainanensis* was gradually reduced during the transformation of acid rain type from sulfuric acid type to nitric acid type, However, in the

Table 4
Effects of different types of acid rain on chlorophyll fluorescence parameters of *H. hainanensis* saplings.

Types of acid rain	pH	$F_v:F_m$	$F_v:F_o$	Φ_{PSII}	qP	NPQ
CK	6.5	0.807 ± 0.006a	4.204 ± 0.160a	0.368 ± 0.005b	0.566 ± 0.004a	1.167 ± 0.076d
SAR	2.5	0.730 ± 0.001de	2.710 ± 0.001e	0.135 ± 0.011e	0.300 ± 0.022d	1.759 ± 0.021a
	4.0	0.746 ± 0.006c	2.949 ± 0.102cd	0.216 ± 0.003d	0.392 ± 0.004c	1.592 ± 0.058bc
	5.6	0.806 ± 0.003a	4.154 ± 0.070a	0.391 ± 0.012a	0.568 ± 0.013a	1.149 ± 0.034d
NAR	2.5	0.733 ± 0.001d	2.742 ± 0.016de	0.131 ± 0.006e	0.310 ± 0.014d	1.658 ± 0.026 ab
	4.0	0.766 ± 0.001b	3.265 ± 0.001b	0.261 ± 0.004c	0.466 ± 0.006b	1.515 ± 0.015c
	5.6	0.809 ± 0.001a	4.232 ± 0.024a	0.404 ± 0.006a	0.582 ± 0.007a	1.108 ± 0.011d
MAR	2.5	0.721 ± 0.001e	2.590 ± 0.019e	0.137 ± 0.003e	0.305 ± 0.011d	1.728 ± 0.021a
	4.0	0.752 ± 0.004c	3.029 ± 0.007c	0.225 ± 0.013d	0.416 ± 0.021c	1.542 ± 0.015c
	5.6	0.809 ± 0.003a	4.227 ± 0.087a	0.397 ± 0.003a	0.576 ± 0.003a	1.117 ± 0.025d
P_{Type}		0.005	0.040	0.023	0.015	0.067
P_{pH}		8.217E-18	1.301E-16	1.325E-20	2.314E-16	3.046E-14
$P_{Type \times pH}$		0.029	0.197	0.638	0.104	0.904

Note: The experimental treatments are: CK = control check; SAR = sulfuric acid rain; NAR = nitric acid rain; MAR = mixed acid rain. Different letters indicate significant difference ($p < 0.05$) among different acid rain treatments on one-way ANOVA, followed by a Duncan test. Type, type of acid rain; pH, acid rain pH. The general linear model analysis was applied to indicate significant difference among variances.

process of acid rain type change, the adverse effects of acid rain on growth and physiology of roots of *H. hainanensis* are aggravating. In summary, SAR primarily damages the leaves and stems of *H. hainanensis*, whereas MAR and NAR mainly damage the roots. Therefore, with the change of acid rain type, the key protective part of *H. hainanensis* should be transferred from the above-ground part to the below-ground in the work of protecting this species.

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