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Allelopathic effects of phenolic acids on seedling growth and photosynthesis in *Rhododendron delavayi* Franch.

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Abstract

The effects of different concentrations of ferulic acid, chlorogenic acid, and protocatechuic acid were studied in a pot experiment to assess the response of *Rhododendron delavayi* seedlings. The results showed that three kinds of phenols promoted increases in chlorophyll (Chl) *a*, Chl *b*, total Chl, and carotenoid contents, but inhibited the accumulation of biomass. Low concentrations of ferulic acid significantly inhibited stomatal opening, the stomatal opening ratio, stomatal length and width. Chlorogenic acid and the moderate and high concentrations of ferulic acid also significantly inhibited stomatal density. Ferulic acid significantly inhibited net photosynthetic rate and stomatal conductance in seedlings, whereas chlorogenic acid significantly inhibited stomatal conductance. The low and moderate concentrations of chlorogenic acid significantly inhibited transpiration rate, and high concentrations of ferulic acid significantly inhibited the stomatal limitation value. The moderate concentration of protocatechuic acid significantly inhibited net photosynthetic rate and stomatal conductance. Ferulic acid exhibited a greater toxic effect than that of chlorogenic acid and protocatechuic acid for *R. delavayi* seedlings.

Additional key words: allelopathy; gas-exchange parameters; photosynthetic pigment content; redundancy analysis.

Introduction

Plant allelopathy is a phenomenon involving direct or indirect harmful or beneficial effects through the release of chemical compounds (Rice 1984, 1995; Li *et al*. 2010, Zhou *et al*. 2012). Allelopathy has become a hot topic in crop intercropping, barren soil, forest community succession, vegetation restoration, agriculture, and silviculture research (Li *et al*. 2010, John 2012). Previous studies have shown that many plants affect the growth and development of seedlings by releasing harmful allelopathic substances, which interfere with seed germination, seedling growth, biomass accumulation, photosynthesis, and stomatal development of forest plants, resulting in failed tree species regeneration and reconstruction efforts (Li *et al*. 2010, Zhang *et al*. 2010, John 2012). Therefore, the allelopathic effects of trees have important scientific implications for assessing their potential effect on forest communities.

Phenolic acids are common secondary metabolites of plants and fungi that are released through leaching, volatilisation, root exudation or tissue decomposition (Li *et al*. 2010, Zhang *et al*. 2010, Singh *et al*. 2014, Ribeiro *et al*. 2016, Shang *et al*. 2017). Studies indicate that phenolic compounds have important health-promoting and disease-preventing value in humans, including antioxidant, antimicrobial, antidiabetic, anti-inflammatory, anticancer, and metabolic regulatory properties (Roleira *et al*. 2015, Shahidi and Ambigaipalan 2015). In the forest system, such phenolic compounds inhibit functions of plant root systems, the development of stomata, water and nutrient uptake, and reduce chlorophyll (Chl) content and photosynthesis to affect plant growth rate and biomass

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Abbreviations: AB – aboveground biomass; CA – chlorogenic acid; C_a – atmospheric CO_2 concentration; Car – carotenoid; Chl – chlorophyll; C_i – intercellular CO_2 concentration; CK – normal management with distilled water; E – transpiration rate; FA – ferulic acid; GD – ground diameter; g_s – stomatal conductance; HAS – horizontal axes of stomata; L_s – stomatal limitation value (= $1 - C_i/C_a$); PA – protocatechuic acid; PH – plant height; P_N – net photosynthetic rate; RDA – redundancy analysis; SA – stomatal aperture; SD – stomatal density; SOR – stomatal opening ratio; UB – underground biomass; VAS – vertical axes of stomata; WUE – water-use efficiency (= P_N/E).

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accumulation (Khan *et al*. 2003, Lu *et al*. 2018). Ferulic acid, chlorogenic acid, and protocatechuic acid are the three most common phenolic compounds (John 2012). Previous reports have shown that ferulic acid inhibits photosynthesis at a concentration of 100 µM (Mersie and Singh 1993). Some research has shown that ferulic acid severely affects root growth (Singh *et al*. 2014); however, protocatechuic acid advances root knot formation and root growth (Mandal *et al*. 2009, Shang *et al*. 2017). A previous study showed that chlorogenic acid decreases the stomatal aperture in tobacco and sunflower (Einhellig *et al*. 1979).

Guizhou Baili Rhododendron National Nature Reserve is located in the highlands of northwest Guizhou Province. *Rhododendron delavayi* Franch. is the main dominant species in this area with a high uniformity (Gu *et al*. 2005, Chen *et al*. 2007); however, the vertical structure of the forest community is simple, the diversity index is low, and new *Rhododendron* seedlings are rare. Artificial removal of the litter layer under forest sowing and planting regimes have resulted in very low seed germination rates and slow seedling growth. A repeat field investigation reported that the annual number of *Rhododendron* seeds remains high, and plants are generally mature, so there is no seed-source problem.

Phenolic acids are the most common allelochemicals in agroforestry ecosystems; they are the main chemical compounds causing natural regeneration of *Rhododendron*. The effects of different concentrations of ferulic acid (FA), chlorogenic acid (CA), and protocatechuic acid (PA) were assessed on growth, photosynthetic characteristics, Chl, and stomatal characteristics of *R. delavayi* seedlings.

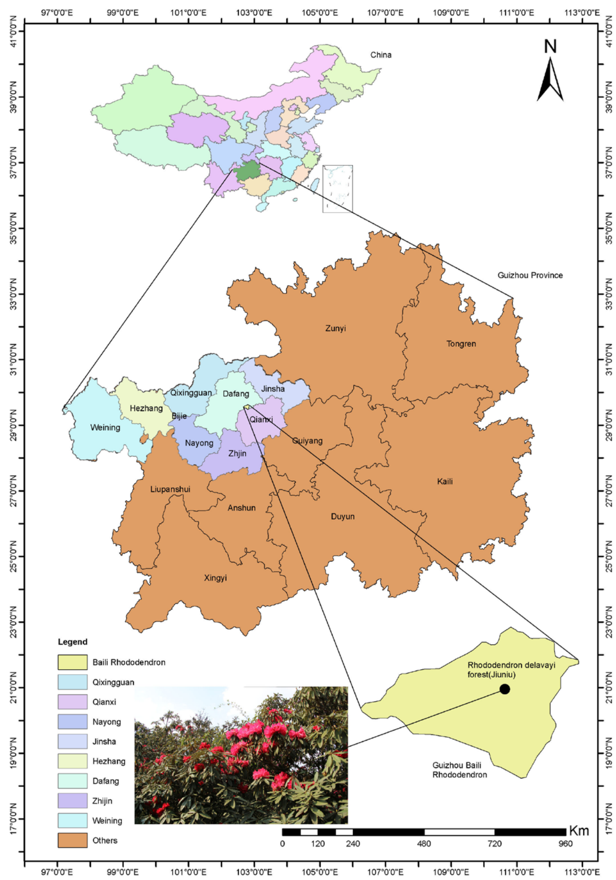

Fig. 1. Distribution of *Rhododendron delavayi* in Baili Rhododendron National Nature Reserve.

Materials and methods

Study area: Guizhou Baili Rhododendron National Nature Reserve is located in northwest Guizhou Province Wumeng Mountain hinterland at latitude 27°10' to 27°15'N and longitude 105°50' to 106°00'E and covers a surface area of 125.8 km^2. The climate is humid subtropical monsoon, and average relative humidity is 84%. The range of altitudes is 1,500–1,800 m, mean average annual precipitation is 1,000–1,100 mm, spring and summer precipitation account for 70% of the annual total amount, and the park contains more than 30 species of *Rhododendron* (Chen et *al*. 2010). This area is known as the largest natural *Rhododendron* forest belt in China. It is also known as the natural garden on the plateau and the world largest natural garden. *R. delavayi* is distributed across the entire flowering area of the park (Fig. 1).

Phenolic acids: Three allelochemicals, such as ferulic acid, chlorogenic acid, and protocatechuic acid, were the analytical reagents (analytic grade; *Sigma*, St. Louis, MO, USA). We accurately weighed 0.25 g (accuracy ± 0.0001 g) of ferulic acid (FA), chlorogenic acid (CA), and protocatechuic acid (PA) into 50-mL beakers with a small amount of ethanol until they were fully dissolved. The solutions were poured into 250-mL volumetric flasks and the beakers were rinsed with distilled water three or four times. We prepared solutions of 1 g L^{-1} FA, CA, and PA solution and stored them at 4°C. We sprayed seedlings with FA, CA, and PA at various concentrations, using distilled water as a control treatment (CK). The seedlings were irrigated as stated in experimental design section.

Experimental design: The seedlings were grown in plastic pots with a diameter of 30 cm and a depth of 40 cm. The seedlings were grown in a humic:perlite (3:1) compost, and were kept in a greenhouse 30 d before the experiment, where they received routine management. The *R. delavayi* seedlings were randomly divided into ten seedlings per treatment. Each treatment contained three replicates [with concentrations of 1,000 µg L^{-1} (low), 2,000 µg L^{-1} (moderate), and 4,000 µg L^{-1} (high) FA; 200 µg L^{-1} (low), 400 µg L^{-1} (moderate), and 800 µg L^{-1} (high) CA; and 50 µg L^{-1} (low), 250 µg L^{-1} (moderate), and 1,250 µg L^{-1} (high) PA] applied to roots. The control (CK) group was irrigated with 50 mL of distilled water per plant. The pots were treated once per week and were watered as needed during the test period. Six seedlings with different phenolic acid contents were selected to determine gas-exchange parameters two months after the phenolic acid stress. Six seedlings from each treatment were used to measure plant height, ground diameter, and fresh mass. Seedling height and ground diameter were measured with a tape (accuracy of 0.1 cm) and Vernier calipers (accuracy of 0.01 mm), respectively. Fresh mass (FM) was determined with an electronic balance (accuracy of 0.0001 g).

Gas-exchange parameters: An *LI-6400* portable photosynthesis system (*LI-COR Inc.*, Lincoln, NE, USA) was used to measure the photosynthetic gas-exchange parameters of functional leaves in the centre of *R. delavayi* leaves at 9:00–12:00 h on a sunny day. The light sources were red and blue, the PAR was natural active radiation (the average PAR of 1,200 µmol m^{-2} s^{-1}), leaf temperature was 25°C, CO_2 concentration was the atmospheric CO_2 concentration (C_a), and the gas-flow rate was 500 mmol s^{-1}. Stomatal conductance (g_s), intercellular CO_2 concentration (C_i), net photosynthetic rate (P_N), and transpiration rate (E) were measured. We also calculated water-use efficiency (WUE = P_N/E) and the stomatal limitation value ($L_s = 1 - C_i/C_a$).

Photosynthetic pigments: The content of photosynthetic pigments was determined after measuring the photosynthetic gas exchange parameters. Photosynthetic pigments (Chls and Cars) were calculated using using a UV-Visible spectrophotometer (*Cary 100*, *Varian*, Palo Alto, CA, USA) according to Arnon (1949) and Jensen (1978).

Stomatal characteristics: We wiped the mature leaves with absorbent cotton and used a blade to take a sample of about 3 × 3 mm from the leaves. The leaf was fixed on the sample table using double-sided adhesive tape and coated with platinum particles (*JFC-1600*, *JEOL*, Japan) for 30 s, and viewed under a scanning electron microscope (*KYKY-1000B*, *Shimadzu*, Kyoto, Japan), imaged with a digital microscope imaging system and digitised with distance detection software (*Motic Images Advanced 3.0*). Three fields were observed on each leaf. Stomatal length, stomatal width, and stomatal density were measured according to Zhou *et al*. (2017). Stomatal opening and the stomatal opening ratio were compared with data from Yang *et al*. (2012).

Data analysis: Data were processed with *MS-Excel® 2013* (*Microsoft Inc.*, Redmond, WA, USA) and *SPSS 19.0* (*SPSS Inc.*, Chicago, IL, USA) software. One-way analysis of variance (*ANOVA*) and the least significant difference test (*LSD*) were used for significance testing ($p<0.05$). The redundancy analysis (RDA) was run using the *CANOCO* software package (*version 4.5*). In this analysis, the response variables were the growth and biomass indices and the explanatory variables were the Chl and stomatal characteristics. A *Pearson*'s coefficient correlation analysis was conducted to test the significance of the relationships between parameters and data using *SPSS 19.0* software. The drawing was completed by *Sigmaplot 13.0* software (*Systat Software Inc.*, San Jose, CA) and a distribution map of *R. delavayi* was drawn using *ArcGIS 10.2* software.

Results

Morphological indexes: The low concentration of FA slightly inhibited the height of seedlings and did not change the ground diameter. The moderate concentration of FA significantly restrained the height and ground diameter of seedlings. The high concentration of FA significantly reduced the height, but enhanced ground diameter of seedlings. The height and ground diameter growth of seedlings was slightly suppressed by CA, and increasing the concentration led to a restraint of basal growth. No variation in ground diameter growth of seedlings was observed between the low concentration of CA and CK. The moderate concentration of PA restrained the height and ground diameter growth of seedlings. The low concentration of PA stimulated ground diameter growth of the seedlings. The high concentration of PA promoted the height and inhibited ground diameter growth of seedlings (Fig. 2*A*,*B*).

The three phenolic acids signi-ficantly reduced aboveground and underground biomass compared with the CK, and had the greatest effect on the underground biomass. The effect of the phenolic acids on biomass increased gradually with increasing concentration (Fig. 2*C*,*D*). The effect of different concentrations of CA and PA on the underground biomass was significantly different. The effect of the different phenolic acids on the aboveground biomass was significant.

Gas-exchange parameters: Increasing the phenolic acid concentration caused decreases in P_N, WUE, and L_s (Fig. 3*A*,*E*,*F*), and increases in g_s, C_i, and E after the FA treatment (Fig. 3*B*–*D*). High concentrations of FA increased C_i and E. No significant difference was observed between L_s, C_i, E under the moderate concentrations of FA treatment, compared to CK.

CA significantly inhibited g_s (Fig. 3*B*). The inhibitory effects were weakened on P_N, C_i, and E with increasing CA concentration, and no differences were seen at the high concentration (Fig. 3*A*,*C*,*D*), compared to CK. WUE and L_s decreased gradually with increasing CA concentration (Fig. 3*E*,*F*); the low and medium concentrations of CA promoted the WUE and L_s; WUE was lower and L_s was slightly higher than CK after applying the high concentration of CA.

P_N, g_s, C_i, E, and WUE decreased and then increased gradually under the PA treatment (Fig. 3*A*–*E*). In contrast, L_s increased and then decreased (Fig. 3*F*). P_N, g_s, C_i, and L_s presented no significant variations from CK at the low concentration of PA. P_N and g_s were restrained and C_i was promoted by the moderate and high concentrations of PA, respectively. The high concentration of PA significantly increased E. No clear effect of PA on WUE was observed at any of the concentrations tested.

Photosynthetic pigment: The relative contents of Chl *a*, Chl *b*, total Chl, and carotenoids (Car) were enhanced by the three kinds of phenolic acids (Fig. 4*A*–*D*). Chl *a*, Chl *b*, total Chl, and Car contents increased first and then decreased with increasing FA concentration. Pigment contents first decreased, and then increased with increasing CA concentration, while they gradually increased with increasing PA concentration.

The stimulating effect of FA on Chl was the highest,

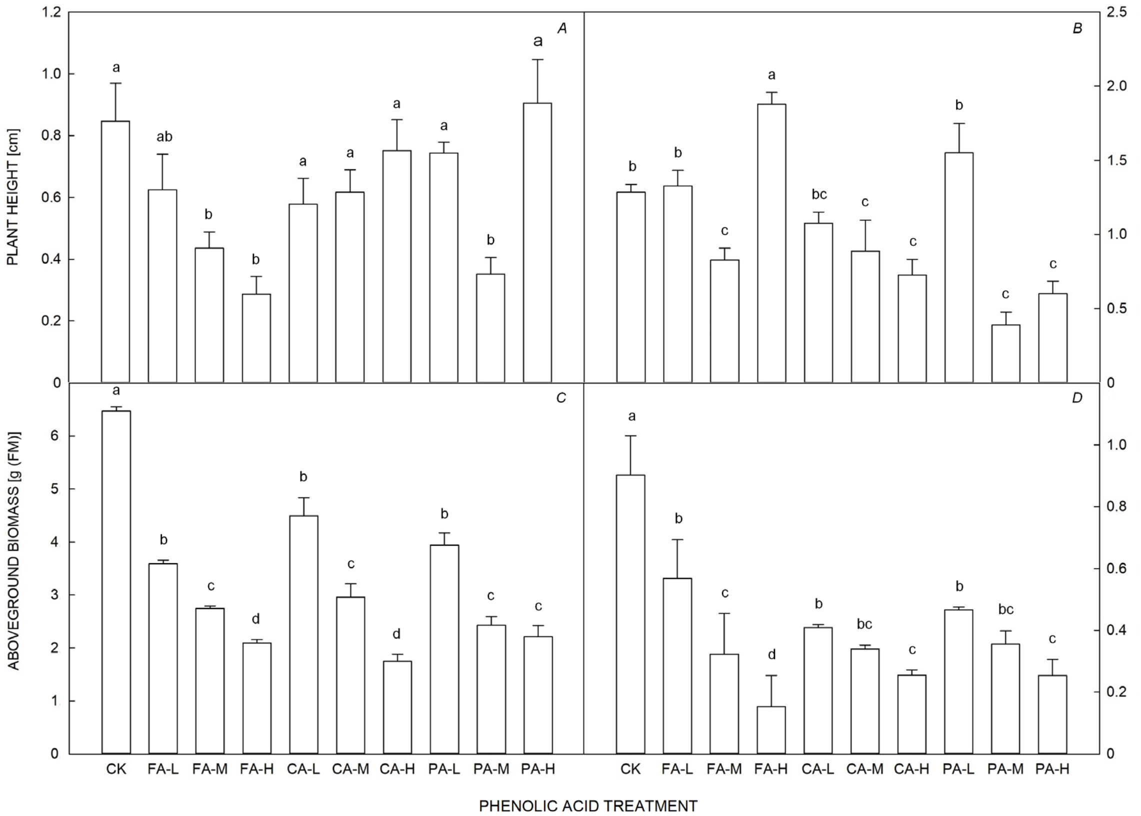

Fig. 2. Effects of phenolic acids treatments on seedlings growth and biomass of *Rhododendron delavayi*. Values are means ± SD, $n = 6$. The *different lowercase letters* indicate significant difference ($p<0.05$). CA – chlorogenic acid, L, M, H represent the concentrations of 200, 400, 800 μg L^{-1}, respectively; FA – ferulic acid, L, M, H represent the concentrations of 1,000, 2,000, 4,000 μg L^{-1}, respectively; PA – protocatechuic acid, L, M, H represent the concentrations of 50, 250, 1,250 μg L^{-1}, respectively; CK – normal management with distilled water. (*C*) and (*D*): g per plant.

and Chl *a*, Chl *b*, total Chl, and Car increased 3.41, 3.35, 2.70, and 3.44 times compared with CK, respectively. The effects of the phenolic acids on the relative contents of Chl were significantly different from those of CK. The effect of the moderate concentration of FA and the low concentration of CA on Car were significantly different from that of CK. No significant differences were observed in the other concentrations of phenolic acids tested.

Stomatal characteristics: The moderate and high concentrations of FA and CA significantly reduced stomatal density compared with the CK (Figs. 5*A*, 6*C–G*). The low concentration of FA significantly inhibited stomata opening, their length and width (Figs. 5*B–D*, 6*B*); the low and high concentrations of FA significantly inhibited the stomatal opening ratio (Figs. 5*E*; 6*B*,*D*). The moderate concentrations of FA and CA promoted the stomatal length and opening ratio (Figs. 5*C*,*E*; 6*C*,*E–G*), and the high concentration of CA significantly promoted stomatal width (Figs. 5*D*, 6*G*). However, there were no significant differences in stomatal density, opening degree, length and width, opening ratio rate between the other phenolic acids concentrations and CK.

Discussion

Morphological indexes: Plant growth rates are affected by phenolic acid stress (Khan *et al*. 2003). The growth of *R. delavayi* was inhibited by FA, which was basically in agreement with Singh *et al*. (2014). P_N is the main driving factor affecting seedling growth (Fig. 7*A*), and it is positively correlated with underground and aboveground biomass as well as plant height (Fig. 7*A*; Table 1S, *supplement*). P_N is the dominant factor influencing seedling growth and biomass, which was similar with Mateo *et al*. (2006) findings that the growth retardation of the *Arabidopsis* salicylic acid mutant is caused by photosynthesis damage. Under CA stress, g_s was the main contributor to seedling growth (Fig. 7*C*), which was positively correlated with seedling height (Table 1S), indicating that CA caused stomatal closure which then decreased the C_i, carbon assimilation, and photosynthetic activity, and hindered seedling growth (Mori *et al*. 2001). However, the growth, *i.e.*, the height of seedlings, under CA stress was insignificant, which was attributed to reduced carbon assimilation causing a failure to reach the threshold value for seedling growth. A previous study revealed that PA improves synthesis of indole acetic acid (IAA) (Mandal *et al*. 2009). Under PA treatment, *E* was the main factor affecting the seedling growth (Fig. 7*E*),

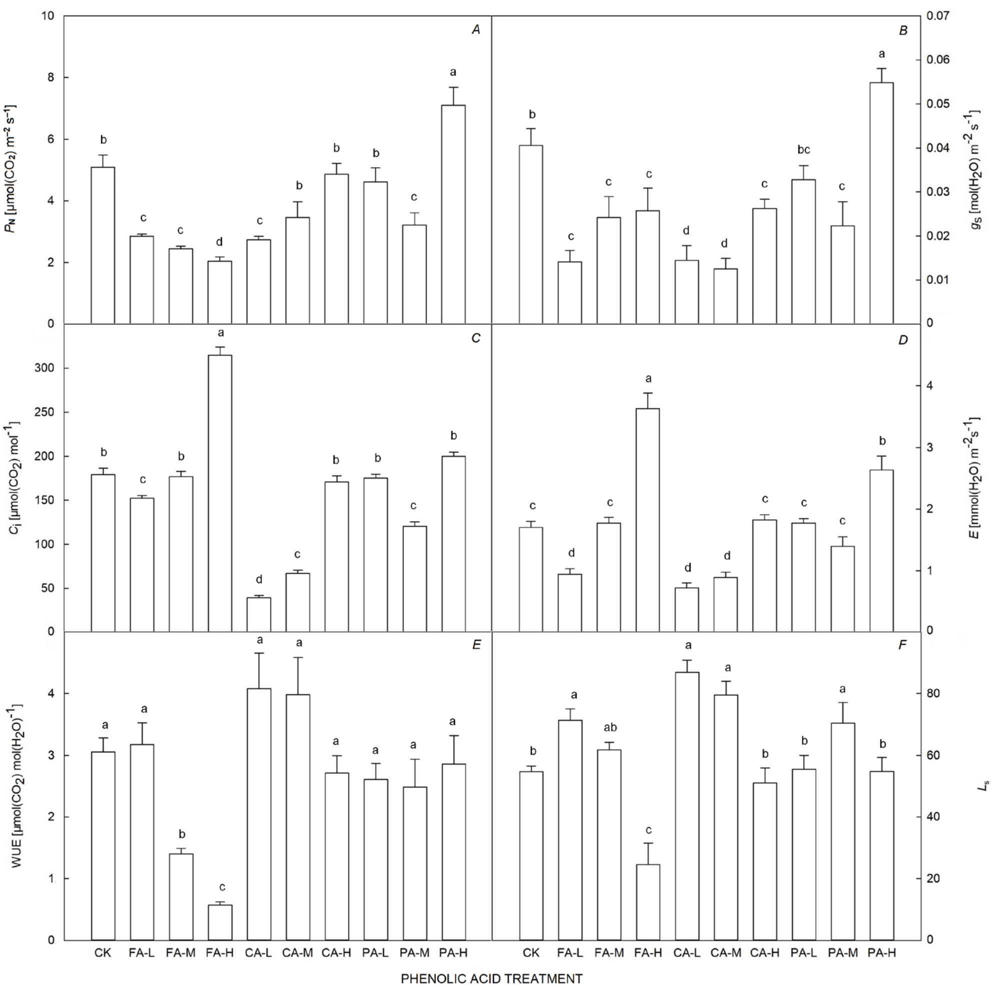

Fig. 3. Effects of phenolic acids treatments on photosynthesis parameters of *R. delavayi*. Values are means ± SD, *n* = 6. The *different lowercase letters* indicate significant difference ($p<0.05$). CA – chlorogenic acid, L, M, H represent the concentrations of 200, 400, 800 μg L^{-1}, respectively; FA – ferulic acid, L, M, H represent the concentrations of 1,000, 2,000, 4,000 μg L^{-1}, respectively; PA – protocatechuic acid, L, M, H represent the concentrations of 50, 250, 1,250 μg L^{-1}, respectively; g_s – stomatal conductance; L_s – stomatal limitation value (= $1 - C_i/C_a$); P_N – net photosynthetic rate; E – transpiration rate; WUE – water-use efficiency (= P_N/E); C_i – intercellular CO_2 concentration; CK – normal management with distilled water.

and positively correlated with the height of seedlings, but the correlation was not significant (Table 1S). P_N, C_i, and g_s showed significant positive correlations with the height of seedlings (Fig. 7*E*; Table 1S), indicating that PA mainly affected stomatal closure which then decreased the C_i, E, and P_N, thereby affecting growth. The low PA concentration inhibited growth of seedlings, while the high concentration showed the opposite effect, which was probably due to seedlings developing a number of stress-resistant substances, such as osmotic regulation substances that are resistant to changes in the external environment (Baziramakenga *et al*. 1994) and facilitate the synthesis of IAA (Mandal *et al*. 2009) to stimulate growth. A similar result was found for PA, which promotes root growth (Singh *et al*. 2014).

Roots are more sensitive to the phenolic acid reaction than aerial parts of plants (Chon *et al*. 2002). In this study, we found that FA, CA, and PA had greater inhibitory effects on root biomass than that on aboveground biomass (Fig. 2*C*,*D*). This result is similar to the results of previous studies (Chon *et al*. 2002, Khattak *et al*. 2015). At the same time, this inhibitory effect also increased with increasing phenolic concentration (*see* Djurdjevic *et al*. 2004).

Photosynthetic pigments: Chl *a*, Chl *b*, and Car are the most important pigments in photosynthesis. Chl *b* and Car contents can reflect plant growth status and photosynthetic capacity (Ronzhina *et al*. 2004). Studies have shown that plant photosynthetic pigments decrease significantly as phenolic acid concentration increases, and the decrease in photosynthetic pigments leads to the transformation of light energy and the supply of energy, which affects normal photosynthesis (Lu *et al*. 2018). Studies have also shown that the plant root system of *Rhododendron* and some fungi form ericoid mycorrhiza (ERM) in natural habitats (Read 1996), and the ERM increases photosynthetic pigment contents in *Rhododendron* leaves, strengthening resistance to stress factors (Cairney and Meharg 2003, Jing 2013). In

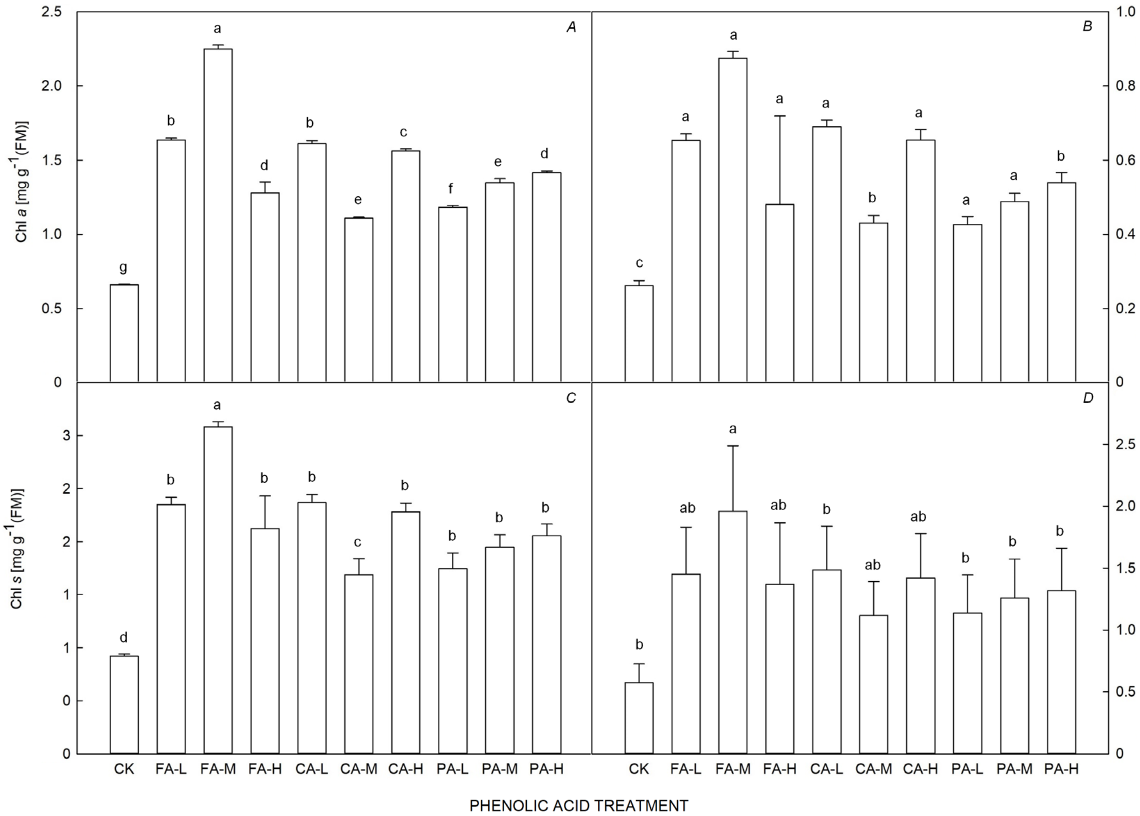

Fig. 4. Effects of phenolic acids treatments on photosynthetic pigment contents of *R. delavayi*. Values are means ± SD, $n = 6$. The *different lowercase letters* indicate significant difference ($p<0.05$). CA – chlorogenic acid, L, M, H represent the concentrations of 200, 400, 800 µg L^{-1}, respectively; FA – ferulic acid, L, M, H represent the concentrations of 1,000, 2,000, 4,000 µg L^{-1}, respectively; PA – protocatechuic acid, L, M, H represent the concentrations of 50, 250, 1,250 µg L^{-1}, respectively; Chl – chlorophyll; Chl *s* – total chlorophyll; Car – carotenoids; CK – normal management with distilled water. mg g^{-1}(FM),

this study, the three phenolic acids enhanced the relative contents of Chl *a*, Chl *b*, and Car, unlike the results of most studies (John 2012, Lu *et al*. 2018). This finding may be related to the promotion of Chl content by *R. delavayi* mycorrhizal fungi (Lin *et al*. 2011, Jing 2013). Under normal circumstances, P_N decreases along with the Chl content (Wang 2015, Xie *et al*. 2018). In this study, under the three kinds of phenolic stress, Chl *a*, Chl *b*, and total Chl were extremely negatively correlated with P_N, and the results of the RDA analysis showed that Chl *a* was the main factor influencing the photosynthetic characteristics (Fig. 7*B*,*D*,*F*) and ensured the capture of light energy. The results showed that the effect of the three phenolic acids on photosynthesis in *R. delavayi* seedlings was probably caused by some other factors, not only by the Chl loss. The reason may be that under the phenolic acid treatment, *R. delavayi* alleviated the influence of external phenolic acid stress by increasing the content of photosynthetic pigments under declining photosynthetic capacity (Chen *et al*. 2007, Cai *et al*. 2014). The photosynthetic pigment content increased, while P_N decreased under CA stress conditions. Car is not only a photosynthetic pigment, but also an antioxidant that plays a role of scavenger of reactive oxygen species, preventing membrane oxidation, and protecting chloroplasts (Efeoğlu *et al*. 2009). In this study, the changes in Car content under treatment with the three phenolic acids were consistent with those of Chl *a* and Chl *b*, indicating that Car perform essential photoprotective roles in chloroplasts by scavenging singlet oxygen and other reactive oxygen species under stress (Choudhury and Behera 2001), thus Car have a substantial role in the Chl protection of *R. delavayi* seedlings under the phenolic stress conditions.

Stomatal characteristics: The pores in leaves form a channel for external CO_2 input and water output, and the density, opening, and opening ratio of the pores directly affect leaf P_N and *E* (Zhou *et al*. 2014). In this study, CA significantly reduced stomatal density of *R. delavayi* leaves. In this study, g_s was significantly positively correlated with stomatal density (Table 2S, supplement), and P_N had significant and extremely significant positive correlations with g_s and *E* (Table 3S, supplement). As a result, the decrease in g_s caused by stomatal density may be one of the main factors leading to P_N stomatal limitations and the decline in *E* (Nilsen and Orcutt 1996). The low concentration of FA significantly inhibited stomatal opening and the opening ratio of *R. delavayi* leaves. A significant positive correlation was observed between the stomatal aperture with *E* and

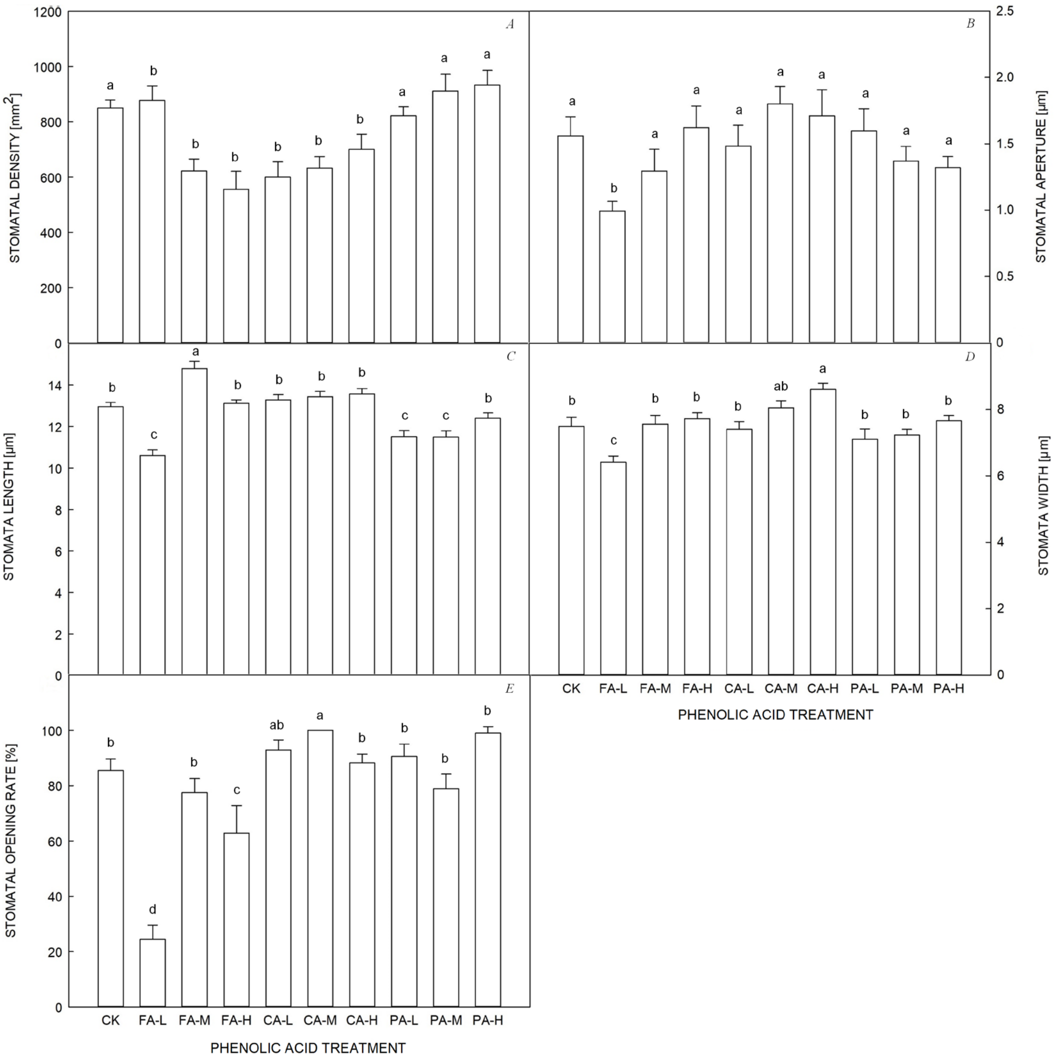

Fig. 5. Effects of phenolic acids treatments on stomatal characteristics of *R. delavayi*. Values are means ± SD, n = 18. The *different lowercase letters* indicate significant difference (p<0.05). CA – chlorogenic acid, L, M, H represent the concentrations of 200, 400, 800 µg L^{-1}, respectively; FA – ferulic acid, L, M, H represent the concentrations of 1,000, 2,000, 4,000 µg L^{-1}, respectively; PA – protocatechuic acid, L, M, H represent the concentrations of 50, 250, 1,250 µg L^{-1}, respectively; CK – normal management with distilled water.

the stomatal opening ratio with g_s (Table 2S), indicating that the low concentration of FA inhibits photosynthesis by stomatal mechanism, apparent from stomatal aperture and stomatal opening ratio. The moderate and high concentrations of FA significantly reduced leaf stomatal density. Stomatal density was significantly positively and negatively correlated with P_N and C_i, respectively (Table 2S), and P_N was significantly negatively correlated with C_i but positively correlated with g_s, but the correlation was not significant (Table 3S), indicating that the decrease in g_s caused by stomatal density is not a key factor causing the decline in P_N (Farquhar and Sharkey 1982, Yang *et al* 2012). Photosynthesis may be inhibited by reducing the amount of functional reaction centers of PSII (Dwyer *et al.* 2012). The maximum quantum yield of the PSII primary photochemistry was depressed, which has yet to be studied (Lu *et al*. 2018, Xie *et al*. 2018). PA had no effect on the leaf stomata of *R. delavayi*. The stomatal opening ratio was significantly positively correlated with P_N, g_s, and C_i, respectively (Table 2S), and P_N was significantly positively correlated with g_s and C_i (Table 3S). Thus, PA affected P_N, g_s, and C_i only when its concentration reached a certain value (medium concentration), while the results were opposite in the case of the excessive concentration.

Photosynthetic characteristics: Previous studies have shown that phenolic acids affect plant growth by reducing leaf photosynthesis, Chl content, protein synthesis, enzymatic activity, *E*, and g_s (Mathesius 2001, Blum and Geric 2005, Heleno *et al.* 2015). The main pathways through which phenolic acid affects plant P_N are stomatal and nonstomatal limitations (John 2012, Lu *et al*. 2018). Previous studies have indicated that the concentration of CO_2 (C_i) and the limiting value of stomata (L_s) can

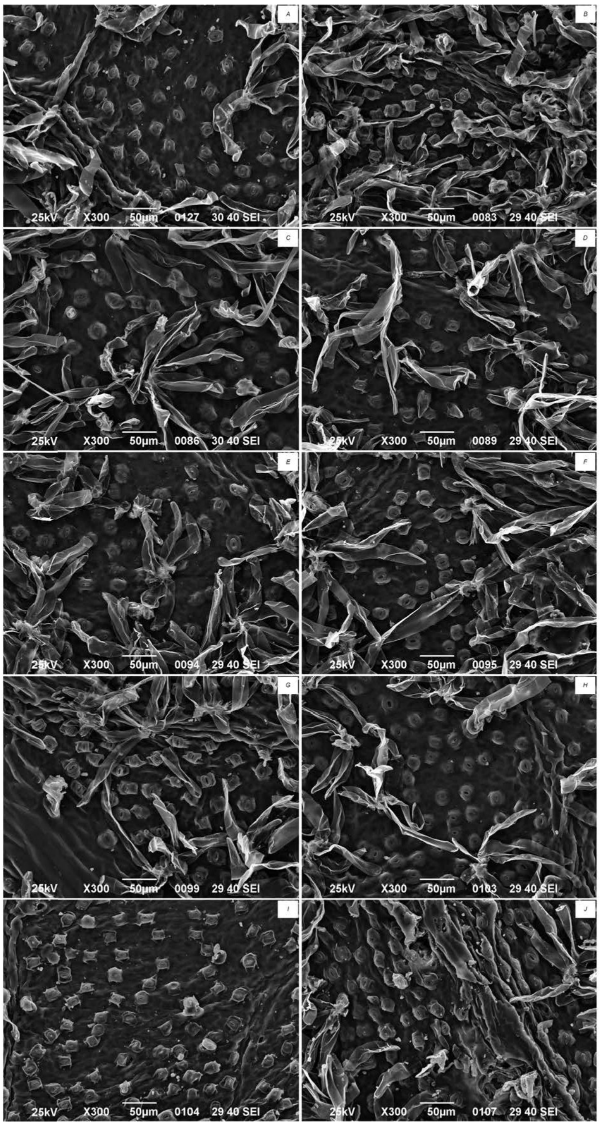

Fig. 6. Epidermal stomata of *R. delavayi* leaf under phenolic acids treatments. (*A*) normal management with water; (*B*) 1,000 μg L^{-1} ferulic acid; (*C*) 2,000 μg L^{-1} ferulic acid; (*D*) 4,000 μg L^{-1} ferulic acid; (*E*) 200 μg L^{-1} chlorogenic acid; (*F*) 400 μg L^{-1} chlorogenic acid; (*G*) 800 μg L^{-1} chlorogenic acid; (*H*) 50 μg L^{-1} protocatechuic acid; (*I*) 250 μg L^{-1} protocatechuic acid; and (*J*) 1,250 μg L^{-1} protocatechuic acid.

predict stomatal or nonstomatal limitations as a cause for a decrease of P_N (Farquhar and Sharkey 1982). In this study, FA significantly restrained the P_N in *R. delavayi*, which was in accordance with Hussain and Hussain (2011). C_i and L_s were inhibited at the low FA concentration, and L_s and C_i were promoted at the high concentration, which led to a stomatal limitation and nonstomatal limitation of *R. delavayi*, respectively. A reduction in g_s leads to a lower CO_2 concentration in cells (Verma *et al*. 2014) and a decrease in substrate concentration of related enzymes in photosynthesis and ultimately to reduced P_N (Zhao *et al*. 2014). g_s was apparently lower than CK at the moderate concentration of FA; C_i and L_s exhibited no clear difference from CK, which was caused by stomatal closure of *R. delavayi* due to the decrease in P_N. Stomatal density was the main driving factor causing the decrease in P_N in *R. delavayi* (Fig. 7*B*). This finding was consistent with the results pertaining to the stomatal characteristics, which were significantly positively correlated with WUE and strongly negatively correlated with *E* (Fig 7*B*; Table 2S). FA mainly caused a stomatal limitation at the low concentration (Marchiosi *et al.* 2016, Lu *et al.* 2017), the reduction of P_N at the high concentration was caused by nonstomatal factors (Hui and Qi 2002, John 2012).

Under CA stress, P_N was negatively correlated with the concentration, which was in accordance with Mersie and Singh (1993) and Sumbele *et al.* (2012). Under the low and moderate concentrations of CA, P_N, C_i, *E*, and g_s in the seedlings of *R. delavayi* were significantly lower than that of CK; however, g_s, *E*, WUE, and L_s showed no significant difference between the low and moderate concentrations of CA (Fig. 5*B*,*D-F*). The higher concentration of CA may promote water conductivity in *R. delavayi*, which helped maintain a certain photosynthetic rate (Li *et al*. 2010, John 2012). In addition, C_i, P_N, and L_s increased with increasing concentration of CA only to decrease thereafter. No significant differences in P_N, C_i, *E*, WUE, or L_s were observed between the high concentration of CA and the CK, which also indicated that the decrease in P_N was caused by stomatal limitations. The high concentration of CA did not increase photosynthetic ability in *R. delavayi* (Muscolo *et al*. 2002, Marchiosi *et al*. 2016). The positive correlation was significant between g_s and stomatal density (Fig. 7*D*; Table 2S), which was confirmed by *Pearson*'s correlation analysis and was consistent with the analysis of stomata characteristics.

Exogenous phenolic acids have antioxidant activity and can protect photosynthetic apparatus (Mateo *et al*. 2006). P_N, g_s, C_i, *E*, WUE, and L_s did not change between the low concentration of PA and CK, indicating that a low concentration of PA did not affect the physiological response of *R. delavayi* seedlings. Under the medium concentration of PA, C_i, g_s, and L_s were significantly lower and higher than that of CK, indicating that the moderate concentration of PA affected P_N through a stomatal limitation of leaves as shown by the RDA analysis and *Pearson*'s correlation analysis; g_s, the vertical axes of stomata, and the stomatal opening ratios were significantly correlated (Fig. 7*F*; Table 2S). g_s and C_i were significantly higher under the moderate concentration of PA than that of CK; no obvious difference was found for L_s and WUE compared with CK, whereas *E* and P_N increased significantly under the high concentration of PA. The high concentration of PA showed potent antioxidant activities and could alleviate the stress on photosynthetic organ damage (Mori *et al*. 2001). The *R. delavayi* seedlings were sensitive to the high concentration of PA.

Conclusion: Three kinds of phenolic acids decreased the photosynthetic pigment contents in *R. delavayi* leaves, and inhibited the accumulation of biomass. Chlorogenic acid and the medium and high ferulic acid concentrations

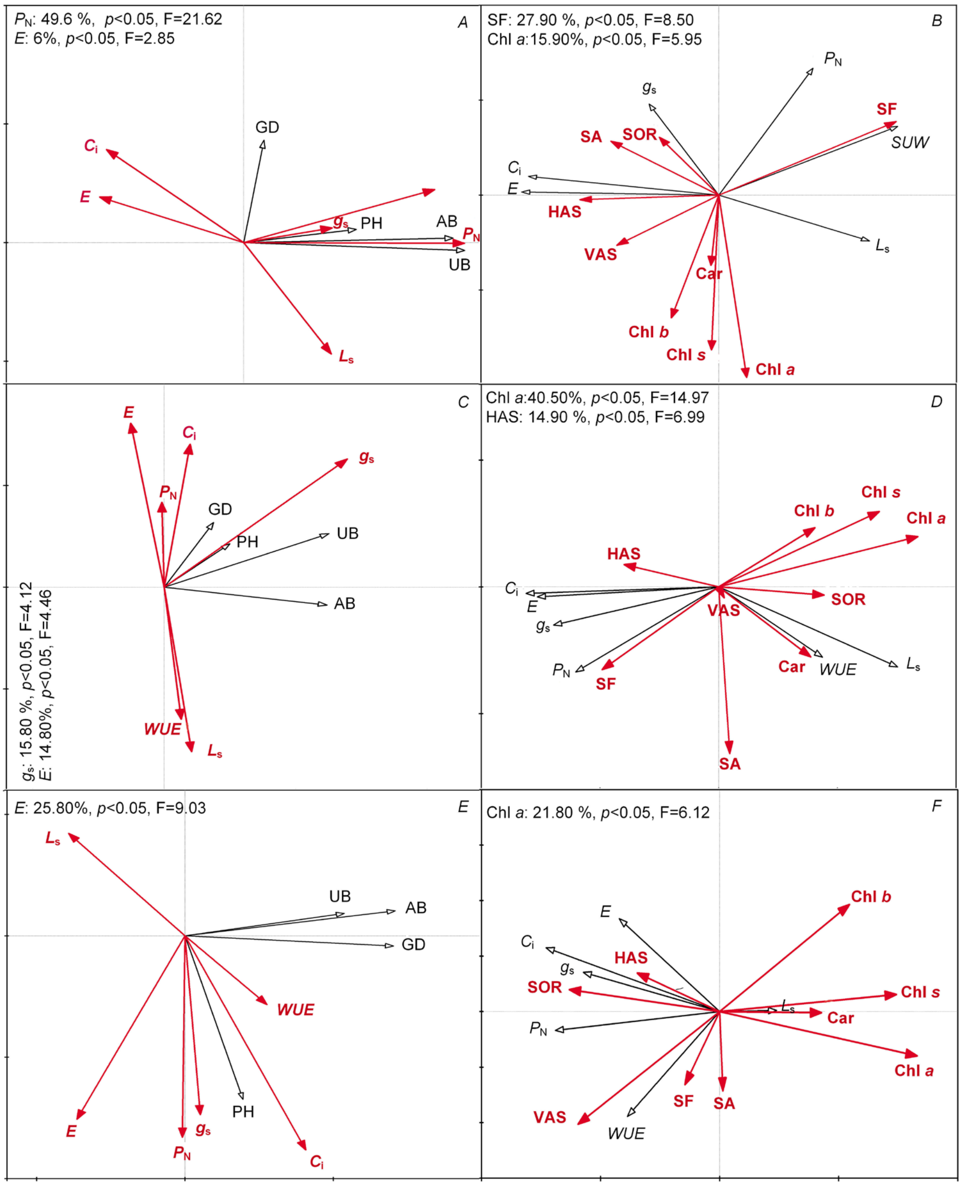

Fig. 7. RDA analysis of photosynthetic characteristics, seedling growth and biomass, stomata, and pigments under phenolic acid treatments. PH – plant height; GD – ground diameter; AB – aboveground biomass; UB – underground biomass; Chl – chlorophyll; Chl *s* – total chlorophyll; Car – carotenoids; P_N – net photosynthetic rate; g_s – stomatal conductance; C_i – intercellular CO_2 concentration; E – transpiration rate; WUE – water-use efficiency (= P_N/E); L_s – stomatal limitation value (= $1 - C_i/C_a$); SF – stomatal density; SA – stomatal aperture; VAS – vertical axes of stomata; HAS – horizontal axes of stomata; SOR – stomatal opening ratio.

decreased stomatal density. The low ferulic acid concentration decreased stomatal opening and the stomatal opening ratio in leaves, thereby affecting photosynthesis and growth of *R. delavayi* seedlings. Photosynthesis was negatively affected under the medium concentration of protocatechuic acid, while the high concentration showed an opposite effect. Ferulic acid had the strongest effect, whereas protocatechuic acid had the weakest effect on *R. delavayi* seedlings.

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