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Diaporthe cotoneastri and *Ilyonectria cyclaminicola* endophytes promote the growth, development, and accumulation of active components of *Epimedium koreanum* Nakai host plants in field experiments

Jiawen Chen¹, Xing Hu¹, Yang Bai¹, Hanning Liu¹, Xin Zhuang¹, Junjie Guo¹ and Jinglei Xiao^{1*} 

Abstract

Purpose This study aims to explore the effects of two endophytes of *Epimedium koreanum* Nakai on host plant growth and development and medicinal material quality and to promote the growth and development of ingredients derived from *Epimedium koreanum* Nakai.

Methods The effects on the biomass, photosynthesis parameters, chlorophyll fluorescence parameters, activity of key enzymes, and active components in the process of growth and development in Korea were examined.

Results From the analysis of biomass and photosynthesis data, both the S3 (*Diaporthe cotoneastri*) and S10 (*Ilyonectria cyclaminicola*) strains promoted the growth of *Epimedium koreanum* Nakai. Redundancy analysis (RDA) showed that the activity of S3 and the content of epimedin A were negatively correlated and that the peroxidase (POD) activity was positively correlated with the total flavonoid content.

Conclusion The application of the S3 (*Diaporthe cotoneastri*) and S10 (*Ilyonectria cyclaminicola*) strains can significantly increase the biomass and contents of total flavonoids, icariin, epimedin B, and epimedin C, but not epimedin A, in *Epimedium koreanum* Nakai host plants grown in field conditions. The S10 strain promotes faster growth of *Epimedium koreanum* Nakai than the S3 strain, and flavonoid 3-hydroxylase (F3H) catalyzes the synthesis of epimedin A and icariin by different pathways using the same substrate. POD plays a key role in total flavonoid synthesis in *Epimedium koreanum* Nakai.

Keywords Flavonoid 3-hydroxylase, *Epimedium koreanum* Nakai, Total flavonoids, Epimedin A, Icariin

Introduction

Endophytes are a classification of microbes present in healthy plants that do not cause infection to the host (Guo, L.D.; Huang, G.R.; Wang, Y. 2008). Many studies have shown that some endophytes can shorten the growth and development cycle of their host plants, but can also enhance the competitiveness of host plants against other plant species. For example, under extreme conditions, strain S61 (*Trichoderma atroviride*)

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promoted the rapid growth of *Astragalus membranaceus* (Fisch.) Bge. root and significantly increased the root-to-crown ratio, further promoting the accumulation of glucoside and miscaridin, which are the active components of *Astragalus* root isoflavones. Endophytes of host plants have attracted increased research attention in recent years, demonstrating that inoculation of endophytes could improve the overall host plant microbial biome, plant growth, and resistance against plant diseases and insect pests, providing new directions for improving the growth and metabolic regulation of medicinal plants. One mechanism by which plant endophytes promote host plant growth is via the production of biomass such as phytohormones, including auxin and ethylene (Patton et al. 2002). Studies have shown that some strains of *Pseudomonas* and nitrogen-fixing bacteria (*Azotobacter*) can promote plant growth and development (Leifert et al. 1994, Holland, M.A. 1997 and Bashan et al. 1997) by producing plant growth regulators such as ethylene, auxin, or cytokinin. For example, the strains isolated from Oli could secrete the auxin indole-3-acetic acid (IAA), thereby expanding the root surface area by absorbing the water and minerals from the soil, thus improving the yield and quality of Oli (Kim et al. 2011). Moreover, some plant-associated bacteria have been suggested to promote plant growth based on the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminases. For example, eight endophytes with ACC deaminase were isolated in rice, which were found to reduce the damage (Matsuoka et al. 2016, Barnawal et al. 2013) caused by ethylene to plants by reducing the content of ACC and ethylene-induced stress in the roots, and could also promote (Singh et al. 2017, Glick, B.R. 1998) and improve the crop yield (Bashan et al. 1997).

Endophytes can increase plant resistance to abiotic stress such as cold, drought, heavy metal pollution, and insect and herbivore feeding (Redman et al. 2002), as well as biological stress such as diseases and insect pests. Endophytes were also found to enhance the degree of plant tolerance to drought stress (Barnawal et al. 2013). Studies have also found that plants inoculated with endophytes under aluminum (Al^{3+}) stress conditions had a higher root growth rate, root biomass, and total biomass (Liu et al. 1996). Endophytes have also been found to play a role in the intrinsic mechanism of the plant resistance response (Rangel de Souza et al. 2016, Del Amor et al. 2012). Inoculation with endophytes can increase the chlorophyll content of plants, accelerate the rate of plant photosynthesis, and increase water utilization by controlling the opening and closing of stomata. Narisawa et al. (2004) found that the application of endophytes to Chinese cabbage could effectively prevent root swelling disease and verticillium wilt. One potential mechanism by

which endophytes help in the fight against plant pests is by inhibiting the growth of plant pathogens via producing physiologically active substances such as antibiotics, antibacterial substances, and cell wall hydrolytic enzymes (Seo et al. 2012, Lodewyckx et al. 2001, and Liu et al. 2006). Endophytes have also been suggested to enhance the adaptability of plants to extreme conditions by improving the plant's absorption capacity of minerals and organic substances, whereas others have suggested that the mechanism involves regulation of signal transduction (Bordiee et al. 2011). However, the specific mechanism remains to be elucidated and thus requires further study.

Diaporthe genus is among the most abundant endophytic fungi that can be recovered from diverse plant hosts (Carvalho et al. 2012, Gomes et al. 2013), including species that are known to produce compounds with anticancer (Kumaran, R.S.; Hur, B. 2009), antimicrobial (Bandre et al. 1977), lignocellulolytic (Jordaan et al. 2006), and bioherbicidal (Ash, G.J et al. 2010) activities. Inoculation with *Diaporthe* promoted the growth of grasses, increasing leaf biomass (by 84% in *Tritordeum* and by 29% in perennial ryegrass), root biomass, nutrient content (N, Ca, Mg, and Fe), and the production of IAA, regardless of salinity treatment. Improved growth and nutrient uptake could be attributed to the production of several extracellular enzymes capable of recycling organic nutrient pools by *Diaporthe*. In addition, the fungus was shown to produce IAA in vitro and could modulate the production of this phytohormone in the plant. Under salinity treatment, the activity of *Diaporthe* ameliorated the response to stress, increasing proline, nutrient uptake in the roots, gibberellins, and IAA, which in turn translated into improved growth (Peer W.A.; Murphy A. S 2006). Thus, the advantages of this fungus in its original habitat can potentially be transferred to alternative hosts.

We previously isolated the *Epimedium koreanum Nakai* endophyte strains *Diaporthe cotoneastri* (strain S3) and *Ilyonectria cyclaminicola* (strain S10) (unpublished study, patent pending). The results of a sterile cultivation experiment showed that the S3 and S10 endophytes could promote the growth of the host plant (*Epimedium koreanum Nakai*). We assume that the application of the S3 and S10 fungal agents promotes the growth, development, and accumulation of the active ingredients of Korean *Epimedium* and that the active components of ABC and icariin are positively correlated with F3H and POD.

Materials and methods

Plant material, experimental treatments, and design

All field experiments were conducted in the experimental practice base of Changchun University of Chinese Medicine, 2021–2022, China. Each plot surface was 14.4 m² (12 m × 1.2 m), and each group of *Epimedium* plants

was planted in a community, including three treatments: S3 (only the S3 fungal fluid was applied), S10 (only the S10 fungal fluid was applied), and CK (no fertilizer was applied). The trial adopted a randomized block design and was repeated three times. There were 120 plants in each plot (three rows, 40 plants in each row). In the spring of 2021, the row spacing was 25 cm, and the plant spacing was 4.5 cm. The field was not fertilized to restore growth. Each strain was used as a test group, using potato dextrose broth (PDB) liquid medium; samples without fungi served as the control group. This field study was conducted in an *Epimedium koreanum Nakai* plantation in China (125.43 E, 43.84 N) at an altitude of approximately 260 m above sea level. This region has a temperate continental climate, with an average annual temperature of 4.3 °C, average annual rainfall of 683 mm, relative humidity of 69%, and depression of 0.6–0.8. The soil (Haplic Phaeozem) consisted of 11.54 g/kg organic matter, 33.14 mg/kg alkali nitrogen, 62.89 mg/kg soluble phosphorus, and 99.16 mg/kg quick potassium. The microorganisms contained in the soil were tested before the experiment.

The samples were divided into three groups. Samples were collected every 15 days a total of five times and recorded as P1–P5. After the first stage of sampling, an equal amount of fungal fluid was applied to the rhizosphere of Korean *Epimedium koreanum Nakai*, and each group was separated by blocking plates to prevent mutual influence. The test strains were cultured in an equal volume of PDB liquid medium and incubated in a thermostatic culture oscillator at 150 rpm for 7 days at 28 °C. The fungal solution was diluted tenfold with sterile water, and a hemacytometer was used to calculate the fungal concentration.

Growth index determination

The leaves of *Epimedium koreanum Nakai* were harvested at each sample period (P1–P5) and the fresh weight, dry weight, and leaf area were measured. Only one leaf was picked from each Korean *Epimedium* during each period, causing no harm to their growth status.

Photosynthetic assays

On sunny days, photosynthetic activity measurements were taken from 09:30 to 11:30 using a dark adaptation clip to first adapt the leaf to the dark for 30 min by Chlorophyll fluorescence parameter analyzer, and then the leaf clip was released to measure the chlorophyll fluorescence parameters. Each leaf was measured three times and the average value was obtained. The photosynthesis apparatus uses an open gas path with a CO₂ concentration of approximately 400 μmol/L and an artificial light source.

Enzyme activity in the leaves

Fresh leaves were taken from each test group, and the enzyme activity was measured three times for each index.

Peroxidase activity assay

Korean *Epimedium* leaves were measured according to the peroxidase (POD) activity detection kit, and the POD enzyme activity was calculated according to the formula.

One gram of Korean *Epimedium* leaves was added to 1 mL of extract for ice bath homogenization and centrifuged at 8000 g at 4 °C for 10 min. The supernatant was removed and placed on ice for testing.

For enzyme solution treatment, the reagents included in Table 1 were added to EP tubes, mixed, and timed, and 200 μL of liquid was added to 96-well plates to record absorbent A1 at 470 nm for 30 s and absorbent A2 after 90 s. Calculate $\Delta A = A2 - A1$.

POD enzyme activity was calculated according to the formula: POD viability (U/g fresh weight) = 9800 ΔA sample weight.

Superoxide dismutase activity assay

Epimedium leaves were measured according to the superoxide dismutase (SOD) activity detection kit, and SOD enzyme activity was calculated according to the formula.

- (1) A total of 0.1 g of Korean *Epimedium* leaves was added to 1 mL of extract for ice bath homogenization and centrifuged at 8000 g at 4 °C for 10 min. The supernatant was removed and placed on ice for testing.
- (2) In addition, the reagents in Table 2 were added to a 96-well plate. After 30 min, the absorbance of each tube was measured at 560 nm. Calculate ΔA determination = A determination - A control, ΔA blank = A1 blank - A2 blank.
- (3) SOD enzyme activity was calculated according to the formula inhibition percentage = $(\Delta A \text{ blank} - \Delta A \text{ measurement}) / \Delta A \text{ blank} \times 100\%$, SOD vitality (U/g fresh weight) = 11.11 inhibition percentage (1 - inhibition percentage) dilution multiple of sample quality, and the SOD vitality of *Epimedium* leaves was calculated.

Table 1 Operation table of POD enzyme activity determination

Reagent name	Volume in tube (μL)
Reagent 1	120
Reagent 2	30
Reagent 3	30
Distilled water	60
Sample	5

Table 2 Operation table of SOD enzyme activity determination

Reagent name	Volume in tube (μL)	Contras tube volume (μL)	Blank tube 1 (μL)	Blank tube 2 (μL)
Sample	18	18	-	-
Reagent 1	45	45	45	45
Reagent 2	2	-	2	-
Reagent 3	35	35	35	35
Double distilled water	90	92	108	110
Reagent 4	10	10	10	10

Table 3 Operation table of PAL enzyme activity determination

Reagent name	Volume in sample tube (μL)	Blank tube (μL)
Sample	20	-
Reagent 1	780	800
Reagent 2	200	200
Reagents were mixed well and placed in a 30 °C accurate water bath for 30 min		
Reagent 3	40	40

Phenylalanine lyase activity assay

The leaves of *Epimedium* were measured according to the phenylalanine ammonia-lyase (PAL) activity detection kit, and the PAL enzyme activity was calculated according to the formula.

Taking 0.1 g of Korean *Epimedium* leaves, 1 mL of extract was added to an ice bath homogenate and centrifuged at 8000 g at 4 °C for 10 min. The supernatant was removed and placed on ice for testing.

For the enzyme solution, the reagents in Table 3 were added to 96-well plates, mixed, and incubated for 10 min, and A2 was recorded at 290 nm, $\Delta A = A1 - A2$.

The PAL enzyme activity was calculated according to the following formula: PAL vitality (U/g fresh weight) = $17.3 \Delta AW$.

Flavonin synthase activity and flavonoid 3-hydroxylase activity were measured

Then, 0.1 g of Korean *Epimedium* leaves was weighed with 1 mL of PBS buffer, and the supernatant was removed by centrifugation at 3000 r for 20 min. Then, 50 μL of the standard at varying concentrations was added to the standard well, and 40 μL of the sample dilution was added to the sample well with an additional 10 μL of the sample. Then, 100 μL of label reagent was added to each well except for the blank wells. The sealing plate

was incubated at 37 °C for 60 min, the liquid was poured, the washing liquid was added to each well, the liquid was discarded for 30 s, and this process was repeated 5 times. Fifty microliters of chromogens A and B were added to each well, incubated at 37 °C in the dark for 15 min and 50 μL of terminator solution. The absorbance was zeroed with a blank well, and the absorbance of each well was measured in sequence at a wavelength of 450 nm.

Determination of total flavonoid content

A 0.5-mL sample of the test solution under the total flavonol analysis was placed in a 50-mL measuring vial, methanol was added, and the mixture was shaken well. In addition, an appropriate amount of icariin control product was added to methanol to prepare a solution of 10 μg/mL, which served as the control product solution. The test solution, control solution, and corresponding reagent as the blank were subject to ultraviolet-visible spectrophotometry (Tu-1810 spectrophotometer), and the absorbance was measured at a wavelength of 270 nm to obtain the total flavonoid content.

Measurement of flavonol glycoside components

The flavonol glycoside components were identified by high-performance liquid chromatography (HPLC). The control solution was prepared with a mixture of 0.102 mg/mL icariin, 0.103 mg/mL epimedin A, 0.101 mg/mL epimedin B, and 0.105 mg/mL epimedin C. The test solution was prepared with 0.2 g of the test sample, to which 20 mL of diluted ethanol was added. The mixture was weighed and sonicated for 1 h, weighed again, and loss of weight was adjusted with diluted ethanol. The mixture was shaken well, filtered, and the filtrate was obtained for HPLC using a Shim-pack VP-ODS column (4.6 mm × 250 mm × 5 μm). The mobile phase was acetonitrile (B) and water (A). Gradient elution was performed as follows: 0–37 min 24% B, 37–47 min 24–38% B, 47–60 min 38–24% B. The wavelength was 270 nm, the column temperature was 30 °C, the flow rate was 1.0 mL/min, and the injection volume was 10 μL.

Linear relationship

The mixed control solution at 2 μL, 4 μL, 6 μL, 8 μL, 10 μL, and 12 μL was injected into the HPLC column, and chromatography was performed under the conditions described in the “Superoxide dismutase activity assay” section. The linear relationship was calculated by regression with the injection quantity (μg) as the abscissa and the peak area (A) as the ordinate. The regression equations for icariin, epimedin A, epimedin B, and epimedin C were $Y = 1,726,783.89X - 9556.7$,

$Y = 1,619,300.32X - 4968.76$, $Y = 2,467,127.86X - 1937.23$, and $Y = 1,419,631.90X - 5895.03$, respectively; the correlation coefficient (r) was 0.9999 and the linear range was 0.204–1.224 μg , 0.206–1.236 μg , 0.202–1.212 μg , and 0.210–1.260 μg , respectively.

Repeatability test

Six samples of the powder at approximately 0.2 g each were subjected to HPLC as described above, and the peak area values of icariin, epimedin A, epimedin B, and epimedin C were calculated. The relative standard deviation (RSD) values of the calculated mass fraction were 1.27%, 1.87%, 1.05%, and 1.98%, respectively. Thus, all of the RSD values were less than 2%, indicating good reproducibility of the analysis method.

Sample recovery test

Six sample powder samples with known content were accurately weighed, and four corresponding controls were added to calculate the sample recovery rate and RSD of each component. The average sample recovery rates of icariin, epimedin A, epimedin B, and epimedin C were 100.71%, 100.37%, 99.96%, and 101.57%, and the RSD values were 0.96%, 1.24%, 1.48%, and 1.74%, respectively. The RSD values were less than 2%, indicating good accuracy of the method. The liquid phase peak table is presented in Table 4.

Statistical analysis

The boxplots were plotted in R (version 3.6.0). Correlation matrix heatmaps were drawn using the R corrplot package (version 0.84). The R version 3.6.0 package was used for redundancy analysis (RDA). Statistical analyses were performed using the IBM SPSS Statistics program (version 21). Comparisons of distributions between 3 groups were made by factor analysis of variance, and correlations were tested with Spearman's rho correlation coefficients. Graphs were prepared in GraphPad Prism (version 7.02).

Results

Effect of strains S3 and S10 on biomass and photosynthesis in *Epimedium koreanum Nakai* grown

After application of the S3 and S10 strains, an increase ($P < 0.05$) in the fresh leaf weight (Fig. 1a), dry weight

(Fig. 1b), and leaf area (Fig. 1c) biomass was detected in the five sample periods in the order $S10 > S3 > CK$. At P2, the chlorophyll fluorescence values of the applied S3 biofertilizer were significantly greater than those of the blank and S10 groups. Over time, the fastest increase in the fluorescence parameter values was found in the treatment applying S10 as a biofertilizer. The fluorescence parameters of S3 and S10 at P5 were significantly greater than those of the blank group ($P < 0.05$) (Fig. 2).

As shown in Table 5, application of the S10 strain increased the net photosynthetic rate at each period and the water utilization rate also increased significantly. The net photosynthetic rate of the S3-treated group was lower than that of the blank group at P2, the water use efficiency was lower than that of the blank group at P3, whereas these values were higher than those of the blank group in the subsequent periods. The fastest photosynthesis rate, largest water utilization value, and slowest transpiration rate for all groups were found in P2.

Effect of S3 and S10 strains on enzyme activity of *Epimedium koreanum Nakai*

As shown in Fig. 3, the POD activity of each group increased after applying the S3 and S10 strains, with a large change at the end of the study period. Each treatment showed the same peak at P4, with the highest peak detected for the S10 treatment group. The PAL activity differences were large at the early and middle stages of strain application, with a large range of change between stages. In the later period, the PAL activity did not significantly differ, and the change range was small, appearing during the P2 period of S3 treatment. Application of the S10 strain enhanced PAL enzyme activity in *Epimedium koreanum Nakai* plants at most stages. Both the S3 and S10 strains increased F3H activity at each stage, with the highest activity detected at P1 and the lowest activity detected at P4, although both were greater than the F3H activity of the blank control group. At P5, only the S10 treatment group had higher F3H activity than that of the blank control group. Except at P3, the FNS content of the S3 and S10 treatment groups was greater than that of the blank group.

Effects of S3 and S10 strains on the accumulation of active components of host plants

As shown in Fig. 4, the S3 and S10 strains significantly increased the total flavonoid content of the Korean *Epimedium koreanum Nakai* host plant. Strain S3 could increase the total flavonoid content of *Epimedium koreanum Nakai* at all stages except for P3, and the total flavonoid content in the S10 group was greater than that in the blank control group. After application of the S3 and S10 strains, the content of epimedin A was lower at P2

Table 4 Peak table

Peak number	Retention time	Area	Resolution (USP)	Height
Epimedin A	27.006	213,692	19.046	4643
Epimedin C	35.152	166,658	3.826	2947
Icariin	39.018	427,687	2.547	6825

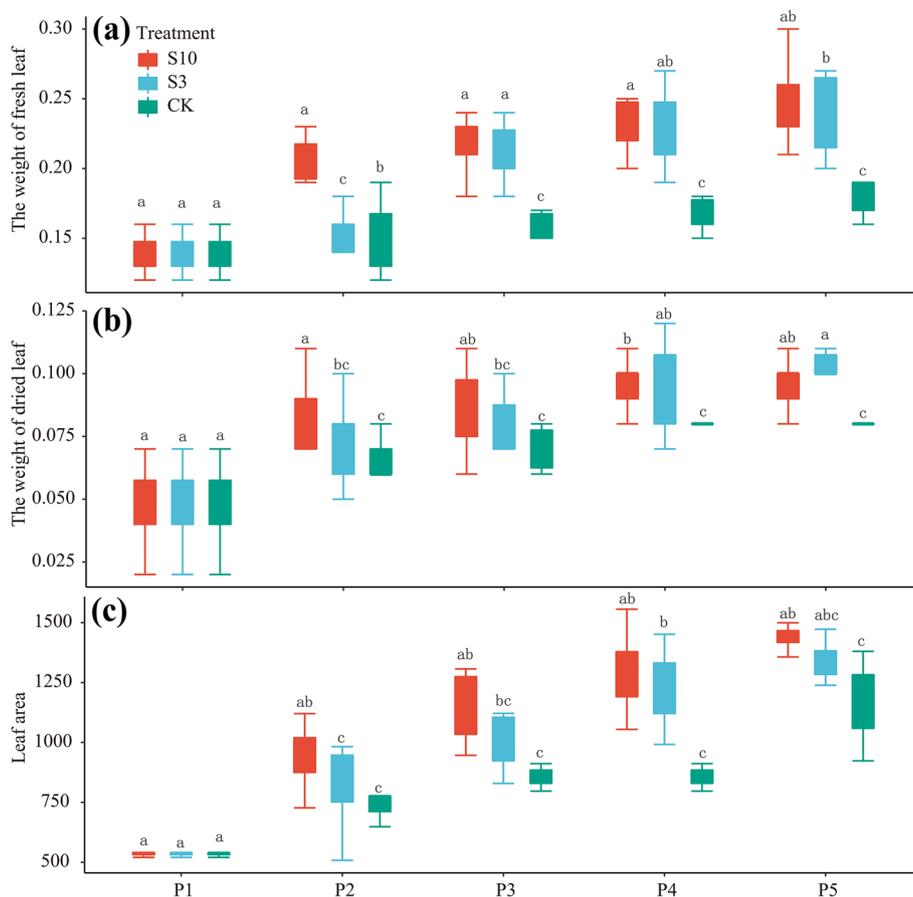


Fig. 1 Boxplots of the **a** fresh weight, **b** dry weight, and **c** leaf area of *Epimediium koreanum Nakai* leaves with application of the S3, S10, and CK strains at P1–P5 (different lowercase letters at the same stage in the same column indicate significant differences ($P < 0.05$))

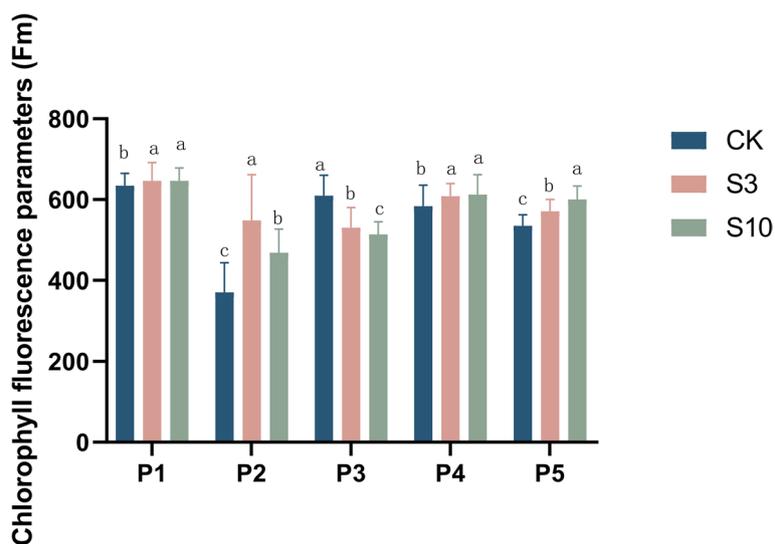


Fig. 2 Chlorophyll fluorescence parameters (Fm) of *Epimediium koreanum Nakai* treated with strains CK, S3, and S10 measured at five growth time points (P1–P5) (different lowercase letters at the same stage in the same column indicate significant differences ($P < 0.05$))

Table 5 Effects of experimental treatments on the photosynthetic parameters of *Epimedium koreanum* Nakai

Group	Time (day)	Net photosynthetic rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Transpiration rate ($\text{mmol m}^{-2} \text{s}^{-1}$)	Cleaf ($\text{mmol m}^{-2} \text{s}^{-1}$)	$\text{CO}_{2\text{in}}$ ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Water use efficiency (mmol/mol)
CK	P1	2.35 ± 0.17 ^a	0.21 ± 0.01 ^a	3.47 ± 0.36 ^a	487.43 ± 16.94 ^a	6.42 ± 0.51 ^a
	P2	4.39 ± 0.80 ^{bc}	0.53 ± 0.01 ^a	6.43 ± 0.37 ^a	507.56 ± 9.22 ^{de}	10.96 ± 0.93 ^d
	P3	2.88 ± 0.38 ^c	0.43 ± 0.08 ^a	4.67 ± 0.30 ^c	731.71 ± 24.13 ^b	7.65 ± 0.47 ^d
	P4	2.97 ± 0.60 ^{bc}	0.34 ± 0.07 ^{abc}	3.28 ± 0.03 ^b	807.24 ± 23.39 ^a	5.92 ± 0.39 ^c
	P5	0.75 ± 0.19 ^d	0.23 ± 0.05 ^{ab}	2.42 ± 0.09 ^{de}	499.29 ± 14.67 ^a	2.21 ± 0.47 ^d
S ₃	P1	2.38 ± 0.33 ^a	0.19 ± 0.01 ^a	3.43 ± 0.23 ^a	479.48 ± 11.09 ^a	6.67 ± 0.46 ^a
	P2	2.97 ± 0.41 ^d	0.2 ± 0.05 ^{bcd}	2.60 ± 0.09 ^d	496.05 ± 20.99 ^e	13.27 ± 0.69 ^{bc}
	P3	3.71 ± 0.41 ^{bc}	0.49 ± 0.04 ^a	6.75 ± 0.22 ^a	644.90 ± 8.51 ^c	6.84 ± 0.66 ^d
	P4	4.85 ± 0.26 ^a	0.37 ± 0.03 ^{ab}	4.48 ± 0.19 ^a	743.21 ± 2.33 ^b	11.21 ± 0.73 ^a
	P5	1.58 ± 0.31 ^c	0.23 ± 0.05 ^{ab}	2.35 ± 0.21 ^e	513.05 ± 21.52 ^a	7.28 ± 0.26 ^b
S ₁₀	P1	2.38 ± 0.12 ^a	0.21 ± 0.01 ^a	3.49 ± 0.32 ^a	482.57 ± 16.81 ^a	6.68 ± 0.23 ^a
	P2	4.04 ± 0.48 ^{cd}	0.26 ± 0.05 ^b	3.39 ± 0.14 ^b	538.71 ± 19.13 ^{cd}	13.17 ± 0.55 ^c
	P3	4.93 ± 0.53 ^a	0.48 ± 0.09 ^a	5.71 ± 0.31 ^b	681.28 ± 24.47 ^c	10.33 ± 0.89 ^c
	P4	2.33 ± 0.65 ^c	0.27 ± 0.05 ^{bc}	4.08 ± 0.33 ^a	803.85 ± 15.39 ^a	8.90 ± 0.51 ^b
	P5	0.81 ± 0.11 ^d	0.19 ± 0.02 ^b	2.7 ± 0.10 ^{cd}	499.56 ± 17.28 ^a	3.39 ± 0.08 ^c

Different lowercase letters at the same stage in the same column indicate significant differences ($P < 0.05$)

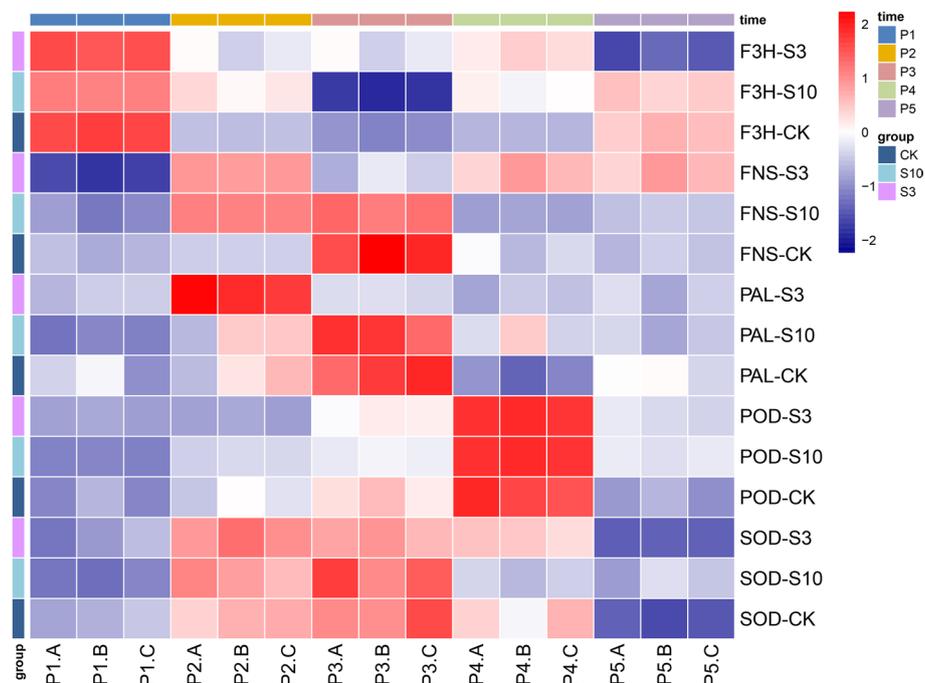


Fig. 3 Heatmap of the enzyme activities of flavanone 3-hydroxylase (F3H), phenylalanine ammonia-lyase (PAL), peroxidase (POD), superoxide dismutase (SOD), and flavone synthase (FNS) in *Epimedium koreanum* Nakai during application of the S3, CK, and S10 strains. Colors represent the period and grouping, in the top right corner of the picture

and P3, and the overall content of epimedin A at P4 and P5 was lower than that at P3; however, the relative content in the S3-treated plants was higher at P4 and P5. The content of epimedin B significantly increased at P2

in the S10 group and at P5 in the S3 group. The changes in the icaricin and epimedin C levels after application of the endophyte strains followed the same pattern as found for epimedin B. The total flavonol glycoside content was

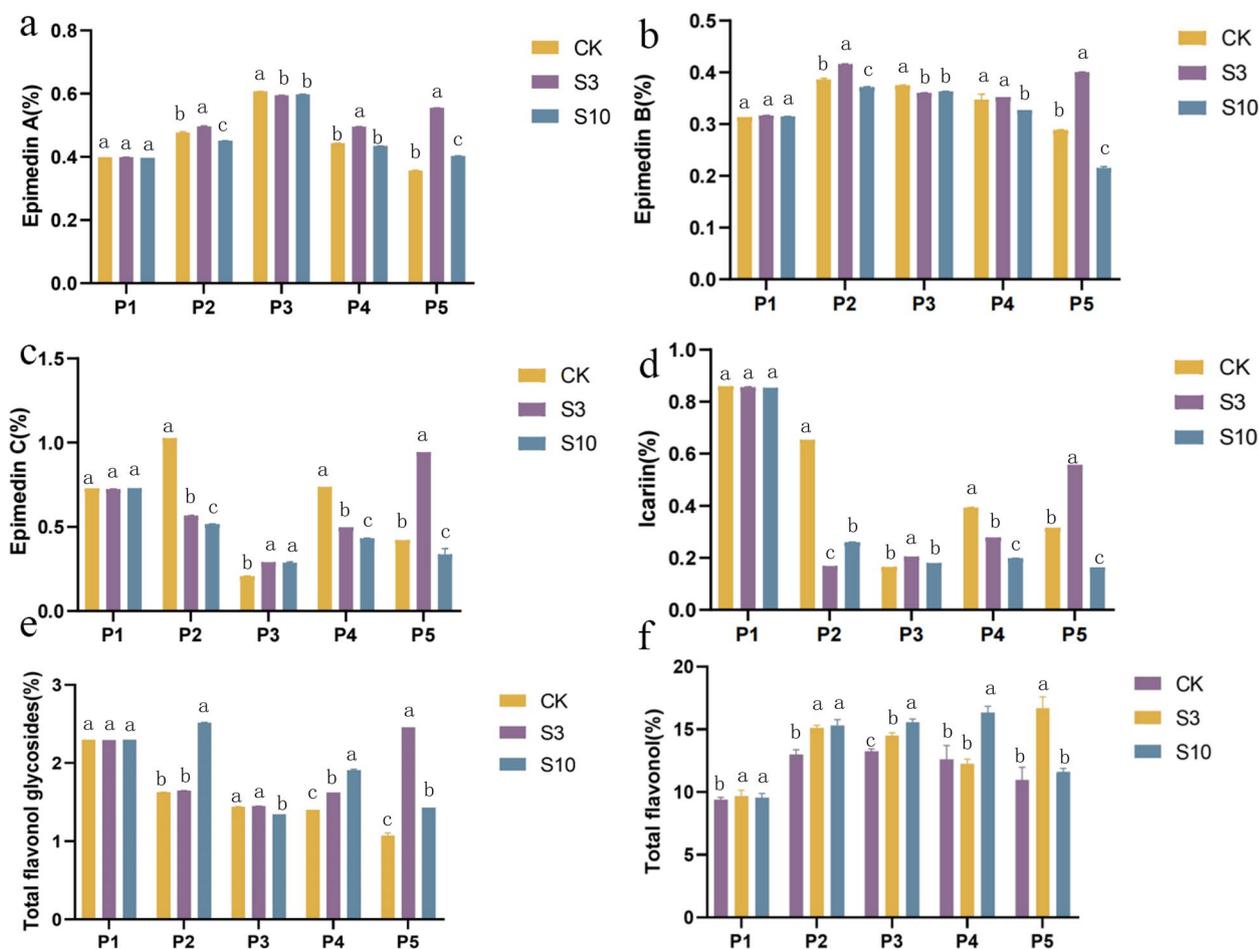


Fig. 4 Comparison of the active ingredient contents of **a** the total flavonoids, **b** total flavonol glycoside, **c** epimedin A, **d** epimedin B, **e** epimedin C, and **f** icariin in *Epimedium koreanum Nakai* after application of the CK, S3, and S10 strains at five sampling periods (P1–P5) (different lowercase letters at the same stage in the same column indicate significant differences ($P < 0.05$))

Table 6 Percent total flavonol glycosides in the five periods of *Epimedium koreanum Nakai*

	CK	S3	S10
P1	2.2979%	2.2944%	2.2995%
P2	1.6285%	1.6485%	2.5215%
P3	1.4356%	1.4501%	1.3455%
P4	1.4010%	1.6240%	1.9174%
P5	1.0486%	2.4562%	1.43 00%

Different lowercase letters at the same stage in the same column indicate significant differences ($P < 0.05$)

measured as the total sum of epimedin A, epimedin B, epimedin C, and icariin. In Table 6, the S3 and S10 strains increased the total flavonol glycoside content at each period, increasing from P2 to P5 by 0.02%, 0.01%, and 0.22%, and by 1.41%, 0.47%, 0.41%, 0.43%, and 0.70% compared with that of the blank control group,

respectively. The changes due to S10 compared with the blank group were larger than those induced by S3.

Associations of enzyme activity and accumulation of active components induced by S3 and S10

Icarosin, hopanin, hopanin (cahuoside), and hopanin (diphyllaside) are dihydroflavonols (dihydroflavonols) (Zhang et al. 2006). The substrate (intermediate precursor) is formed through a series of enzymatic reactions. According to the redundancy analysis (Figs. 5, 6, and 7), the content of epimedin A after application of S3 strain showed a significantly positive correlation with the level of FNS, a significant negative correlation with F3H activity, and different degrees of positive correlation with PAL, SOD, and POD activities. The contents of epimedin B and total flavonoids were positively correlated with FNS, PAL, SOD, and POD activities, and the correlation was in the order of FNS > PAL > SOD > POD, whereas epimedin B and total flavonoids were significantly negatively

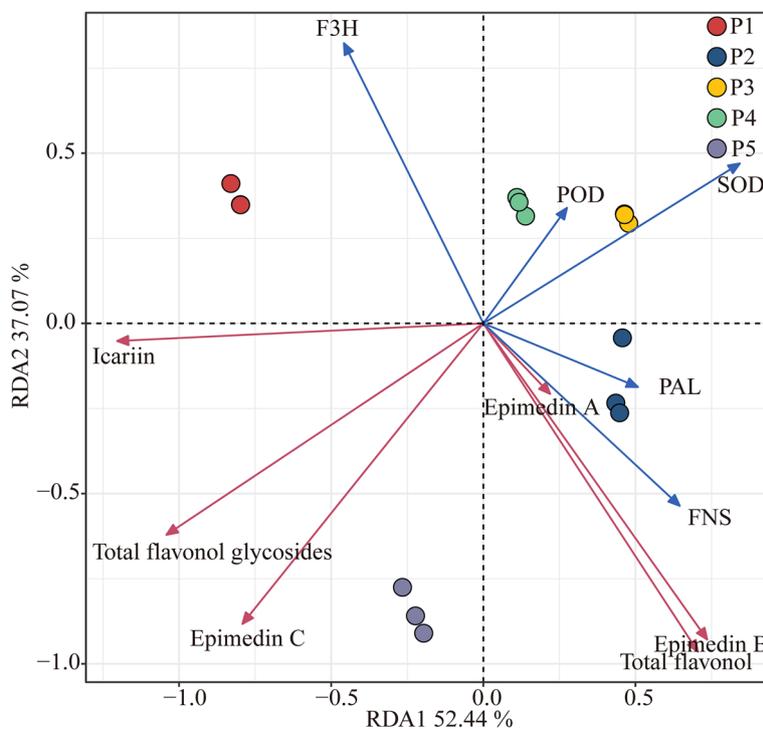


Fig. 5 Redundancy analysis (RDA) plot of F3H, FNS, PAL, POD, and SOD enzyme activities and active components (flavonoids) of *Epimedium koreanum Nakai* at different sampling periods (P1–P5) with treatment of S3

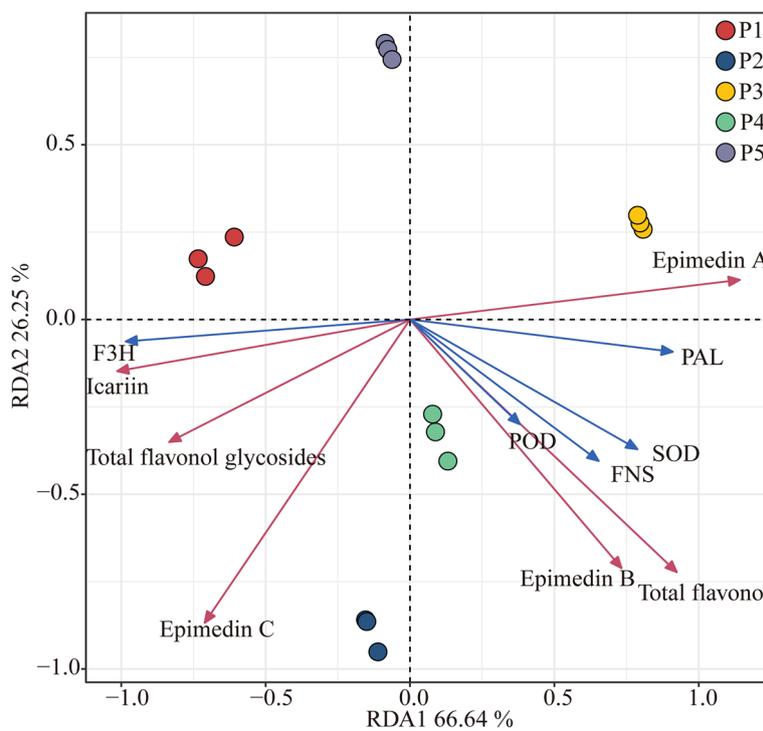


Fig. 6 Redundancy analysis (RDA) plots of F3H, FNS, PAL, POD, and SOD enzyme activities and active components (flavonoids) of *Epimedium koreanum Nakai* at different sampling periods (P1–P5) after treatment with strain S10

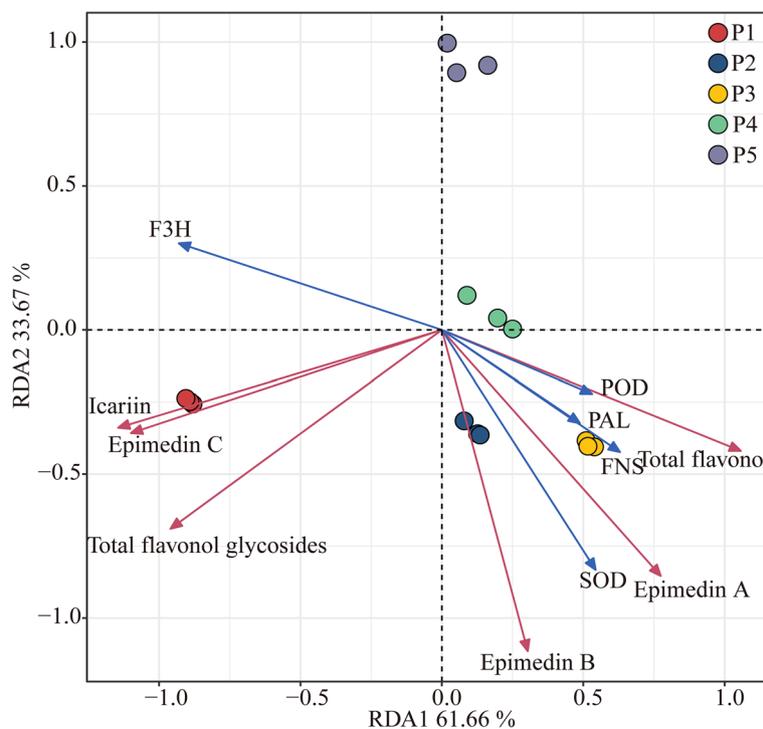


Fig. 7 Redundancy analysis (RDA) plots of F3H, FNS, PAL, POD, and SOD enzyme activities and active components (flavonoids) of *Epimedium koreanum Nakai* in the blank control (CK) group at each sampling period (P1–P5)

associated with F3H activity. The epimedinin C and total flavonol glycoside contents were negatively correlated with F3H, POD, and SOD activities and positively correlated with PAL and FNS activities. Icariin was negatively correlated with the activities of all enzymes. After application of S10 strain, the content of epimedinin A was significantly negatively correlated with F3H activity and was positively correlated with FNS, PAL, SOD, and POD activities. The degree of correlation was in the order PAL > SOD > FNS > POD. The contents of epimedinin B and total flavonoids were negatively correlated with F3H activity and positively correlated with FNS, PAL, SOD, and POD activities, and the degree of correlation was in the order POD > FNS > SOD > PAL. Icariin, epimedinin C, and total flavonol contents were positively correlated with F3H activity, and showed different degrees of negative correlation with FNS, PAL, SOD, and POD activities in the order POD > FNS > SOD > PAL. In the blank group, epimedinin A, epimedinin B, epimedinin C, and total flavonoids were positively correlated with F3H and were negatively correlated with POD, FNS, SOD, and POD activities.

Figure 8 shows that F3H enzyme activity was negatively associated with epimedinin A after application of the S3 and S10 strains, and showed a general positive correlation with icariin. As shown in Fig. 9a, the blank, S3,

and S10 groups showed a significant positive correlation between the POD enzyme content and the total flavonoid content of *Epimedium koreanum Nakai*. Figure 9b shows that the total flavonoid content after applying the S3 strain increased relative to that of the blank group from P1 to P3, decreased at P4, and then increased at P5. However, the S10 strain showed the same trend in flavonoid content as found for the blank group.

Discussion

The genus *Diaporthe* comprises plant pathogens and endophytes, and it is a source of secondary metabolites. These metabolites have been explored for their potential applications in health care (e.g., antioxidant and anti-inflammatory properties), pharmacology (e.g., clinical toxicology assessment), and biomedicine (e.g., drug development) (Hen et al. 2022). However, there is still a lack of information on the phytotoxins produced by species of *Diaporthe*, which should be explored given their potential application in agriculture as promising candidates for the development of natural herbicides (Hen et al. 2022). Moreover, endophytic *Diaporthe* species have been reported to produce antimicrobial compounds to control plant pathogens and to be promising agents in the development of biofertilizers to promote plant growth (Abramczyk B., Marzec-Grządziel A., Grządziel

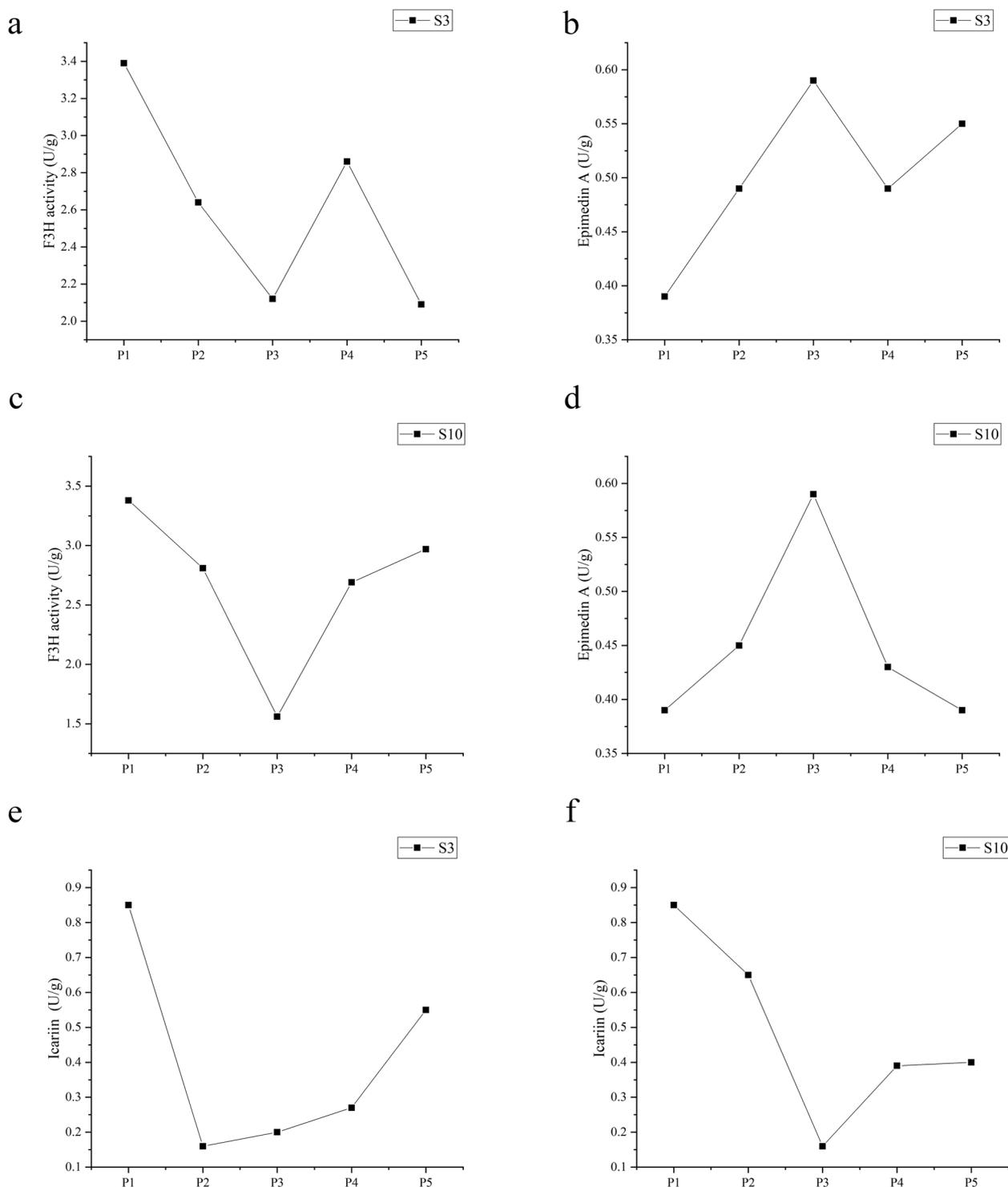


Fig. 8 **a** F3H enzyme activity after application of strain S3. **b** Change in epimedin A levels after application of strain S3. **c** Change in F3H enzyme activity after application of strain S10. **d** Change in epimedin A levels after application of strain S10. **e** Change in icariin levels after application of strain S3. **f** Change in icariin levels after application of strain S10

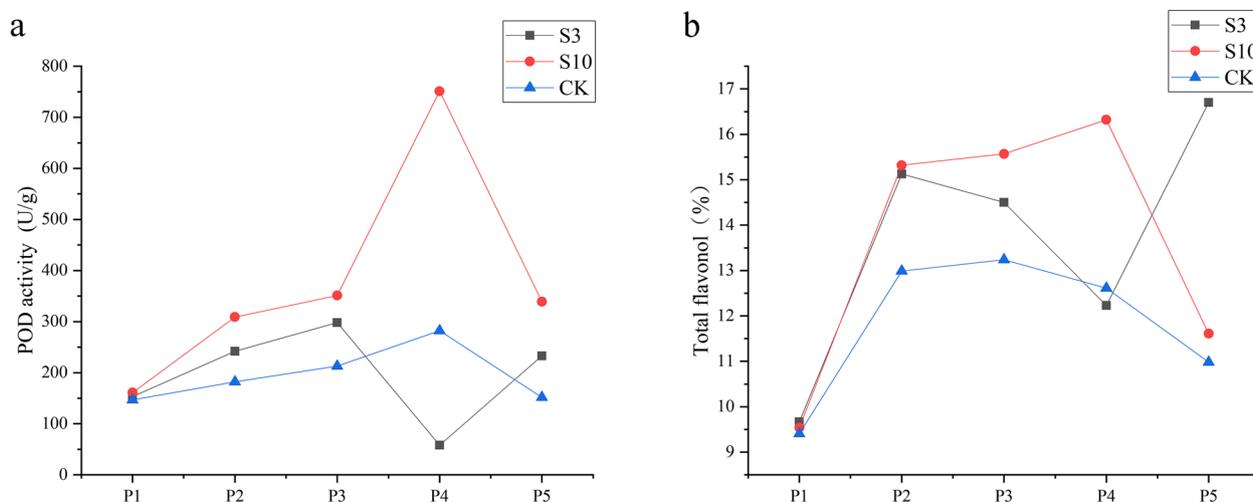


Fig. 9 **a** Change of POD activity over time in the blank group (CK) and after application of the S3 and S10 strains. **b** Change of total flavonoid content over time in the blank group (CK) and after application of the S3 and S10 strains

J et al. 2022). However, the team found that it promotes the growth of *Epimedium koreanum Nakai*. *Ilyonectria cyclaminicola* is a new fungus isolated from *Epimedium koreanum Nakai*, and no reports have been published on it. This paper is the first report on the promotion of *Epimedium koreanum Nakai* growth. According to the analysis of the S3 and S10 strains, the application of S10 biofertilizer had the best effect on the leaf area, dry weight, and fresh weight of *Epimedium koreanum Nakai* plants grown in their natural environment. The application of strain S3 caused the greatest inactivation or destruction of the photosynthesis reaction center and resulted in the highest chlorophyll content. Moreover, the chlorophyll fluorescence values increased the fastest over time after applying the endophytic fungal strain S10 as a biofertilizer, indicating that S10 acts through temporal accumulation in *Epimedium koreanum Nakai*. The change in the F0 value is related to the photosynthesis reaction center condition and is a possible photoprotection mechanism, which also reflects the leaf chlorophyll content, where a higher F0 value indicates higher chlorophyll content and stronger photosynthesis in the leaves. The fluorescence parameters after the application of S3 and S10 were significantly greater than those of the control group at P5, indicating that these endophyte strains used as biological fertilizer significantly promote photosynthesis toward the end of the *Epimedium koreanum Nakai* growth period, which is also reflected by the increase in the dry and fresh weights of the leaves and the leaf area. All groups showed the fastest photosynthesis rate, the highest water utilization rate, and the slowest transpiration rate at P2. These results suggest that strains S3 and S10 increase the leaf dry weight and leaf area by

accelerating the leaf photosynthesis rate and water utilization and increase the leaf fresh weight by reducing the transpiration rate. The application of strain S10 significantly increased water availability in the leaves at all time points. However, the comprehensive comparative analysis showed that the ability of the S10 strain to promote the growth of *Epimedium koreanum Nakai* was greater than that of the S3 strain.

Flavonoid compounds are permanent components in plant tissues that participate in respiration and ontogenesis, play an essential role in oxidation–reduction and defense reactions, influence membrane permeability, and are substrates for various enzymes (Toghueo et al. 2022). F3H is a key enzyme in the flavonoid biosynthetic pathway, which is involved in the biosynthesis of five of the 12 main classes of flavonoid compounds by catalyzing the conversion of flavanones into dihydroflavonols (Winkel, S.B. 2001, Zaprometov, M.N. 1974). In most plants, 3,4-dihydroxylation of flavonoid B rings is achieved by modification of F3 H to dihydroflavonols or dihydroflavonols (Punyasiri et al. 2004). Redundancy analysis showed that F3H enzyme activity was negatively associated with epimedin A contents after applying the S3 and S10 strains and showed a generally positive correlation with icariin contents. In the flavonoid synthesis pathway in *Epimedium koreanum Nakai*, F3H can catalyze the synthesis of dihydroflavonol by dihydroflavone, with the synthesis of dihydroflavonol occurring in one pathway and the synthesis of icariin occurring in another pathway. Therefore, we compared the changes in icariin content with respect to the content of epimedin A and found no association after the application of the S3 strain, whereas a positive association was found after applying the S10

strain. The F3h2 gene is specifically transcribed in the roots of wheat and rye and is likely functionally distinct from the F3h1 gene; however, its specific biological function is not yet known (Shoeva, O. Y. and E. K. Khlestkina 2013). Lyu et al. (2020) identified a novel flavonoid-3-O rhamnose glycosyltransferase that catalyzes the production of icariin by kaempferol and quercetin. Collectively, these findings suggest that the S10 strain can use dihydroflavonols to synthesize epimedin A alone, although the mechanism is not clear. POD is an enzyme that utilizes an electron donor to reduce the H₂O₂ generated by SOD, resulting in water and an oxidized product of the hydrogen donor. In plant hormone signaling pathways, POD acts as a stress-responsive enzyme. These pathways, which are controlled by abscisic acid, methyl jasmonate, and reactive oxygen species signaling, are important in biotic and abiotic stress signaling crosstalk (Fujita et al. 2006). POD promotes the oxidation of phenolic compounds in the presence of hydrogen peroxide and can explain the differences observed in elderberry phenolic content (Tomás-Barberán, F.A.; Espín, J.C. 2001). We found a significant positive correlation between POD enzyme activity and the total flavonoid content of *Epimedium koreanum Nakai* in the control, S3 strain, and S10 strain groups. Overall, these results indicated that POD can affect the content of total flavonoids and that the S3 and S10 epiphyte strains can produce changes in the POD content of the host plant, thereby influencing the total flavonoid content.

Conclusions

The S10 strain promoted the growth of *Epimedium koreanum Nakai* better than the S3 strain. The F3H enzyme is critical for the synthesis of active components in *Epimedium koreanum Nakai*. Icariin and epimedin A are alternative synthesis pathways for the same dihydroflavonol. POD plays a key role in total flavonoid synthesis in *Epimedium koreanum Nakai*.

Abbreviations

Cleaf	Stomatal conductance
CK	Control check
CO _{2in}	Intercellular carbon dioxide concentration
F ₀	Minimal fluorescence
F _m	Maximal fluorescence
F3H	Flavanone 3-hydroxylase
FNS	Flavone synthase
PAL	Phenylalanin ammonia-lyase
PBS	Phosphate-buffered saline
PDB	Potato dextrose broth
POD	Peroxidase
Pn	Net photosynthetic rate
SOD	Superoxide dismutase
Tr	Transpiration rate
We	Water use efficiency

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Authors' contributions

XJ designed the entire experiment, and CJ and BY completed all the picture production, data analysis, and writing the manuscript. ZX, GJ, and HX collected the experimental samples.

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Availability of data and materials

The data that supports the findings of this study is available in this article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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