Effects of NPK Fertilizers on Physiology and Chlorogenic Acid Synthesis and Accumulation in *Pyrrosia petiolosa* (Postprint)

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Abstract

To investigate the effects of nitrogen, phosphorus, and potassium nutrients on the physiology and the synthesis and accumulation of the active component chlorogenic acid in *Pyrrosia petiolosa*, this study utilized tissue-cultured seedlings of *P. petiolosa* as experimental material. Three nutrient concentration gradients were established: low nutrient (no fertilization: N0, P0, K0), normal fertilization (N: 0.2 $g \cdot kg^{-1}$, P: 0.15 $g \cdot kg^{-1}$, K: 0.15 $g \cdot kg^{-1}$), and high nutrient (N1: 0.4 $g \cdot kg^{-1}$, P1: 0.3 $g \cdot kg^{-1}$, K1: 0.3 $g \cdot kg^{-1}$). Seven treatments were designed: NPK, N0PK, N1PK, NP0K, NP1K, NPK0, and NPK1. Stress resistance physiological indicators, chlorogenic acid content, and activities of key enzymes involved in chlorogenic acid synthesis were measured under different treatments. The results demonstrated: (1) NPK fertilization significantly affected the stress resistance physiology of *P. petiolosa*, with superoxide dismutase (SOD) activity increasing significantly under high nitrogen and low potassium treatments, while both low- and high-concentration treatments of the three nutrients induced significant elevation of catalase (CAT) activity; (2) Different nitrogen, phosphorus, and potassium concentrations significantly influenced chlorogenic acid content, which peaked at 12.92 $mg \cdot g^{-1}$ under normal fertilization and reached its minimum of 7.79 $mg \cdot g^{-1}$ under high potassium fertilization, with potassium fertilizer exerting the most pronounced effect; (3) Activities of key enzymes for chlorogenic acid synthesis varied significantly among fertilization treatments, with chlorogenic acid content showing significant positive correlations with hydroxycinnamoyl-CoA quinate transferase (HQT) and 4-coumarate:CoA ligase (4CL) activities, and a significant negative correlation with hydroxycinnamoyl-CoA shikimate transferase (HCT) activity. HQT, 4CL, and HCT were identified as the key factors responsible for variations in chlorogenic acid content. These findings provide a theoretical foundation for the artificial cultivation of *P. petiolosa* as a medicinal plant.

Full Text

Effects of Nitrogen, Phosphorus, and Potassium Fertilizers on Physiology and Chlorogenic Acid Synthesis and Accumulation in *Pyrrosia petiolosa*

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Abstract

To investigate the effects of nitrogen (N), phosphorus (P), and potassium (K) on the physiology and chlorogenic acid (CGA) synthesis in *Pyrrosia petiolosa*, tissue-cultured seedlings were subjected to three nutrient levels: low (no fertilization: N0, P0, K0), normal (N: 0.2 $g \cdot kg^{-1}$, P: 0.15 $g \cdot kg^{-1}$, K: 0.15 $g \cdot kg^{-1}$), and high (N1: 0.4 $g \cdot kg^{-1}$, P1: 0.3 $g \cdot kg^{-1}$, K1: 0.3 $g \cdot kg^{-1}$). Seven treatment combinations were established: NPK, N0PK, N1PK, NP0K, NP1K, NPK0, and NPK1. Resistance physiological indices, CGA content, and key enzyme activities in the CGA synthesis pathway were measured. The results showed: (1) NPK fertilizers significantly affected the resistance physiology of *P. petiolosa*. Superoxide dismutase (SOD) activity increased markedly under high nitrogen and low potassium treatments, while both low and high concentrations of all three nutrients significantly elevated catalase (CAT) activity. (2) Different N, P, and K levels significantly influenced CGA accumulation. Normal fertilization yielded the highest CGA content (12.92 $mg \cdot g^{-1}$), whereas high potassium fertilization produced the lowest (7.79 $mg \cdot g^{-1}$), with potassium showing the most pronounced effect. (3) Key enzyme activities in CGA synthesis varied significantly among treatments. CGA content was positively correlated with quinate hydroxycinnamoyl transferase (HQT) and coumaroyl-CoA ligase (4CL) activities, but negatively correlated with shikimate hydroxycinnamoyl transferase (HCT) activity. HQT, 4CL, and HCT were identified as critical factors driving CGA content variation. These findings provide a theoretical basis for the artificial cultivation of *P. petiolosa*.

Keywords: *Pyrrosia petiolosa*, nitrogen-phosphorus-potassium fertilizer, physiology, chlorogenic acid, accumulation

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Introduction

Pyrrosia petiolosa (Christ) Ching, a member of the Polypodiaceae family, is one of the botanical sources of the traditional Chinese medicine "Shiwei" recorded

in the 2020 edition of the Chinese Pharmacopoeia. The dried leaves, characterized by bitter-sweet taste and slightly cold properties, are used to promote diuresis, clear lung heat, and arrest bleeding. Studies have shown that *P. petiolosa* contains various bioactive compounds including phenolic acids, flavonoids, and triterpenoids, with chlorogenic acid (CGA) serving as a key quality control marker. Currently, *P. petiolosa* is primarily harvested from wild populations. However, overexploitation and increasing clinical demand have led to rapid resource depletion and mounting supply shortages. Moreover, inconsistent quality among wild-collected materials compromises therapeutic efficacy. Therefore, developing domestication and artificial cultivation protocols is imperative to meet market demands and ensure stable medicinal quality.

Artificial cultivation of *P. petiolosa* remains in its infancy, facing challenges such as low spore germination rates, poor seedling uniformity, low survival rates, slow natural growth, and undefined fertilization protocols. Fertilization represents a critical factor in medicinal plant cultivation that directly influences growth, yield, and active constituent accumulation. To date, no scientific literature has reported optimal fertilization strategies for *P. petiolosa*. Consequently, elucidating how fertilization management regulates growth and CGA accumulation is essential for successful cultivation.

Numerous studies demonstrate that appropriate fertilization promotes medicinal plant development and secondary metabolite synthesis, whereas nutrient deficiency or excess creates stress conditions that disrupt metabolic balance, reducing yield and bioactive content. For instance, nitrogen application negatively affected tanshinone IIA accumulation in *Salvia miltiorrhiza*, with content decreasing as nitrogen levels increased, while phosphorus enhanced tanshinone IIA and danshensu accumulation. In *Panax quinquefolius*, appropriate nitrogen increased ginsenoside Rb3 content, but excessive nitrogen reduced it. CGA in *P. petiolosa* belongs to phenolic acids synthesized via the phenylpropanoid pathway, whose precursors are aromatic amino acids (phenylalanine and tyrosine). Nitrogen availability influences phenylpropanoid content by affecting aromatic amino acid pools. In *Lonicera japonica*, CGA content correlated negatively with nitrogen rate, while moderate fertilization upregulated key CGA synthesis genes including *PAL*, *C4H*, *4CL*, and *HQT*. Phosphorus also affects phenolic metabolism; *PAL* and *4CL* genes show differential expression under phosphorus stress, and high phosphorus significantly reduced phenolic acids such as isochlorogenic acid C, cryptochlorogenic acid, and isochlorogenic acid B. Unlike nitrogen and phosphorus, potassium is not incorporated into organic compounds but functions as a major cation and enzyme cofactor in physiological and biochemical processes including enzyme activation, ion homeostasis, osmotic regulation, and protein synthesis. However, no studies have examined how fertilization affects phenolic acid accumulation in *P. petiolosa*.

This study investigated physiological responses and CGA synthesis patterns in *P. petiolosa* under varying NPK concentrations by measuring antioxidant enzymes (SOD, CAT), osmotic regulators (proline), key CGA synthesis enzymes

(PAL, C4H, 4CL, HCT, HQT, C3H), and CGA content. The objective was to clarify fertilization effects on physiology and CGA accumulation to guide domestication and cultivation practices.

Materials and Methods

1.1 Plant Materials The experiment was conducted from July to September 2022 in a greenhouse at Guangxi University of Chinese Medicine. One-year-old tissue-cultured *P. petiolosa* seedlings of uniform growth status were selected. Roots were rinsed clean, soaked in 1,000× carbendazim for 15 minutes, then transplanted into the greenhouse. Urea (Tianjin Oubokai Chemical Co., Ltd.), calcium dihydrogen phosphate (Tianjin Damao Chemical Reagent Factory), and potassium chloride (Tianjin Beichen Fangzheng Reagent Factory) were used for N, P, and K treatments, respectively.

1.2 Substrate Preparation The acidic red soil substrate was sterilized, air-dried, crushed, and mixed uniformly for pot experiments. The mixture consisted of red soil: nursery substrate (organic matter + humic acid): perlite at a 2:2:1 ratio. The substrate had the following properties: pH 4.9, total N 0.1%, total P 0.12%, total K 2.5%, organic matter 16.0 $g \cdot kg^{-1}$, hydrolyzable N 73.0 $mg \cdot kg^{-1}$, available P 8.8 $mg \cdot kg^{-1}$, and available K 247 $mg \cdot kg^{-1}$.

1.3 Experimental Design Analytical-grade urea, calcium dihydrogen phosphate, and potassium chloride were applied at three levels for each nutrient: low (no fertilization), normal, and high. Seven treatment combinations were established: NPK, N0PK, N1PK, NP0K, NP1K, NPK0, and NPK1. The nitrogen treatments comprised N0PK, NPK, and N1PK; phosphorus treatments included NP0K, NPK, and NP1K; and potassium treatments consisted of NPK0, NPK, and NPK1. The specific rates were: N0/P0/K0 = no fertilizer; normal rates = N: 0.2 $g \cdot kg^{-1}$, P: 0.15 $g \cdot kg^{-1}$, K: 0.15 $g \cdot kg^{-1}$; high rates = N1: 0.4 $g \cdot kg^{-1}$, P1: 0.3 $g \cdot kg^{-1}$, K1: 0.3 $g \cdot kg^{-1}$.

Seedlings were transplanted into cultivation pots (30 cm × 15 cm × 10 cm) containing 2.8 kg of substrate. Each treatment had three replicates with 15 plants per replicate. Fertilizer was applied in two split doses: half at day 0 and half at day 30. The experiment lasted 60 days, with tap water irrigation every three days and uniform management across all treatments.

1.4 Physiological Index Determination Superoxide dismutase (SOD) activity was measured using the nitroblue tetrazolium (NBT) photochemical reduction method. Fresh samples (0.100 g) were weighed on an analytical balance, ground in 1 mL extraction buffer, and centrifuged at 8,000 $r \cdot min^{-1}$ for 10 minutes at 4 °C. The supernatant was processed and absorbance measured at 560 nm using a microplate reader. One SOD unit was defined as the enzyme

amount causing 50% inhibition in the xanthine oxidase-linked reaction system. Catalase (CAT) activity was determined spectrophotometrically using the same extraction procedure. One CAT unit was defined as the amount catalyzing the degradation of 1 mol H_2O_2 per minute per gram of tissue. Proline content was measured using the ninhydrin method.

1.5 CGA Content Determination CGA content was determined following the Chinese Pharmacopoeia (2020) with minor modifications. Dried and powdered plant material (0.2 g) was placed in a 50 mL centrifuge tube, extracted with 15 mL of 50% methanol for 24 hours, then re-extracted with an additional 10 mL of 50% methanol. The mixture was sonicated for 45 minutes, cooled to room temperature, adjusted to weight, and centrifuged at 10,000 $r \cdot min^{-1}$ for 10 minutes. The supernatant was filtered through a 0.22 m membrane for analysis.

A CGA standard (Solarbio Science & Technology, Beijing; batch No.: 929 N022; 2.17 mg) was dissolved in 50% methanol to prepare a 0.217 $mg \cdot mL^{-1}$ stock solution, which was serially diluted to concentrations of 0.0217, 0.0434, 0.0868, 0.1302, 0.1736, and 0.217 $mg \cdot mL^{-1}$. The calibration curve yielded the linear regression equation $y = 3.00424 \times 10^7 x - 32943$ ($R^2 = 0.9992$). Chromatographic conditions: XBridge C18 column (4.6 × 250 mm, 5 m), detection wavelength 326 nm, column temperature 25 °C, flow rate 1.0 $mL \cdot min^{-1}$, gradient elution with acetonitrile (C) and 0.1% phosphoric acid (A): 0–5 min, 10–15% C; 5–12 min, 15–19% C; 12–18 min, 19–27% C; 18–40 min, 27–80% C; 40–60 min, 80–10% C.

1.6 Key Enzyme Activity Assays Key CGA synthesis enzymes were measured using enzyme-linked immunosorbent assay (ELISA) kits (Guangxi Junqi Biotechnology Co., Ltd., 202301). Fresh samples (0.1 g) were ground in 1 mL PBS buffer (pH 7.2–7.4) at 4 °C, then centrifuged at 10,000 $r \cdot min^{-1}$ for 20 minutes at 4 °C to obtain enzyme extracts. Sample wells received sample diluent, while blank wells received none. Standard and sample wells (except blanks) were incubated with horseradish peroxidase (HRP)-conjugated detection antibodies at 37 °C for 60 minutes. After washing five times, substrate was added and incubated at 37 °C for 15 minutes in darkness. The reaction was terminated with sulfuric acid, and optical density measured at 450 nm. Enzyme activity was defined as the amount degrading 1 mol tetramethylbenzidine (TMB) per minute per gram of protein.

1.7 Statistical Analysis Data were processed using Microsoft Excel 2019 and presented as means ± standard deviation. SPSS software was used for statistical analysis (details incomplete in original).

Results

2.1.1 Effects of Nitrogen Concentration on Resistance Physiology As shown in [Figure 1: see original paper], SOD activity under high nitrogen treatment was 2.18-fold higher than under low nitrogen, with no significant difference between normal and low nitrogen treatments [Figure 1: see original paper]A. CAT activity was lowest under normal nitrogen, showing significant differences compared to both low and high nitrogen treatments [Figure 1: see original paper]B. Proline content was lowest under low nitrogen (42.09 $g \cdot g^{-1}$), significantly different from normal nitrogen but not from high nitrogen. Proline content increased initially then decreased with rising nitrogen levels [Figure 1: see original paper]C.

2.1.2 Effects of Phosphorus Concentration on Resistance Physiology Under phosphorus treatments, SOD activity peaked at normal phosphorus, with no significant difference between low and high phosphorus [Figure 1: see original paper]A. CAT activity showed the opposite trend, reaching its minimum at normal phosphorus. Low and high phosphorus treatments increased CAT activity by 82.64% and 77.41%, respectively, compared to normal phosphorus (both significant) [Figure 1: see original paper]B. Proline content was highest under normal phosphorus, with no significant difference between low and high phosphorus treatments [Figure 1: see original paper]C.

2.1.3 Effects of Potassium Concentration on Resistance Physiology SOD activity differed significantly among potassium treatments, being highest under low potassium and lowest under high potassium [Figure 1: see original paper]A. CAT activity was minimal under normal potassium, with low potassium treatment showing significantly higher activity than both normal and high potassium. Potassium effects on proline content mirrored nitrogen and phosphorus patterns: normal potassium was significantly higher than low and high potassium, which did not differ from each other [Figure 1: see original paper]C.

Note: Different letters indicate significant differences among treatments ($P < 0.05$). The same notation applies below.

2.2.1 Effects of NPK Fertilizers on CGA Content NPK levels significantly affected CGA accumulation. Normal nitrogen produced the highest CGA content (12.92 $mg \cdot g^{-1}$), with low and high nitrogen reducing content by 15.87% and 19.66%, respectively (both significant) [Figure 1: see original paper]D. High phosphorus and high potassium were detrimental to CGA accumulation, decreasing content by 28.33% and 39.71% compared to their respective normal levels. Potassium concentration exhibited the most significant effect on CGA content.

2.2.2 Effects of Nitrogen Treatment on Key CGA Synthesis Enzymes PAL activity was highest under low nitrogen, with no significant difference be-

tween normal and high nitrogen [Figure 2: see original paper]A. Low nitrogen reduced C4H activity by 23.39% compared to normal nitrogen [Figure 2: see original paper]B. High nitrogen decreased 4CL activity by 13.23% relative to normal nitrogen [Figure 2: see original paper]C. HCT activity under high nitrogen was 21.05% higher than under normal nitrogen, which showed the lowest HCT activity. High nitrogen also reduced HQT activity by 12.6% compared to normal nitrogen (both significant). Nitrogen concentration did not significantly affect C3H activity.

2.2.3 Effects of Phosphorus Treatment on Key CGA Synthesis Enzymes High phosphorus yielded the lowest PAL activity (12.85% lower than normal phosphorus), while low phosphorus showed no significant difference [Figure 3: see original paper]A. Low phosphorus did not significantly affect 4CL activity, but high phosphorus reduced it by 14.39% [Figure 3: see original paper]C. Normal phosphorus produced the lowest HCT activity, with low and high phosphorus increasing activity by 20.15% and 15.67%, respectively [Figure 3: see original paper]D. HQT activity increased then decreased with phosphorus concentration, with high phosphorus reducing activity by 24.83% compared to normal phosphorus [Figure 3: see original paper]E. Phosphorus treatments did not significantly affect C3H activity [Figure 3: see original paper]F.

2.2.4 Effects of Potassium Treatment on Key CGA Synthesis Enzymes Potassium concentration did not significantly affect PAL activity [Figure 4: see original paper]A. Low and high potassium reduced C4H activity by 23.44% and 31.72%, respectively, compared to normal potassium [Figure 4: see original paper]B. While low potassium did not significantly affect 4CL activity, high potassium decreased it by 22.40%. High potassium increased HCT activity by 28.94% compared to normal potassium [Figure 4: see original paper]D. HQT activity under high potassium was 22.45% lower than under normal potassium, with no significant difference between low and normal potassium [Figure 4: see original paper]E. C3H activity remained unchanged across potassium treatments [Figure 4: see original paper]F.

2.3 Correlation Analysis Between CGA Content and Key Enzymes Correlation analysis revealed that 4CL, an upstream key enzyme, was extremely significantly positively correlated with CGA content. The downstream enzyme HQT showed a significant positive correlation, while the downstream enzyme HCT exhibited a significant negative correlation with CGA content .

Table 1 Pearson correlation between CGA content and key CGA synthesis enzymes in *Pyrrosia petiolosa*

Item	PAL	C4H	4CL	HCT	HQT	C3H
CGA content	0.911**	-0.851*	0.843*	0.926**		

*Note: ** indicates extremely significant correlation ($P < 0.01$); * indicates significant correlation ($P < 0.05$); n = 7.*

Discussion

Both insufficient and excessive nitrogen create different stress conditions that alter antioxidant enzyme activities. Plants enhance reactive oxygen species scavenging by upregulating antioxidant enzymes to maintain normal growth. In this study, high nitrogen created a stressful environment for *P. petiolosa*, which responded by increasing SOD and CAT activities—consistent with findings in *Notopterygium incisum* under nitrogen treatments. The antioxidant enzyme system requires coordinated action to eliminate harmful free radicals. Plants increased CAT activity to cope with oxidative damage from low and high phosphorus stress. SOD activity decreased with increasing potassium concentration, aligning with antioxidant system changes in *Tagetes patula* under low potassium stress. Overall, low and high nutrient concentrations stressed *P. petiolosa*, but CAT and SOD responded differently: SOD primarily reacted to high nitrogen and low potassium, while CAT responded significantly to both low and high concentrations of all three nutrients.

Proline plays vital roles in plant development, with its synthesis and metabolism linked to photosynthesis, mitochondrial respiration, redox balance, and energy homeostasis. Research indicates proline also serves as an energy source, influencing oxidative pentose phosphate pathway carbon flux by mediating $NADP^+$/NADPH ratios and providing erythrose-4-phosphate precursors for phenylpropanoid synthesis under stress. This may explain why proline content patterns resembled CGA accumulation trends in this study.

NPK fertilizers significantly affected CGA accumulation in *P. petiolosa*. Studies on *Hypericum perforatum* showed reduced CGA content under high nitrogen, with optimal nitrogen promoting accumulation—possibly due to downregulated CGA synthesis-related enzyme gene expression under high nitrogen. Similarly, low phosphorus stress downregulates *PAL* and *4CL* genes involved in phenylpropanoid biosynthesis, likely contributing to significantly reduced CGA content under low phosphorus in this study. Research on apple leaves demonstrated upregulated *PAL*, *C4H*, and *4CL* expression under potassium deficiency and downregulated *PAL* under high potassium, with the flavonoid pathway playing important roles in potassium responses. The lowest CGA content under high potassium in our study aligns with findings that high potassium is detrimental to phenolic accumulation in *Tetrastigma hemsleyanum*. This may result from antagonistic interactions between potassium and cations like calcium and magnesium, where excessive potassium reduces Ca/K and Mg/K ratios, causing nutrient imbalance and affecting secondary metabolism.

Key CGA synthesis enzymes including PAL, 4CL, C4H, HCT, C3H, and HQT have been validated in plants such as *Lonicera japonica* and *Morus alba*, though

their relative importance varies. In *P. petiolosa*, CGA content and key enzyme activities differed significantly among fertilizer treatments. Correlation analysis showed CGA content was significantly positively correlated with upstream enzyme 4CL and downstream rate-limiting enzyme HQT, but significantly negatively correlated with HCT activity. HCT is upstream of HQT, and their substrate-product relationship involves feedback and negative feedback regulation. Thus, 4CL, HQT, and HCT are key enzymes through which NPK nutrients influence CGA accumulation, though the underlying mechanisms require further investigation.

In summary, NPK fertilizers significantly affected SOD and CAT activities in *P. petiolosa*, with SOD primarily responding to high nitrogen and low potassium, while CAT responded to both low and high concentrations of all three nutrients. Different N, P, and K levels significantly influenced CGA content, with normal fertilization achieving the highest level (12.92 $mg \cdot g^{-1}$) and high potassium the lowest (7.79 $mg \cdot g^{-1}$). Potassium exhibited the most significant effect on CGA content. HQT, 4CL, and HCT were identified as the key enzymes responsible for CGA content variation under different fertilization regimes.

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Note: Figure translations are in progress. See original paper for figures.