Postprint: Comparative Metabolomics of Calophaca and Soybean by LC-MS

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Abstract

Calophaca sinica is a rare wild plant endemic to North China. To investigate its nutritional value, this study conducted a comparative metabolomics analysis of its seeds using liquid chromatography-mass spectrometry, with soybean as the reference group. The results showed that: (1) A total of 1,857 metabolites were detected in both Calophaca sinica and soybean, among which 1,698 metabolites (>90%) had identical composition and similar content, while 159 metabolites (<10%) were differential metabolites. (2) Among the differential metabolites, 9 showed compositional differences, of which 5 were unique to Calophaca sinica, and the remaining 150 showed content differences, with 48 (approximately 30%) having higher content in Calophaca sinica than in soybean. (3) KEGG annotation identified 8 pathways with significant enrichment of differential metabolites ($P < 0.1$), mainly including various amino acid biosynthesis pathways for primary metabolites and biosynthesis pathways for secondary metabolites such as matairesinol, arachidonic acid, and diterpenoids. (4) The chemical components with lower content in Calophaca sinica than in soybean were primarily primary metabolites, while those with higher content were mainly secondary metabolites, which play positive roles in physiological processes such as blood glucose regulation, bone damage repair, immune enhancement, and anti-inflammatory and anti-cancer activities. In summary, this study suggests that Calophaca sinica has nutritional value similar to soybean and exerts positive effects on improving human sub-health conditions; furthermore, this paper provides a comprehensive understanding of the nutritional value and metabolic composition of Calophaca sinica, while also furnishing essential data for the in-depth development and efficient utilization of Calophaca sinica resources.

Full Text

Preamble

Comparative Metabolomic Analysis of *Calophaca sinica* and Soybean Based on LC-MS Technology

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Abstract: *Calophaca sinica* is a rare wild plant endemic to northern China. To evaluate its nutritional value, this study conducted a comparative metabolomic analysis of its seeds using liquid chromatography-mass spectrometry (LC-MS), with soybean as a reference. The results showed: (1) A total of 1,857 metabolites were detected in both species, with 1,698 metabolites (>90%) showing similar composition and content, while 159 metabolites (<10%) were differentially expressed. (2) Among the differential metabolites, nine differed in composition, including five unique to *C. sinica*; the remaining 150 differed in content, with 48 (approximately 30%) showing higher levels in *C. sinica* than in soybean. (3) KEGG annotation identified eight pathways significantly enriched ($P < 0.1$) with differential metabolites, primarily including amino acid biosynthesis pathways for primary metabolites and biosynthesis pathways for secondary metabolites such as matairesinol, arachidonic acid, and diterpenoids. (4) The chemical components with lower content in *C. sinica* compared to soybean were mainly primary metabolites, while those with higher content were mainly secondary metabolites, which play positive roles in regulating blood glucose, repairing bone damage, enhancing immunity, and exerting anti-inflammatory and anticancer effects. In conclusion, this study demonstrates that *C. sinica* has nutritional value comparable to soybean and positively impacts human sub-health conditions. Furthermore, this work provides a comprehensive understanding of the nutritional value and metabolic composition of *C. sinica*, establishing a necessary data foundation for its deep development and efficient utilization.

Keywords: *Calophaca sinica*, soybean, LC-MS, metabolome, component analysis

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Introduction

Calophaca sinica is a perennial erect shrub, an entomophilous cross-pollinated plant belonging to the genus *Calophaca* Fisch. ex DC. in the Fabaceae family (Papilionoideae). This genus comprises approximately 10 species, three of which are distributed in China: *C. sinica*, *C. chinensis*, and *C. soongorica* (Li and Chen, 2019). *C. sinica* is exclusively distributed in the southern Yinshan Mountains of Inner Mongolia and central-southern Shanxi Province, with its

distribution range and population size continuing to decline (Ma, 2012), making it a rare and vulnerable endemic plant in northern China (Chinese Academy of Sciences Flora Committee, 1993). Research has shown that *C. sinica* seeds have high nutritional value, being rich in protein, carbohydrates, sugars, and other essential nutrients, indicating significant development potential (Li and Chen, 2019). Additionally, *C. sinica* exhibits characteristics of cold, drought, and poor soil tolerance, enabling survival in harsh environments while contributing to soil and water conservation (Wu et al., 2017). However, despite its dual economic and ecological value and vulnerable status, this plant remains insufficiently understood, particularly regarding the chemical composition of its seed metabolome using metabolomic approaches.

Plant metabolomics is an emerging interdisciplinary field (Guo et al., 2017) and a major branch of systems biology (Zeng et al., 2017). It enables unbiased, holistic analysis of endogenous small molecules (relative molecular weight <1,000) involved in organismal metabolism (Zhang, 2018). Metabolomic methodologies have evolved through mass spectrometry (MS) (Julian et al., 2003), high-performance liquid chromatography (HPLC) (Lin et al., 2007), and nuclear magnetic resonance (NMR) techniques. The current state-of-the-art approach is LC-MS-based metabolomics, which can identify trends among similar species and investigate metabolic differences holistically. This technology has been widely applied in botany (Duan and Qi, 2015), microbiology, and medicine (Tamas et al., 2011). For example, Rafael et al. (2019) used LC-MS metabolomics to analyze metabolic differences among chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), and white bean (*Phaseolus vulgaris*), identifying three discriminatory compounds from 43 differential metabolites. Gong et al. (2020) analyzed the intrinsic mechanisms underlying eating quality in three rice varieties, revealing core factors for taste and flavor differences. Gao et al. (2022) characterized over 400 chemical components in *Coilia nasus* ovary using LC-MS, identifying 47 differential metabolites between marine and freshwater populations and revealing divergence in ovarian development. Wu et al. (2022) identified 973 metabolites in *Rhododendron delavayi* across different periods, revealing metabolic changes from flowering to withering. Jiang et al. (2021) analyzed chemical constituents in different *Polygonatum cyrtonema* germplasms using LC-MS, screening 22 metabolites, with 15 showing higher expression in red-stemmed versus green-stemmed varieties, providing theoretical support for superior variety selection. Yuan et al. (2021) investigated differential metabolites between wild and cultivated watermelon, identifying 431 differential metabolites and providing a data foundation for watermelon improvement. These studies demonstrate that LC-MS-based metabolomics offers an efficient method for analyzing metabolic differences and nutritional value comparisons among plants.

As a rare wild plant resource endemic to northern China (Xie et al., 2018), *C. sinica* urgently requires comprehensive understanding of its metabolic composition to evaluate its nutritional value and provide scientific basis for resource development and utilization. Soybean (*Glycine max*) is a close relative of *C. sinica*, both belonging to Fabaceae Papilionoideae, and represents one of the

most important sources of plant protein (Wu, 2022). Its various bioactive components help prevent cardiovascular and cerebrovascular diseases, diabetes, and cancer (Kumar et al., 2013). Therefore, this study selected soybean as a control and employed LC-MS-based comparative metabolomics to compare metabolites between *C. sinica* and soybean, conducting metabolic pathway enrichment analysis through the KEGG database to evaluate the nutritional value and application prospects of *C. sinica* comprehensively. The results will provide a data foundation for *C. sinica* resource development and future comprehensive revelation of its metabolome composition.

[Figure 1: see original paper] Photos of (a) plant and (b) seeds of *Calophaca sinica*

Materials and Methods

1.1 Materials

In this study, soybean consisted of common yellow soybeans purchased from a local supermarket, while *C. sinica* seeds were collected from native habitats in the shrublands of Tianlong Mountain Nature Reserve in Taiyuan, Shanxi (112°25' 10.56" E, 37°43' 22.44" N) at 1,180 m elevation. Tianlong Mountain Nature Reserve is located in the uplift zone at the junction of the Loess Plateau and North China Plain, featuring large elevation differences and diverse climate zones. It has a warm temperate continental monsoon climate, with rainy summers/autumns and dry winters/springs dominated by northwest winds. The average annual precipitation is 487 mm (Guo and Li, 2022), unevenly distributed and concentrated in July, August, and September. The reserve has rich plant resources with high and relatively stable vegetation coverage, typical of the north temperate zone (Guo, 2021). Following ecological sampling methods, we collected seeds from 50 *C. sinica* individuals. After transport to the laboratory, 3-5 plump seeds from each individual were randomly selected to create one pooled sample for subsequent metabolomic analysis.

1.2 Metabolite Extraction

The main processing procedure was as follows: (1) 10 g of soybean and *C. sinica* seeds (approximately 100 seeds each) were ground to powder using a grinder; (2) 100 L (approximately 100 mg) of sample was added to a 1.5 mL EP tube, followed by 300 L methanol and 20 L internal standard, vortexed for 30 s; (3) sonicated in an ice bath for 10 min; (4) stored at -20°C for 1 h; (5) centrifuged at 13,000 $r \cdot min^{-1}$ at 4°C for 15 min; (6) 200 L of supernatant was carefully transferred to a 2 mL sample vial, and 200 L QC (quality control) sample was prepared for instrumental analysis.

1.3 Instrumental Analysis

The LC-MS system consisted of a Waters Acquity I-Class PLUS ultra-high-performance liquid chromatograph coupled with a Waters Xevo G2-XS QTof high-resolution mass spectrometer, using a Waters Acquity UPLC HSS T3 column (1.8 m, 2.1$×$100 mm).

Positive ion mode: mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile. Negative ion mode: mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile. Injection volume was 1 L.

Liquid chromatography mobile phase conditions

1.4 Metabolite Quantification

Raw data acquired by MassLynx V4.2 were processed using Progenesis QI software for peak extraction and alignment. Metabolite identification was performed based on the online METLIN database and a custom database from Biomarker Technologies (Beijing), with simultaneous theoretical fragment recognition. Data were normalized by total peak area normalization (each metabolite peak area divided by the total peak area of its sample) before analysis.

1.5 KEGG Analysis

All identified metabolites were compiled into a *.txt file and uploaded to the KEGG database (https://www.kegg.jp/kegg/) for metabolic pathway annotation. The top 20 KO pathway level2 entries with the most annotations were selected for plotting.

Results

2.1 Metabolic Composition Analysis of *C. sinica* and Soybean

A total of 1,857 metabolic components were detected in *C. sinica* and soybean, belonging to 68 categories (Figure 2 [Figure 2: see original paper]). Among these, “others” comprised the largest group with 1,271 chemical components. Of the remaining 586 components, carboxylic acids and derivatives (amino acids) were most abundant with 135 types, followed by fatty acyls (68), prenol lipids (43), organooxygen compounds (40), steroids and derivatives (34), and glycerophospholipids (28). These results indicate that *C. sinica*, like soybean, contains diverse amino acid compounds with excellent nutritional value.

[Figure 2: see original paper] Categorical statistics of metabolic composition of *Calophaca sinica* and *Glycine max*

2.2 Differential Metabolite Analysis

Pairwise comparison of metabolite types and contents between *C. sinica* and soybean identified 159 differential metabolites (Table 2). Approximately 30% (48) of these showed higher content in *C. sinica*, including five unique components, while about 70% (111) showed lower content, with four components absent in *C. sinica* (unique to soybean). These results demonstrate that the two species differ by less than 10% in metabolite content, with over 90% of *C. sinica* metabolites showing similar levels to soybean, indicating comparable nutritional value. Regarding differential metabolite composition, *C. sinica* contained only four fewer types than soybean but possessed five unique components, suggesting distinct nutritional advantages.

Differential metabolites of *Calophaca sinica* and *Glycine max*

Among the 159 differential metabolites, 43 chemical components showed higher content in *C. sinica* than soybean (Table 3). Notably, fewer than 10 components were primary metabolites, such as L-phenylalanyl-L-proline and serinyl-histidine from amino acid metabolism, Gypsogenin 3-O-rhamnosylglucosiduronic acid and β-citraurinene from carbohydrate metabolism. L-phenylalanyl-L-proline content in *C. sinica* was approximately 40-fold higher than in soybean, while β-citraurinene was 10-fold higher. Over 75% of the elevated components were secondary metabolites. For instance, 12 components in fatty acyls, benzene derivatives, and other categories exceeded soybean levels by more than 10-fold, including Δ12-prostaglandin J2, quinate, almotriptan, N-methyltyramine, ampicillin, ethosuximide, methyl 4-hydroxybenzoate, abscisic acid, and ferulic acid. Additionally, *C. sinica* contained five unique components: 3-guanidinopropanoate, baccatin III, matairesinol, adynerin, and desacetylvindoline, all secondary metabolites. These findings indicate that *C. sinica*' s superior chemical components are concentrated in secondary metabolites, with L-phenylalanyl-L-proline being the only primary metabolite showing significant advantage.

Chemical constituents with higher content in *Calophaca sinica* compared to *Glycine max*

2.3 KEGG Functional Annotation and Enrichment Analysis of Differential Metabolites

Differential metabolites between *C. sinica* and soybean were distributed across 20 metabolic pathways, with eight showing $P<0.1$: diterpenoid biosynthesis, valine/leucine/isoleucine degradation, penicillin and cephalosporin biosynthesis, inositol phosphate metabolism, glycosylphosphatidylinositol-anchor biosynthesis, autophagy, lysine biosynthesis, and biosynthesis of amino acids (Figure 3 [Figure 3: see original paper]a).

The most enriched primary metabolic pathway was biosynthesis of amino acids (Figure 3b), including L-valine↓, L-saccharopine↓, phenylalanolamine↓,

and homocitrate↓. Lysine biosynthesis included L-saccharopine↓ and homocitrate↓. Phenylalanine, tyrosine and tryptophan biosynthesis included quinate↑ and pipemidic acid↓. The most enriched secondary metabolic pathway was biosynthesis of various secondary metabolites, including matairesinol↑ and pipemidic acid↓. Other enriched pathways included purine metabolism (indole-3-carboxaldehyde↑, guanosine↓), glycerophospholipid metabolism (phosphatidylinositol↓, LysoPC(18:3(6Z,9Z,12Z))↓), arachidonic acid metabolism (Δ12-prostaglandin J2↑, 15-deoxy-Δ12,14-prostaglandin J2↓), and diterpenoid biosynthesis (baccatin III↑, ent-kaur-16-en-19-al↓). These results demonstrate that primary metabolic differences concentrate in amino acid biosynthesis (mostly downregulated), while secondary metabolic differences focus on matairesinol, arachidonic acid, and diterpenoid biosynthesis (mostly upregulated).

[Figure 3: see original paper] Differential metabolites (a) pathway classification map and (b) KEGG enrichment map

Discussion and Conclusion

Metabolites are organic compounds produced or consumed during metabolism, whose composition and content determine plant nutritional value (Li et al., 2017). Our comparative metabolomic analysis detected 1,857 metabolites in both species, with over 90% showing similar composition and content, likely reflecting their close phylogenetic relationship and indicating that *C. sinica* possesses high nutritional value comparable to soybean, with potential as a second source of plant protein.

Among 159 differential chemical components, 48 (approximately 30%) showed higher content in *C. sinica*. KEGG annotation revealed these were primarily secondary metabolites concentrated in matairesinol, arachidonic acid, and diterpenoid biosynthesis. For example, Δ12-prostaglandin J2 (Δ12-PGJ2) was 107-fold more abundant in *C. sinica*. Studies show that Δ12-PGJ2-loaded polylactic-co-glycolic acid nanoparticles can upregulate gene and protein expression in bone defect regions, showing potential for periodontal disease treatment and bone repair (Chen et al., 2015). Quinate was 68-fold more abundant and can inhibit platelet aggregation, prevent cerebral thrombosis, be converted into antiviral and anticancer agents, and reduce pain and inflammation (Zhou, 2013). Almotriptan, 40-fold higher in *C. sinica*, is an effective acute migraine treatment with high vascular selectivity and good tolerance (Wang et al., 2006). N-methyltyramine, 31-fold higher, treats shock (Yan et al., 1983; Dai and Xiong, 1989). Ampicillin, 13-fold higher, is an antibiotic treating various bacterial infections (Yang et al., 2022). Ethosuximide, 12-fold higher, treats presbycusis (Su, 2014) and childhood absence epilepsy (Fan and Chen, 2013). Methyl 4-hydroxybenzoate and abscisic acid, both 11-fold higher, can delay age-related physiological decline (Mi et al., 2018) and inhibit cancer cell proliferation while promoting stem cell growth (Guo, 2017), respectively. Ferulic acid, over 10-fold higher, inhibits inflammatory responses to treat atherosclerosis (Yang et

al., 2021).

Additionally, we identified *C. sinica*-unique components. β-guanidinopropanoate helps regulate blood glucose and improve hyperglycemia, serving as a water-soluble supplement for diabetic patients (Baumgarner et al., 2015). Baccatin III, a taxol precursor, significantly enhances immunoglobulin production and induces Th1/Th2 immune responses, representing an ideal vaccine adjuvant candidate (Yuan et al., 2014). Matairesinol, a common plant lignan, exhibits anti-angiogenic, antifungal, antioxidant, and anticancer properties (Xu et al., 2017).

In conclusion, *C. sinica* has nutritional value similar to soybean, with enriched secondary metabolites that positively impact various human diseases. The seeds contain abundant metabolites, particularly important secondary metabolites with high development value, providing references for future nutritional research and scientific cultivation planning.

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