

Ectomycorrhizal symbiosis shapes root exudation across discrete stages and phosphorus and nitrogen limitation in *Pinus yunnanensis* seedlings

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Abstract

Mycorrhiza, a mutualistic plant-fungal symbiosis, is essential for plant nutrients acquisition and stresses resistance, with root exudates playing crucial roles in these processes. Although ectomycorrhizal fungal colonization is known to influence root physiology, its specific effects on exudation profiles remain largely unexplored. In this study, *Lactarius deliciosus*-*Pinus yunnanensis* ectomycorrhizal seedlings were established using pouch and pot systems to compare root exudation profiles between mycorrhizal and non-mycorrhizal seedlings, track exudates across ectomycorrhizal formation stages (signal recognition, initial colonization, rapid development, maturity), and analyze exudative responses to early phosphorus and nitrogen deficiencies. The impacts of P-deficiency-induced ectomycorrhizal root exudates on phosphate-solubilizing bacteria proliferation were also assessed. Results showed *L. deliciosus* inoculation altered exudate compositions by introducing organohalogen and organometallic compounds, without changing total carbon exudation. Stage-specific biomarkers (His-Pro, trichloroacetic acid, sulfobacin b, γ -dodecalactone) were annotated during ectomycorrhizal development. Under early phosphorus/nitrogen deficiency, phenylpropanoids and polyketides were the main differential metabolites. Ectomycorrhizal colonization induced root exudates with antioxidant, antibacterial, and antifungal activities. Notably, P-limitation triggered trans-11-octadecenoic acid and FA20:1, which significantly stimulated phosphate-solubilizing bacteria growth. Trans-11-octadecenoic acid enriched *Pseudomonas* sp. NR6-04, without influencing its extracellular phosphatase or phytase activities. Our findings indicate that colonization by the ectomycorrhizal fungus *L. deliciosus* modified root exudation profiles and their responses to phosphorus and nitrogen limitation in *P. yunnanensis* seedlings. Fungal-symbiosis-associated metabolites significantly enhanced the enrichment of phosphate-mobilizing bacteria and demonstrated key eco-physiological functions. This study reveals new insights underlying the *L. deliciosus*-*P. yunnanensis* symbiosis originated by root exudates, highlighting their roles in ectomycorrhizal ecophysiology.

1. Introduction

Mycorrhiza is a symbiotic structure formed by mycorrhizal fungi on the roots of host plants, where plants provide carbon source to mycorrhizal fungi and mycorrhizal fungi promote plant growth mainly by enhancing plant nutrient acquisitions (Smith and Read 2008). In forests, arbuscular mycorrhizae (AM) and ectomycorrhizae (EM) are two dominant mycorrhizal types. For EM association, fungal hyphae envelop the host root to form a sheath while proliferating intercellularly to establish the Hartig net - a specialized hyphal network that infiltrates between epidermal and cortical cells of EM roots, yet remains external to the host cell plasma membrane (Sardans et al. 2023). The functional roles of ectomycorrhizal (EM) fungi extend beyond symbiosis to critically influence ecosystem processes. They directly enhance host plant water and nutrient uptake through extensive hyphal networks (Lehto and Zwiazek 2011; Pritsch and Garbaye 2011) and provide indirect protection via pathogen-suppressive secondary metabolites (Allen 2007; Guerrero-Galán et al. 2019; Wang et al. 2022). Moreover, EM fungi contribute to soil health through functions including heavy metal remediation (Bellion et al. 2006) and enhanced nutrient cycling (Talbot et al. 2008).

Root exudation represents a dynamic plant adaptation mechanism reflecting physiological status, crucial for establishing plant-microbe symbioses. EM fungal-derived small secreted proteins and soluble or volatile metabolites modulate host defense responses and induce root architectural changes, including increased branching and lateral root formation, thereby facilitating symbiotic establishment (Splivallo et al. 2009; Garcia and Ane 2016). Root exudation of organic acid anions and extracellular enzymes represents a key biochemical strategy for enhancing the bioavailability of geochemically immobilized nutrients, including phosphorus (P), directly supporting plant nutrient acquisition processes in the rhizosphere (Wang and Lambers 2020). Nitrogen (N) and P are essential nutrients for tree growth. Plant-available soil N and P constitute key limiting factors for tree growth. Soil P is dominated by organic phosphorus species (Marschner 1995), most of which is mineral-bound or in high-molecular-weight complexes requiring enzymatic hydrolysis for plant uptake. Plants have evolved three main strategies to cope with P deficiency: adjusting root architecture, forming symbiosis with mycorrhizal fungi, and secreting P-dissolving root exudates (Bucher 2007; Richardson et al. 2009). Although plants directly take up P via the root system, this is dependent on mycorrhizas in most plant species (Bolan 1991). For instance, under nutrient stress conditions, *Glomus mosseae* (T. H. Nicolson & Gerdemann) inoculation improved the root architecture of *Glycyrrhiza uralensis* Fisch. ex DC., and promoted plant uptake of P, K, Mg, Cu, Zn, and Mn (Chen et al. 2017). Moreover, fructose, a metabolite secreted by arbuscular mycorrhizal fungi (AMF), modulates bacterial secretory systems. This modulation enhances phosphatase biosynthesis and efflux into the surrounding milieu, consequently promoting the mineralization of organic phosphorus through mutualistic fungal-bacterial activity (Jiang et al.). Meanwhile, some studies showed that carbon secretion decreases after mycorrhizal symbiosis, and under P-deficient conditions, mycorrhizal plants primarily rely on the hyphal pathway to acquire phosphorus (Ryan et al. 2012; Nazeri et al. 2014; Zhang et al. 2018). However, recent studies suggest that some mycorrhizal plants may also rely on carboxylates release to mobilize P (Zhou et al. 2022, 2024). In addition, some ectomycorrhizal fungi can also utilize organic nitrogen sources in the soil by secreting nitrate reductase or extracellular acid proteases (Bending and Read 1995). Moreover, EM can selectively recruit bacteria that play crucial roles in soil mineral weathering and nutrient cycling, establishing specific microbial communities in the mycorrhizosphere (Calvaruso et al. 2007). Recent evidence further indicates that bacteria associated with ectomycorrhizal (EM) fungi exhibit superior capability in hydrolyzing soil organic phosphorus compared to EM fungi themselves (Yuan et al. 2024). Concurrently, root-exuded carbon compounds constitute critical modulators of rhizosphere microbiota assembly (Sasse et al. 2018). Therefore, it is necessary to analyze the root exudates differences between mycorrhizal and non-mycorrhizal plants, as well as their responses to nutrient deficiencies.

Lactarius deliciosus (L.) Gray. is an obligate symbiotic ectomycorrhizal fungus that primarily forms mutualistic relationships with pine trees. Its fruiting bodies possess high practical and economic value (Wang et al. 2019b). When it forms ectomycorrhizae with pine trees, it not only promotes pine growth and enhances stress resistance but also participates in the decomposition of soil organic matter and nutrient cycling (Nehls and Plassard 2018). However, the root exudation responses to ectomycorrhizal colonization by *L. deliciosus*, and the potential eco-physiological function of the root exudates remain

largely unexplored. In this study, we hypothesize that (1) Composition and concentration of root exudates will be significantly affected by EMF colonization, and P- and N- deficiency will further change EM root exuded compounds; (2) P deficiency induced EM root exudates may enrich phosphate solubilizing bacteria (PSB). To test our hypotheses, *P. yunnanensis* and *L. deliciosus* were used to: (1) Analyze root exudates in EM and non-EM seedlings, and at different developmental stages of EM symbiosis; (2) EM roots exudation profiles in response to early stage of P- and N- deficiency, respectively. Moreover, the effects of P deficiency induced EM root exuded compounds on proliferation and enzyme activities of PSBs were determined. This study aims to determine shifts in the root exudates of *Pinus yunnanensis* Franch. seedlings induced by colonization with the EM fungus *Lactarius deliciosus*, and to evaluate the contribution of these symbiosis-modified exudates to key eco-physiological processes.

2. Materials and methods

2.1 Plant, fungal and bacterial materials

Seeds of *P. yunnanensis*, sourced from Kunming, China, and cultures of the ectomycorrhizal basidiomycete *L. deliciosus*, originating from New Zealand were used in this study. *L. deliciosus* mycelium preserved in the laboratory was activated and propagated using m + p medium (Wang et al. 2019a). The m + p medium composition included (per 1000 mL ultrapure water): NaCl 0.0125 g, CaCl₂ 0.025 g, MgSO₄ 0.05 g, (NH₄)₂HPO₄ 0.125 g, KH₂PO₄ 0.25 g, thiamine (VB₁) 0.025 mg, 1% ferric citrate 0.75 mL, yeast extract 0.25 g, malt extract 0.25 g, glucose (C₆H₁₂O₆) 20 g, fresh potato infusion 100 g, agar 16.5 g (pH adjusted to 5.6–5.7). Aseptically inoculated cultures were incubated at 24°C, with sub-culturing performed every 3 months. Seeds of *P. yunnanensis* were washed and soaked in distilled water and maintained at 4°C for two days. Subsequently, the seeds were sterilized with 30% hydrogen peroxide solution and evenly sown in sterilized growth substrate consisting of a 1:1 (V/V) vermiculite and perlite. Finally, the germinated seeds were transferred to the greenhouse for approximately two months to obtain plant seedlings.

Four phosphate-solubilizing bacterial strains (A17, A34, A58, A61), exhibiting high phosphate mobilization capacity (solubilization index > 1.6; solubilization efficiency > 65%), were selected based on their demonstrated efficacy established in our prior investigation (Yuan et al. 2024). These strains were originally isolated from ectomycorrhizae of *Pinus radiata*-*L. deliciosus* symbiosis and were molecularly identified as *Pseudomonas* sp. NR6-04, *Pseudomonas migulae*, *Acinetobacter calcoaceticus*, and Bacteria (NCBI: txid1869227), respectively.

2.2 *Pinus yunnanensis*-*L. deliciosus* symbiosis and identification

The Pouch (Guerin-Laguette et al. 2000; Tang et al. 2021) and Pot (Wang et al. 2019a) co-culture systems were used to synthesize *P. yunnanensis*-*L. deliciosus* ectomycorrhizal seedlings. For Pouch

system, surface-sterilized *P. yunnanensis* roots were placed on cellophane-overlaid filter paper, followed by inoculation of *L. deliciosus* mycelium onto the roots. The assembly was transferred to a sterile bag containing 30 mL of MES buffer solution. Stems were wrapped with degreasing cotton, and the sealed sterile bags were put in aluminum foil pouches. For pot system, the growth substrate was prepared by mixing vermiculite, perlite, peat, and pine bark at a 4:2:1:1 volumetric ratio. Pots (6.7 cm × 5 cm × 14.2 cm) were filled with one third volume of autoclaved substrate, followed by transplantation of *P. yunnanensis* seedlings. *L. deliciosus* inoculum was applied around the root systems before adding the remaining substrate (Wang et al. 2019a). Inoculated seedlings were cultivated in a climate-controlled greenhouse for 3 months with watering every 3 days.

The macroscopic morphological characteristics of *L. deliciosus* ectomycorrhizal root tips in association with *P. yunnanensis* were investigated using stereomicroscopy (Leica S8AP0, Leica Microsystems, Wetzlar, Germany). For molecular identification, DNA was extracted from collected *L. deliciosus* hyphae and ectomycorrhiza using a DNA extraction kit (Tiangen Biotech DP305, Tiangen Biotech, China); then PCR was carried out on a LifeECO thermocycler (LifeBioer Technology, China), using the primer pair ITS1F and ITS4. Morphological and molecular analyses conclusively demonstrated the establishment of a well-developed symbiotic relationship between *P. yunnanensis* and *L. deliciosus* for mycorrhizal seedlings (Figure S1, S2).

2.3 Sampling of root exudates

Standardized protocols for root exudate sampling currently remain elusive (Wang and Lambers 2020). Consequently, this study utilized an aqueous hydroponic system for exudate collection. Preliminary experimentation demonstrated the suitability of this approach, as inoculated mycorrhizal seedlings exhibited sustained physiological status with no observable adverse effects for up to one-week experimental conditions (data not shown). Moreover, three days of P and N deficiency treatments could result in decreased root P and N concentrations (see the result section). Therefore, we transplanted *P. yunnanensis* seedlings to hydroponics for three days to sample root exudates. For root exudates from mycorrhizal and non-mycorrhizal *P. yunnanensis* seedlings, seedlings of the same age—both mycorrhizal (M, colonized by *L. deliciosus*) and non-mycorrhizal controls (NM)—were cultured in half-strength Hoagland's nutrient solution for 3 days. For ectomycorrhizal root exudates under P and N deficiency, respectively, uniform (similar plant size and EM colonization rates) *P. yunnanensis*-*L. deliciosus* mycorrhizal seedlings were selected and cultured for 3 days in three distinct 0.5× Hoagland's nutrient solutions: complete nutrition (control), P-deficient (-P), and N-deficient (-N) formulations. Root exudates were then collected by immersing the root systems in 150 mL of ultrapure water for 4 hours under controlled climatic conditions identical to those used for plant growth.

To profile root exudates across discrete developmental stages of *L. deliciosus* EM formation, we employed our Pouch system which established EM symbiosis progression at three defined physiological transitional stages: signal recognition [1 day after inoculation (dai)], initial colonization (12 dai), and rapid development (23 dai) (Wang et al. 2019a). Hydroponic culture solutions were collected at 7-day intervals through 28 dai. Exudates were then evaluated in four symbiotic developmental stages: pre-colonization

(0–6 dai), initial colonization (7–13 dai), rapid development stage (14–20 dai), and mature mycorrhiza stabilization (21–28 dai).

To prevent microbial degradation, Micropur was added to the sampled solution at a concentration of 0.01 g·L⁻¹. The collected exudate samples were sequentially filtered through a 0.45 µm Phenex regenerated cellulose syringe filter. The filtrates were immediately frozen at -20°C for analysis.

2.4 Quantification of total organic carbon in root exudates

A 10 mL aliquot of root exudates was lyophilized and reconstituted in 2 mL of deionized water. The solution was centrifuged at 1000 rpm for 10 min, followed by filtration through a 0.45 µm microporous membrane. The total organic carbon (TOC) concentration was quantified at 254 nm wavelength (Huang et al. 2023). A standard curve was generated by measuring OD₂₅₄ values of TOC standard solutions at concentrations of 0, 0.5, 1, 5, 10, 20, and 40 mg L⁻¹, enabling sample concentration determination via linear regression.

2.5 LC-MS/MS-based untargeted metabolomic profiling of root exudates

The 1 mL liquid sample was lyophilized, resuspended in 100 µL pre-cooled ethanol/acetonitrile/water (2:2:1, v/v/v), vortexed, and sonicated for 30 min. After incubating at -20°C for 10 min, the mixture was centrifuged at 14,000×g (4°C) for 20 min. The supernatant was vacuum-dried, reconstituted in 100 µL acetonitrile/water (1:1, v/v), vortexed, centrifuged again at 14,000×g (4°C) for 15 min, and the final supernatant was collected for analysis (Zhan et al. 2023).

The samples were separated using an ultra-high performance liquid chromatography (UHPLC) HILIC column with the following parameters: column temperature maintained at 40°C, flow rate of 0.4 mL/min, and injection volume of 2 µL. The mobile phase consisted of: A: water, 25 mmol/L of ammonium acetate, and 25 mmol/L of ammonia; mobile phase B: acetonitrile. The gradient elution program was established as follows: 95% B was maintained for 0–0.5 min; linearly decreased from 95% to 65% B over 0.5–7 min; then reduced from 65% to 40% B during 7–8 min and held until 9 min; subsequently increased linearly back to 95% B from 9–12 min (Yang et al. 2020).

The electrospray ionization (ESI) source conditions were set as follows (Yang et al. 2020): Ion Source Gas1 60, Ion Source Gas2 60, Curtain gas 30, source temperature 600°C, In positive and negative ion mode, the ion spray voltage fluctuates as: ±5500 V; TOF MS scan m/z range 60–1000 Da, Secondary mass spectrometry was obtained using information dependent acquisition (IDA) and high sensitivity modes, The Declustering potential (DP) is ±60 V and the Collision Energy is 35 ± 15 eV in both positive and negative ion modes.

The raw data in Wiff format was converted to mzXML format using ProteoWizard. Subsequently, the XCMS software was employed for peak alignment, retention time correction, and peak area extraction. The CAMERA (Collection of Algorithms for Metabolite Profile Annotation) tool was used to annotate

isotopes and adducts. Metabolite annotation was performed by comparing m/z values (< 10 ppm) and MS/MS spectra against the mzCloud (www.mzcloud.org/), mzVault, and Masslist databases.

The quality control analysis showed that the response intensities and retention times of each chromatographic peak basically overlap, and the correlation coefficients among the QC (Quality Control) samples were all greater than 0.9, indicating that the analytical system of the experimental instrument has good stability, and the data are stable and reliable (Figure S3).

2.6 Effects of P-deficiency induced root exudation compounds on PSB proliferation and enzyme activities

Five metabolites showing significantly elevated levels under P-deficient conditions were selected for analysis: trans-vaccenic acid (TVA), cis-11, 14-eicosadienoic acid (FA20:2), cis-11-eicosenoic acid (FA20:1), methylprednisolone, and mannitol. Commercial compounds of these metabolites were purchased and dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions, which were stored at 4°C until use. Four P-solubilizing bacterial strains (A17, A34, A58, A61) were separately inoculated into sterilized LB liquid medium (10 g tryptone, 5 g yeast extract, 10 g NaCl, 1000 mL distilled water, pH 7.0 $\pm$ 0.1) and cultured in a shaker at 28°C, 160 rpm until reaching an OD600 of 0.1. Aliquots (2 mL) of bacterial suspension were treated with each metabolite stock solution to a final concentration of 1 $\mu\text{mol}\cdot\text{mL}^{-1}$. Controls received an equivalent volume of DMSO, while sterilized LB medium served as the blank. All cultures were incubated statically at 28°C with three biological replicates. OD600 measurements were taken every 24 hours within three days.

Strain A17 was cultured in sterilized LB liquid medium at 28°C with 160 rpm shaking until reaching an OD600 of 0.1. The bacterial suspension was then aliquoted (2 mL/tube) into 5 mL centrifuge tubes. Trans-vaccenic acid (TVA) stock solution (prepared in DMSO) was added to achieve five concentration gradients: 10, 1, 0.1, 0.01, and 0 $\mu\text{mol}\cdot\text{mL}^{-1}$. Control groups received equivalent volumes of sterilized LB medium. All treatments were incubated statically at 28°C with triplicate replicates. OD600 measurements were performed at 8, 24, 48, and 72 h. Post-incubation, residual cultures were centrifuged (5,000 rpm, 1 min), and supernatants were analyzed for acid phosphatase (Boxbio AKFA017M, Boxbio, China), alkaline phosphatase (Boxbio AKFA018M, Boxbio, China), and phytase (Grace Biotechnology G0905W, Grace Biotechnology, China) activities using their corresponding assay kits.

2.7 Statistical analysis

SIMCA 14.1 and Origin 2021 were employed for multivariate data analysis. Principal component analysis (PCA) was applied for unsupervised data analysis to examine the overall distribution trends and variations among component samples. For supervised discriminant analysis, orthogonal partial least squares-discriminant analysis (OPLS-DA) was utilized, and permutation tests were conducted to prevent model overfitting. Significantly differentially expressed metabolites were annotated by combining variable importance in projection (VIP) values from the OPLS-DA model with Student's t-test results (VIP > 1 , FC > 1.2 or FC < 0.833 , and $p < 0.05$). These metabolites were then qualitatively stratified, clustered,

and subsequently subjected to metabolic pathway analysis using KEGG (www.genome.jp/kegg/) and MetaboAnalyst (www.metaboanalyst.ca/).

3. Results

3.1 Root exudates of mycorrhizal and non-mycorrhizal seedlings

There was no significant difference in the TOC concentration in the root exudates between mycorrhizal and non-mycorrhizal *P. yunnanensis* seedlings (Fig. 1a). A total of 1400 metabolites were annotated, which were classified into 15 super classes, including 264 organoheterocyclic compounds, 220 lipids and lipid-like molecules, and 163 organic acids and derivatives (Fig. 1b, Table S1). Principal Component Analysis (PCA) showed that PC1 accounting for 35.3% and PC2 for 17% of the variance (Fig. 1c). The results demonstrated good intra-group similarity and significant inter-group differences.

A total of 395 differentially expressed metabolites were annotated between the M and NM groups, with 289 metabolites upregulated and 106 downregulated. These differential metabolites were significantly enriched in three metabolic pathways: Pantothenate and CoA biosynthesis, ABC transporters, and Biotin metabolism (Figure S4, and Fig. 1d). The top 15 upregulated and downregulated metabolites respectively were shown in a heatmap (Fig. 1e), and the samples within each group clustered together. The metabolites clustered into several distinct groups, with each group potentially participating in identical metabolic pathways or exhibiting similar patterns of chemical abundance variation. In addition, the M group had 660 unique metabolites compared to the NM group, which were classified into 16 super-classes (Fig. 1f).

Figure 1 Root exudation profiles between mycorrhizal and non-mycorrhizal *P. yunnanensis* seedlings. (a) TOC concentration in root exudates of mycorrhizal and non-mycorrhizal *P. yunnanensis* seedlings. (b) Superclass of annotated metabolites. (c) PCA score plot of metabolites under combined positive-negative ion mode. (d) A bubble diagram showing KEGG pathways for differential metabolites, with each bubble representing a metabolic pathway. (e) A heatmap showing top 15 upregulated and downregulated metabolites respectively, in mycorrhizal root exudates compared to the non-mycorrhizal root exudates. The color bars in the heatmap indicate the concentration level (expressed as the transformation intensity of the original peak). The number on the axis represents the Z – score, $Z = (x - \mu) / \sigma$; x represents the intensity of the original peak; μ represents the average; σ denotes the standard deviation. (f) Number and classification of metabolites specific to mycorrhizal and non – mycorrhizal root exudates.

3.2 Root exudates at different stages of EM symbiosis

A total of 1381 metabolites were annotated and classified into 12 superclasses, including 478 organic acids and derivatives, 195 lipids and lipid-like molecules, and 133 organoheterocyclic compounds (Fig. 2a, Table S1). PCA analysis defined intergroup differences, and some overlap among samples and

high variability within the same group. PC1 and PC2 accounted for 36.5% and 14.4% of the variance, respectively (Figure S5). Multiple differentially expressed metabolites were annotated across discrete symbiotic stages, as defined in the Material and Methods section (Fig. 2b). The differential metabolites were annotated to various metabolic pathways, mainly prodigiosin biosynthesis, mineral absorption, protein digestion and absorption, mTOR signaling pathway, clavulanic acid biosynthesis, chloroalkane and chloroalkene degradation, glycerophospholipid metabolism, and carbapenem biosynthesis (Fig. 2c-e).

Venn analysis revealed 4 common differential metabolites across different stages of EM formation: His-Pro, trichloroacetic acid, sulfobacin b, and γ -dodecalactone (Fig. 2f). Cluster analysis showed that the root exudates of EM7D and EM21D, as well as EM14D and EM28D, exhibited similar metabolic characteristics. The relative concentration of His-Pro showed an overall downward trend throughout the process; sulfobacin b was upregulated in EM14D and EM28D; and γ -dodecalactone was significantly upregulated in EM14D (Fig. 2g). In addition, 74, 36, 32, and 42 unique metabolites were annotated at EM7D, EM14D, EM21D, and EM28D, respectively, which could be classified into up to 9 superclasses (Fig. 2h).

3.3 Impact of N and P deficiency on ectomycorrhizal root exudates

Under P-deficient conditions, P concentrations in both needles and roots of *P. yunnanensis* decreased significantly relative to the control (two-tailed t-test, $P < 0.01$) (Fig. 3a). N-deficiency significantly reduce N concentration compared to the control group (one-tailed t-test, $P < 0.05$), whereas needle N concentration remain unchanged (Fig. 3b). Collectively, these results demonstrate that a three-day N or P deficient treatment reduce tissue-specific N and P concentrations in the roots of *P. yunnanensis*.

Figure 3 Nitrogen and phosphorus concentrations in the leaves and roots of *P. yunnanensis* under P and N deficiency conditions. NS = No significant difference; *, $P < 0.05$; **, $P < 0.01$.

TOC concentration in the ectomycorrhizal root exudates of *P. yunnanensis* seedlings remained unchanged relative to the control under both P and N deficiency treatments (Fig. 4a). Metabolic analysis annotated 931 metabolites across all treatment groups, categorized into 12 superclasses. Predominant superclasses included 283 lipids and lipid-like molecules, 130 phenylpropanoids and polyketides, and 114 benzenoids (Fig. 4c, Table S1). PCA revealed significant separation among treatments (Fig. 4b), with PC1 and PC2 explaining 27.3% and 19.1% of the total variance in EM root exudation profiles, respectively, demonstrating significant differences among the treatments. P deficiency induced 60 upregulated and 12 downregulated compounds relative to the control. Upregulated metabolites under P deficiency were significantly enriched in biosynthesis of unsaturated fatty acids, glycerophospholipid metabolism, and caffeine metabolism (Figure S6, d). Conversely, N deficiency resulted in differential accumulation of 142 upregulated and 11 downregulated compounds. Upregulated metabolites under N deficiency exhibited enrichment in ABC transporter pathways, biosynthesis of unsaturated fatty acids, tropane, piperidine and pyridine alkaloid biosynthesis, histidine metabolism, fatty acid biosynthesis, and caffeine metabolism (Figure S6, e).

Venn analysis revealed seven common differentially expressed metabolites across the three groups: Trans-Vaccenic acid, FA20:2, FA20:1, mannitol, selina-4(14), 7(11)-diene-9-ol, dihydrocodeine, and methylprednisolone (Fig. 4f). The relative abundance of these metabolites was significantly higher in the -P group compared to the CK group (control group) (Fig. 4g).

A total of 96, 148, and 131 unique metabolites were annotated in the CK, -P and -N, respectively. Lipid and lipid-like molecules constituted the most abundant category in both the -P and -N groups (Fig. 4h).

3.4 P-deficiency promotes root exudates that stimulate phosphate solubilizing bacteria proliferation

Trans-vaccenic acid (TVA) significantly stimulated proliferation of strains A17, A34, and A61, inducing peak relative growth increases of 243.6%, 246.7% and 228%, respectively, at 24 hours post-treatment. Growth rates subsequently declined progressively across all three strains (Fig. 5a, b, d). FA20:1 promoted proliferation of strains A17, A34, and A58, achieving relative growth rates of 185.7% for A17 within 48 h, and 155.9% and 151.3% for A34 and A58 within 24 h (Fig. 5a, b, c). In contrast, FA20:2, methylprednisolone, and mannitol exhibited weak effects on phosphate-solubilizing bacterial proliferation (Fig. 5a, b, c, d).

TVA at concentrations of $10 \mu\text{mol}\cdot\text{mL}^{-1}$ and $1 \mu\text{mol}\cdot\text{mL}^{-1}$ enhanced the proliferation of strain A17, with the optimal proliferative response observed at $1 \mu\text{mol}\cdot\text{mL}^{-1}$ (Fig. 6a). Acid phosphatase and phytase showed no statistically significant effect across the tested TVA concentration range (Fig. 6b, d). Alkaline phosphatase activity, however, was significantly reduced relative to the control exclusively at the $0.1 \mu\text{mol}\cdot\text{mL}^{-1}$ concentration. (Fig. 6c).

4. Discussion

4.1 Ectomycorrhizal colonization regulates the root exudation profiles of *P. yunnanensis*

Plant root exudate composition and quantity undergo dynamic alterations under biotic or abiotic stress conditions, with soil microorganisms directly influencing exudate profiles (Técher et al. 2011). The establishment of mycorrhizal symbiosis necessitates continuous bidirectional signaling between host roots and fungal partners, inducing transcriptional and metabolic reprogramming within host roots (Smith and Read 2008). Previous research has predominantly focused on comparative metabolomics between mycorrhizal and non-mycorrhizal plants (Sebastiana et al. 2021; Xia et al. 2023). However, mycorrhiza-induce metabolic shifts likely extend to root exudation patterns. Consistent with this, inoculation with the arbuscular mycorrhizal fungus *Glomus versiforme* (P. Karst.) S.M. Berch significantly altered metabolite levels in both the root tissues and exudates of *Lotus japonicus* (Regel) K.Larsen (Xu et al. 2023). Furthermore, mycorrhizal plants exhibited elevated concentrations of carbohydrates and amino acids in root exudates compared to non-mycorrhizal controls; though no species compounds,

including specific sugars, amino acids, or carboxylic acids, were uniquely absent in the latter (Schwab et al. 1984). Obligate mycorrhizal fungi, lacking phototrophic capacity, depend entirely on plant-derived carbohydrates for carbon. Concurrently, fungal nitrogen demands may be partially met by root-exuded amino acids, which could additionally function as symbiotic signaling molecules (Schwab et al. 1984). In this experiment, a substantial number of unique metabolites were annotated in both mycorrhizal and non-mycorrhizal *P. yunnanensis* seedlings. Notably, 17 amino acids, peptides, and analogs were significantly upregulated in mycorrhizal root exudates. While amino acids can stimulate hyphal growth *in vitro* (Kawagishi et al. 2004), a direct role in mycorrhization *in planta* remains unestablished. Critically, 660 unique metabolites were detected in the root exudates of *P. yunnanensis* seedlings colonized with *L. deliciosus* indicating profound symbiosis-specific metabolic restructuring. Among these, organohalogen compounds were exclusively annotated in exudates from mycorrhizal plants. Organohalogens, biosynthesized by diverse marine and terrestrial bacteria, fungi, and plants (Gribble 2024), play pivotal roles in chemical ecology, e.g., defense, allelopathy, and exhibit significant pharmacological and enzymological relevant functions (Table 1) (Paul and Pohnert 2011; Wang et al. 2021).

Table 1
Potential function of some differential metabolites in different experiments

Group	Metabolite name	Function	References
1	(s) – atpa	AMPA receptor agonist	(Curry and Pajouhesh 1998)
	(8S,19S) – 19,20 – Dihydro – 9,19,20 – trihydroxy – 8-methoxy – 9-epi – fumitremorgin C	Cytotoxicity, antiviral activity, selective ABCG2 inhibitor	(Szolomajer-Csikós et al. 2013; Saraiva et al. 2015; Hamed et al. 2023)
	Subnulatone B	Cytotoxicity	(Duong et al. 2020)
	Excavatin L	Antioxidant, antibacterial activity, cytotoxicity	(Tatsimo et al. 2015; Thant et al. 2021)
	Solstitialin A 13 – acetate	Cytotoxicity, antibacterial and anti-inflammatory activities	(Cheng et al. 1992; Radan et al. 2017)
	Linderazulene	Cytotoxicity, anti-inflammation, anti-oxidation	(Reddy et al. 2005; Bakun et al. 2021; Yetkin et al. 2022)
	Muralatin A	Antioxidant, anti-inflammatory, antibacterial	(Heim et al. 2002; Chagas et al. 2022)
2	3-(2 – hydroxyethoxy)xanthen – 9-one	Antibacterial, anti-inflammatory, anti-tumor	(El-Seedi et al. 2010; Feng et al. 2020; Klein-Júnior et al. 2020)
	γ – dodecalactone	Antifungal; antibacterial	(Kishimoto et al. 2005; Fujita et al. 2021; Mazur and Masłowiec 2022)
	His – Pro	Antifungal and antibacterial; serving as a bacterial quorum sensing signal to regulate the behavioral transition from symbiosis with the host to pathogenicity; inducing a protective response in nerve cells	(de Carvalho and Abraham 2012; Bellezza et al. 2014)
Note: Group 1: Partial differential metabolites between the secretions of <i>Pinus yunnanensis</i>-<i>Lactarius deliciosus</i> ectomycorrhizae and non-mycorrhizae; Group 2: Common differential metabolites in the root exudates of <i>Pinus yunnanensis</i> ectomycorrhizae at different developmental stages after inoculation with <i>Lactarius deliciosus</i>; Group 3: Common differential metabolites among the ectomycorrhizal secretions of <i>Pinus yunnanensis</i>-<i>Lactarius deliciosus</i> under nitrogen/phosphorus stress conditions.			

Group	Metabolite name	Function	References
	Sulfobacin b	Selectively inhibit DNA polymerases of animal origin; possess anti-inflammatory activity and exhibit broad-spectrum antibacterial activity	(Maeda et al. 2010)
3	Trans – Vaccenic acid	Antibacterial; anti-inflammatory	(Reynolds and Roche 2010)
	FA 20:2	Antibacterial activity; anti-inflammatory activity; anti-tumor	(You et al. 2003; Pereira et al. 2014; Lee et al. 2017)
	FA 20:1	Antibacterial; anti-inflammatory	(Pereira et al. 2014; Lee et al. 2017)
	Mannitol	Scavenge free radicals; enhance serum bactericidal activity.	(Dai et al. 2017; Kou et al. 2022)
	Selina – 4(14),7 (11) – diene – 9–ol	Antioxidant, which can reduce oxidative stress by enhancing the cellular antioxidant system; inhibits melanin production	(Chang et al. 2007; Kim et al. 2018; Zhao et al. 2022)
	Dihydrocodeine	Mainly used for pain relief, cough suppression, and the treatment of opioid addiction.	(Schmidt et al. 2002)
	Methylprednisolone	Anti-nflammatory and immunosuppressive effects.	(Möhlmann et al. 2025)
Note: Group 1: Partial ifferential metabolites between the secretions of <i>Pinus yunnanensis-Lactarius deliciosus</i> ectomycorrhizae and non-mycorrhizae; Group 2: Common differential metabolites in the root exudates of <i>Pinus yunnanensis</i> ectomycorrhizae at different developmental stages after inoculation with <i>Lactarius deliciosus</i>; Group 3: Common differential metabolites among the ectomycorrhizal secretions of <i>Pinus yunnanensis-Lactarius deliciosus</i> under nitrogen/phosphorus stress conditions.			

Colonization of host plants by ectomycorrhizal fungi elicits metabolic reprogramming that subsequently modulates root exudate profiles (Tscharplinski et al. 2014; Li et al. 2018). During the pre-symbiotic phase, host roots secrete key metabolites (e.g., flavonoids and hormones) functioning as signaling molecules that induce chemotrophic hyphal growth toward root systems (Martin et al. 2001). At the symbiotic interface establishment stage, root-exuded lipids and lipid-like molecules mediate membrane interface ontogenesis between symbionts (Siebers et al. 2016) or function as interkingdom signaling compounds. Throughout the functional symbiotic phase, upregulated saccharide efflux from the roots, provides fungal carbon substrates (Nehls et al. 2007), while fungi reciprocally translocate acquire nutrients such as N and P to the plant. Our analysis detected diverse lipid molecules and also organic acids, in root exudates, with amino acids constituting a proportionally dominant fraction. We posit that amino acids, serving as carbon and N sources, and enzymatic constituents, may facilitate colonization of mycorrhizal

fungi. Significant transcriptomic and metabolomic differences were observed in *P. yunnanensis* 23 days post-inoculation with *L. deliciosus* (Su et al. 2024). By day 28, we documented substantial compositional and quantitative restructuring of root exudates during ectomycorrhization, reflecting: i) dynamic symbiotic responsiveness, and ii) continuous metabolic pathway remodeling in *P. yunnanensis*. These stage-specific metabolites may function as temporal biomarkers delineating ectomycorrhizal development phases.

Differentially expressed metabolites were predominantly categorized as: lipids and lipid-like molecules, organoheterocyclic compounds, organic acids and derivatives. Notably, linderazulene and muralatin A manifest antibacterial and antioxidant bioactivities (Bakun et al. 2021; Yetkin et al. 2022; Chagas et al. 2022). Both properties significantly influence mycorrhizal synthesis establishment: antibacterial activity reduce rhizosphere pathogen loads, thereby diminishing infection risk and establishing a conducive niche for mycorrhization. Concurrently, antioxidant capacity scavenges reactive oxygen species (ROS) generated during root-fungal growth, mitigating oxidative stress-induced cellular damage and stabilizing redox homeostasis. Furthermore, multiple exudates exhibit pleiotropic functionality: organic acids and amino acids facilitate hyphal proliferation; histamine orchestrates plant immunity and fungal signaling cross-talk; and diethanolamine participates in lipid metabolic pathways (Yar et al. 2013; Roshchina 2022; Khan et al. 2023) (Table 1).

4.2 Shifts in the ectomycorrhizal root exudate profiling induced by N and P limitation

P-deficiency elicits enhanced rhizospheric organic acid exudation in P-demand plants, facilitating solubilization and acquisition of insoluble P pools (Lambers et al. 2006). Analogously, N limitation alters root exudation composition, typically elevating organic acid concentration while modulating amino acid profiles (Carvalhais et al. 2011). This study demonstrates that lipids and lipid-like molecules constitute the predominant category among differential abundant metabolites under both P- and N-deprivation regimes relative to controls, suggesting their functional significance in stress adaptation. Lipids critically regulate cell membrane fluidity and stability, preserving cellular homeostasis in both plants and fungi during abiotic stress (Vishwakarma et al. 2019; Liu et al. 2019). Notably, subcritical cellular P concentrations trigger phospholipid catabolism to release P for essential metabolic processes (Svietlova 2012; Yang et al. 2021). Consistent with this mechanism, P-deficient conditions in our system induced: i) Metabolic enrichment in glycerophospholipid metabolism and unsaturated fatty acids pathways; and ii) a net increase in the total number of root-exuded metabolites. This observations align with membrane phospholipid remodeling under P stress. Correspondingly, N-deficiency upregulated metabolites associated with fatty acid biosynthesis, unsaturated fatty acid production, and histidine metabolism (Xue et al. 2022). Such pathway modulations likely reflect coordinated nutrient stress responses in *P. yunnanensis*-*L. deliciosus* ectomycorrhizae.

While prior studies establish that P limitation enhances organic acid exudation to mobilize recalcitrant soil P (Dinkelaker et al. 1989; Vance et al. 2003), our short-term deficiency model (3-day exposure)

primary captures early-phase physiological adjustments. In this study, under P-deficient conditions, differential metabolites were annotated to pathways including glycerophospholipid metabolism and the biosynthesis of unsaturated fatty acids. A concomitant increase in the total number of root-exuded metabolites increased was observed. This shift potentially reflects adaptative responses to reduced phospholipid levels in cellular membranes. Under N-deficiency, differential metabolites were similarly annotated to metabolic pathways encompassing fatty acid biosynthesis, unsaturated fatty acids biosynthesis, and histidine metabolism (Ryan et al. 2012; Nazeri et al. 2014).

The impact of N and P deficiency stress on plants constitutes a long-term adaptative process. This study employed short-term treatments (3 days duration) for P or N deficiency; consequently the observed alterations in root exudates primarily represent physiological regulatory mechanisms during the initial stress response phase. During this early stage of N and P deficiency, plants modulate the composition and quantity to mitigate environmental stress (Marschner 1995). Prolonged stress exposure elicits further adaptive modifications, including alterations in root architecture alongside sustained adjustments in exudation profiles, facilitating acclimation to chronic nutrient limitation (Lambers et al. 2006). Furthermore, EMF secrete organic acids capable of solubilizing immobilized Pi compounds within the soil matrix. To elucidate the temporal dynamics of plant adaptation to P stress, future research should extend the duration of P-deficiency treatments. Investigating root exudate profiles at subsequent developmental stages (e.g., mid-term and late-term deficiency) would provide a more comprehensive understanding of the physiological and molecular mechanisms underpinning plant acclimation to prolonged P limitation.

4.3 Phosphorus deficiency induced ectomycorrhizal root exudation compounds can enrich P mobilize bacteria

Soil harbors a variety of PSB, which enzymatically convert insoluble P into plant-available forms through the exudation of organic acids and phosphatases (Burgstaller and Schinner 1993). Root exudates function as selective agents, recruiting and enriching these PSB populations, thereby facilitating the formation of a specialized rhizosphere microbiome (Bulgarelli et al. 2013). This microbial enrichment contributes significantly to enhanced soil nutrient cycling and improved plant nutrient acquisition. Furthermore, specific signaling molecules within root exudates, such as fructose, have been demonstrated to modulate bacterial metabolic pathways, potentially augmenting phosphate-solubilizing functionality (Zhang et al. 2018). In the present study, P deficiency was observed to induce the production of several metabolites within the root exudates of *P. yunnanensis* ectomycorrhizal seedlings, notably unsaturated fatty acids. Chemotactic responses of certain Gram-negative bacteria towards various unsaturated fatty acids are well-documented (Eder et al. 2017; Baker et al. 2018; Hobby et al. 2019). Consistent with these reports, experimental data demonstrated that the Trans-Vaccenic acid (TVA) and FA20:1 promote the proliferation of Gram-negative bacteria PSB strains A17, A34, and A58. Regarding P mobilization, the solubilization of organic P within the mycorrhizosphere is predominantly mediated by bacteria-secreted phosphatases, rather than enzymes derived from the EM fungi themselves [26]. Additionally, microbial phytases exhibit the ability to hydrolyze phytate, releasing

inorganic phosphate (Richardson and Simpson 2011). While root exudates are recognized for enriching P-solubilizing microorganisms and enhancing their solubilizing capacity (Wang et al. 2023; Pantigoso et al. 2023), their specific influence on phosphatase and phytase enzymatic activities remains less extensively characterized. Our investigation revealed that TVA, within a defined concentration rate, exerted no significant effect on bacterial-secreted acid phosphatase and phytase activity. However, alkaline phosphatase activity was inhibited at TVA concentrations of 0.1 and 1 $\mu\text{mol}\cdot\text{mL}^{-1}$. It is pertinent to note that bacterial phosphatase activity is also subject to regulation by inorganic and organic P concentrations present in the soil solution (Xu et al. 2025). Therefore, it is proposed that TVA released by EM roots may facilitate the enrichment of PSB at the mycorrhizosphere. Under conditions of low plant-available P, these enriched PSB populations may subsequently enhance soil P mobilization through elevated enzymatic activities.

5. Conclusion

This study demonstrates that the establishment with the mutualistic EM fungus *L. deliciosus* significantly modifies the compositional profiles of root exudates in *P. yunnanensis* seedlings. Furthermore, it reveals that EM root exudation exhibits distinct responses during the early stage of the establishment of the mutualistic relationship under N and P deficiency. Critically, compounds exuded by EM roots under P-limited conditions enhance the proliferation of PSB. These findings advance mechanistic understanding of root exudate-mediated interactions within EM symbiosis and underscore the role of EM-derived exudates in mediating ecosystem-relevant eco-physiological functions.

Declarations

Ethics approval and consent to participate

The plant experiments were conducted in accordance with relevant institutional, national, and international guidelines and legislation, including the collection of plant and fungal materials.

Consent for publication

Not applicable.

Authors' contributions

Wen Zhou and Yuanhao Wang primarily wrote and revised the manuscript text; Wen Zhou and Yuanhao Wang prepared all figures and supplementary materials; Wen Zhou, Jing Yuan, and Guixian Zheng collected and performed all experiments; Yanliang Wang and Fuqiang Yu supervised the work, reviewed and edited the manuscript, with intensive inputs from Jesús Pérez-Moreno and Xinhua He. All authors read and revised the manuscript.

Competing interests

The authors declare no conflicts of interest.

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Availability of data and materials

The published article and its supplementary materials include most of the data generated in this study, and the original datasets are available from the corresponding authors on reasonable request.

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