METABOLOMIC ANALYSIS PROVIDES NEW INSIGHTS INTO THE GROWTH-PROMOTING MECHANISM OF ENDOPHYTE IN RICE SEEDLINGS

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Abstract. The role of endophytes in promoting plant growth has been explored, but the metabolomic perspective of plant-endophyte interaction remains unknown to some extent. Therefore, we studied growth, soluble sugar content and sucrose metabolism-related enzyme activities (sucrose synthase (SS), sucrose phosphate synthase (SPS), invertase (INV), and hexokinase (HXK)) of rice seedlings uninfected and infected by endophyte EF0801, and metabolomic analysis was performed using gas chromatography and mass spectrometry (GC-MS). The results showed that plant height, above-ground dry weight, under-ground dry weight, soluble sugar content, SS, SPS, INV and HXK activities were significantly increased in endophyte-infected rice seedlings. The Kyoto Encyclopedia of Genomes (KEGG) was utilized for functional annotation and enrichment pathway analysis and the results showed that 36 metabolites were significantly changed in endophyte-infected rice seedlings, and were significantly enriched in glyoxylate and dicarboxylate metabolism, starch and sucrose metabolism, citrate cycle, galactose metabolism, fructose and mannose metabolism, pentose phosphate pathway and alanine, aspartate and glutamate metabolism, in which fructose and mannose metabolism and pentose phosphate pathway being the most significantly up-regulated. This study reveals the metabolomic mechanism of endophyte and rice interaction and provides new insights into the appliance of endophyte in promoting plant growth and development.

Keywords: *rice, endophyte infection, GC-MS analysis, sucrose metabolism-related enzyme, plant-growth promotion*

Introduction

Petrini defined endophytes as "organisms inhabiting plant organs that can colonize the internal tissues of a plant at some time in its life without causing harm to the host" (Petrini, 1991). De Bary first proposed the concept of endophytes in 1866 (Herre et al., 2007). It has been reported that when host plants are infected with endophytes, they coevolve with the plants to form mutually beneficial symbiotic relationships, enhancing their stress tolerance (Zarea et al., 2011; Karthikeyan et al., 2012). In addition, endophyte inoculation can promote the growth and development of plants (Larriba et al., 2015; Ren et al., 2022), enhance their antioxidant and photosynthetic capacities (Bu et al., 2012), improve nutrient uptake, and increase endogenous hormone levels (Gond et al., 2015). Ferreira et al. (2019) found that wheat inoculation with *Bacillus* spp. enhanced photosynthesis by producing siderophores and chelated iron, which provided iron for photosynthesis, it also reduced plant iron deficiency, mitigated heavy metal-induced oxidative stress, and prevented oxidative damage. Mohanty et al. (2017) found that inoculation with endophytic bacteria significantly increased shoot and root length

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($P < 0.01$) and enhanced phosphatase and 3-indoleacetic acid activities in seedlings compared to uninoculated maize seeds. Therefore, researching the application of endophytes is crucial.

The concept of metabolomics was first established by the Neiholsonl research group in 1999 (Nicholson et al., 1999). Metabolomics is based on genomics, transcriptomics, and proteomics to identify and quantify low-molecular-weight metabolites in biological samples and provide a comprehensive view, thus revealing physiological changes influenced by external stimuli or intervention (Fuica-Carrasco et al., 2023). Metabolic processes in organisms are studied through changes in metabolite abundance and metabolic pathways. Metabolomics can reveal the close relationship between plants and their environment and also understand the functions of plant genes, metabolic networks, metabolic regulation, and the link between phenotype and growth. Currently, metabolomics has been used to study potato (Lekota et al., 2020), tomato (Mzibra et al., 2021), rice (Ranjitha et al., 2019), wheat (Guo et al., 2020), cucumber (Pan et al., 2022), maize (Deng et al., 2020), and soybean (Yang et al., 2017).

Rice (*Oryza sativa* L.) is one of the world's most widely cultivated food crops. Compared with other cereals, it has a small genome and is considered a model plant (Devos and Gale, 2000). There are many studies on rice genome, transcriptome, proteome, and metabolomics. However, there are few studies on rice metabolomics under endophyte infection. Metabolites are the end products located downstream of gene and protein regulation, and their composition and content directly affect the phenotype. In this experiment, we measured the growth, soluble sugar content and sucrose metabolism-related enzyme activities (SS, SPS, INV, HXK)) of uninfected and endophyte-infected rice seedlings and compared the metabolic changes in rice seedlings under endophyte infection using GC-MS, thus revealing the metabolic mechanism by which the endophytes promote the growth of rice seedlings.

Materials and methods

Culture of endophyte EF0801 and rice seedlings

Endophytic fungus EF0801 was isolated from *Suaeda salsa* at Red Beach, Panjin, which was identified to have 99% similarity with *Sordariomycetes sp*. Endophyte EF0801 were obtained and prepared as described in our previous studies (Bu et al., 2012). They were transferred to sterilized conical flasks and added to 125 mL of potato dextrose agar (PDA) medium and incubated for 12 d in a shaking incubator at 25℃ and 125 rpm. The cultured endophyte EF0801 cocci and liquid were homogenized into a suspension. Based on the results of previous studies (Bu et al., 2012; Ren et al., 2022), we chose 5% endophyte suspension to treat rice in this study.

The experimental material was *Oryza sativa* L. (Liaoxing NO.1). First, healthy rice seeds were selected and sterilized with 1% NaClO for 25 min and rinsed with distilled water. Then 100 seeds were placed in a plastic beaker (700 mL) containing sterilized Hoagland solution in a light incubator with a day/night temperature of 28/22℃, light of 16 h, relative humidity of 80%, and light intensity of 10,000 lux. Rice seedlings were cultivated to the two-leaf-one-heart stage and randomly divided into two groups. Each group was replicated 5 times. Control group (CK): cultured with Hoagland solution. Endophyte-infected group (E+): cultured with Hoagland solution containing 5% endophyte suspension. According method of Liu and Chen (2007), more than 90% of endophyte were colonized (Li et al., 2012).

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Measurement of growth index

After treatment to the three-leaf-one-heart stage, rice seedlings of CK and E+ groups were collected and measured for plant height, root length, and dry weight. Dry weight was determined after drying in an oven at 80°C for 12 h.

Measurement of soluble sugar content

Soluble sugar content was determined according to the anthrone colorimetric method proposed by Zhang et al. (2009). The dried leaf sample was homogenized in 80% ethanol, placed in a water bath at 80°C for 30 min, and then centrifuged at 5000 rpm for 10 min. The supernatant was transferred, 80% ethanol was added to the precipitate, and the extraction process was repeated twice as described above. The reaction mixture of supernatant and anthrone sulfate was incubated in a boiling water bath for 20 min, then content of soluble sugar was determined spectrophotometrically at 620 nm.

Measurement of enzyme activities

Sucrose synthase (SS), sucrose phosphate synthase (SPS) and invertase (INV) activities were determined by the method of Zhang et al. (2009). Leaf sample was homogenized in HEPES-NaOH buffer (50 mM, pH 7.5) and then centrifuged at 12,000 rpm for 10 min at 4°C to obtain the enzyme extract. The mixture contained supernatant, HEPES-NaOH buffer (50 mM; pH 7.5), $MgCl_2$ (50 mM), Fructose (10 mM), and UDPG (10 mM), which was incubated at 37°C for 30 min. The reaction was stopped by adding 30%NaOH, then activity of SS was determined spectrophotometrically at 480 nm. SPS activity was determined in the same way as SS, except Fructose-6-phosphate instead of Fructose.

Leaf sample was homogenized in deionized water, ice water bath for 3 h, centrifuged at 5000 rpm for 15 min, and collected the supernatant. The mixture contained supernatant, phosphate buffer (pH 6.0), 10% sucrose solution, and water bath at 37°C for 30 min. The mixture was added 3,5-dinitro salicylic acid, and incubated in a boiling water bath for 5 min, then the activity of INV was determined spectrophotometrically at 540 nm.

Hexokinase (HXK) activity was determined according to the method of Whittaker et al. (2001). Leaf sample was homogenized in an ice bath by extraction buffer containing EDTA (0.5 mM), KH_2PO_4 (20 mM, pH 7.5) and DTT (5 mM), and centrifuged at 12,000 rpm for 20 min at 4°C and the supernatant collected. The reaction mixture consisted of $MgCl_2$ (2 mM), EDTA (1 mM), NAD (0.4 mM), KH_2PO_4 (100 mM, pH 7.5), ATP (1 mM), 1 IU glucose-6-phosphate dehydrogenase (5 mM), 1 IU glucose phosphate isomerase, glucose (5 mM) and the supernatant, and the reaction was carried out for 5 min at 25°C, then the activity of HXK was determined spectrophotometrically at 340 nm.

Quantitative real-time PCR analysis

Expression levels of endophyte-infected and non-infected rice leaves were examined using qRT-PCR to verify the accuracy of sucrose metabolism-related enzyme activities. TubA is a ubiquitin extension protein homologous gene, and it has good stability, so it was used as internal reference gene. Fresh rice leaves were ground with liquid nitrogen, and total RNA from rice leaves was extracted using the RNA Simple Kit (Tianyuan Bio Beijing Co., Ltd.), and first-strand cDNA was synthesized using the PrimeScript RT

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Reagen Kit (TaKaRa). Design of specific primers through the Primer3plus website (https://www.primer3plus.com/) (*Table 1*). Validated using the LightCycler96 PCR system (Roche Co., Ltd., Basel, Switzerland). $2^{-\Delta\Delta CT}$ was used to calculate carbon metabolism-related gene expression.

Metabolite extraction and GC-MS analysis

Transfer 30 mg of rice leaves to the 1.5 mL Eppendorf (EP) tube and extract with 1 mL of extraction solution, and add ribitol 0.02 mL as an internal quantitative standard. The sample was ground in a grinder at 45 Hz for 4 min, ice water bath was ultrasonic for 5 min, and the repeat was 3 times. Then centrifuged for 15 min at 12,000 rpm, at 4℃. Transfer 200 μL of supernatant into a 1.5 mL EP tube, add 40 μL methoxyamine hydrochloride, and dry under vacuum at 80°C for 2 h, add 60 μL of the BSTFA regent (1% TMCS, v/v) to the sample aliquots, incubated for 1.5 h at 70°C. All samples were used for GC-MS analysis.

Raw data were pre-processed, identification, data baseline filtering, and integration using Chroma TOF software (V4.3x, LECO). Metabolites were identified using the LECO-Fiehn Rtx5 database (Kind et al., 2009). Both mass spectral matching and retention index matching were considered for metabolite identification. Remove peaks detected in QC < 50% or RSD > 30% in QC samples (Dunn et al., 2011). Metabolite data were analyzed using SMICA 14.1 software, including principal component analysis (PCA), orthogonal partial least-squares discrimination analysis (OPLS-DA), and partial least-squares discriminant analysis (PLS-DA). Subsequently, Metabolites were projected to the MetaboAnalyst 6.0 (http://www.genome.jp/keg) websites and KEGG (http://www.genome.jp/keg) database to identify the metabolic pathways involved.

Statistical analysis

Two-tailed Student's t-test analyzed significant differences in growth parameters between CK and E+. Statistical analysis was performed using SPSS 23 software. All data analyses were calculated as the mean ± standard deviation (SD) of three biological replicates.

Table 1. *List of specific primers for qRT-PCR*

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
TubA	TCGCAGCATCAACCCAATC	GCAACCAGTCCTCACCTCAT
OsSUS4 (SS)	TCGAAACTCCCAGCTGACAC	AGCAATCTCACCAGCAGCAT
OsSPS11 (SPS)	CCCGAAGAAGAACGTCACCA	AGGAGAAGCGGTGGATGTTG
OsINV2 (INV)	ATGCTGGACACGAAGACTGG	CACACGCGAGGTAATGCATG
OsHXK1 (HXK)	CCACTGGGAGAGAGGATGGA	TCCCCTGAATTGGGCAACTC

Results

Changes in growth performance

In this experiment, plant height, root length, above-ground dry weight and under-ground dry weight of rice seedlings with endophyte infection significantly changed, and plant height was significantly higher compared with CK group (*Fig. 1*). In contrast, the main roots of E+ rice were significantly shorter but the number of lateral roots was

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significantly increased. Plant height, above-ground dry weight and under-ground dry weight significantly increased by 0.12-fold ($p < 0.01$), 0.06-fold ($p < 0.05$) and 0.10-fold ($p < 0.01$), root length decreased by 0.41-fold ($p < 0.01$) under endophyte infection as compared to control group (*Table 2*).

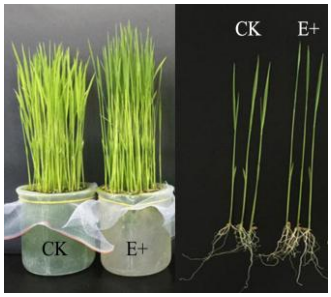

Figure 1. *Plant height and root length of endophyte-infected and -uninfected rice seedlings*

Table 2. *Effect of endophyte infection on growth parameters*

Growth parameters	CK	E+	FC
Plant height (cm)	21.48 ± 1.40	23.27 ± 1.85	0.12**
Root length (cm)	11.47 ± 1.60	8.64 ± 0.90	-0.41**
Above-ground dry weight (g/10 plant)	0.97 ± 0.02	1.11 ± 0.07	0.06*
Under-ground dry weight (g/10 plant)	0.38 ± 0.03	0.47 ± 0.00	0.10**

The data were mean ± SD of the three biological replicates. * and ** indicate significant ($P < 0.05$) and extremely significant ($P < 0.01$), respectively. FC, fold change that the change of growth parameters is represented by $\log_2^{(E+/CK)}$

Changes in physiological parameters

Soluble sugar contents, SS, SPS, HXK, and INV activities of rice seedlings under endophyte infection were increased by 0.71-fold ($p < 0.01$), 0.12-fold ($p < 0.05$), 0.43-fold ($p < 0.05$), 0.1-fold ($p < 0.01$), and 0.6-fold ($p < 0.01$), respectively, as compared to the CK (*Fig. 2*).

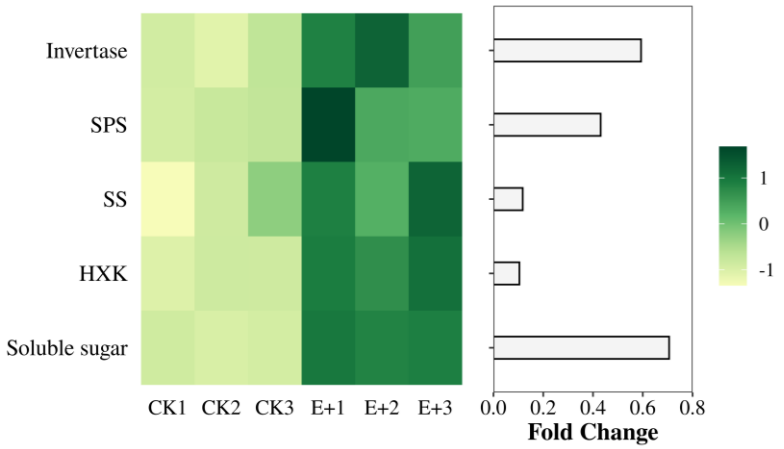

Figure 2. *Heat map of physiological parameters in endophyte-uninfected (CK) vs -infected (E+) rice seedlings*

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Sucrose metabolism-related gene expression

To definitively study the effects of endophyte infection on rice leaves, sucrose metabolism-related gene expression in rice leaves was analyzed. The results showed that relative expression of genes encoding SS, SPS, INV, and HXK increased 0.23-fold, 0.92-fold ($p < 0.01$), 0.78-fold ($p < 0.01$) and 0.38-fold ($p < 0.01$) under endophyte infection (*Fig. 3*).

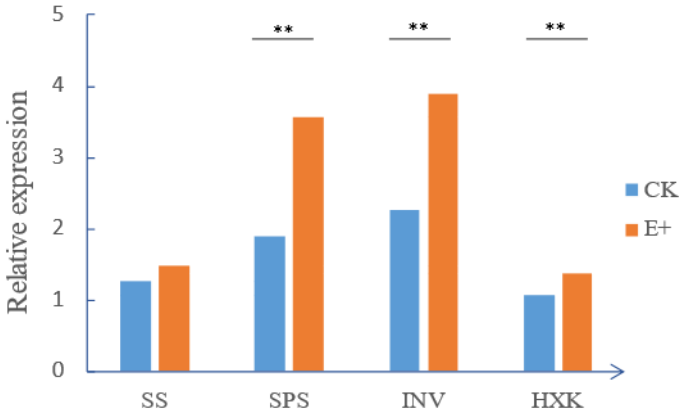

Figure 3. *Sucrose metabolism-related gene expression in endophyte-uninfected (CK) vs -infected (E+) rice leaves*

Metabolomic changes

We performed metabolomics studies on endophyte-infected and -uninfected rice seedlings based on GC-MS. PCA results showed that PC1 was 28.4%, mainly reflecting the significant differences in metabolomics between CK and E+ group. PC2 was 18.4%, indicating that the results were reliable (*Fig. 4A*). The PLS-DA showed that the main contributors to PC1 were fructose, citric acid, sedoheptulose, and pyroglutamic acid. The main contributors to PC2 were oxalic acid, L-serine, L-malic acid, mannose, quinic acid, and 2-Deoxy-D-galactose (*Fig. 4B*). Differential accumulation metabolites (DAMs) were screened for $P < 0.05$, similarity > 500, and VIP > 1. Eventually, a total of 36 DAMs were screened from the metabolites: 6 glycolysis and tricarboxylic acid cycle, 9 amino acid metabolites, 10 carbohydrate and sugar alcohol metabolites, 4 organic acid metabolites, 2 fatty acid metabolites, and 8 organic compound metabolites (*Table 3*).

Metabolic profiles in response to endophyte

Metabolite changes in endophyte-infected and -uninfected rice seedlings were analyzed using GC-MS, resulting in 36 metabolites with significant differences, including 24 up-regulated metabolites and 12 down-regulated metabolites (*Fig. 5*). The key metabolites of the glycolysis pathway glucose-6-phosphate (G-6-p) and fructose-6-phosphate (F-6-p) increased 0.68-fold and 0.71-fold in E+ group. In E+ group malate acid and succinate significantly increased by 0.73-fold and 0.99-fold, respectively, and citrate and aconitate significantly decreased by 0.58-fold and 0.85-fold. The results showed that glycolysis and TCA cycle were enhanced in rice seedlings with endophyte infection. Organic acid and fatty acid metabolites were significantly accumulated in E+

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rice. Compared with CK group, isomaltose, maltose and sucrose contents in carbohydrate and sugar alcohol metabolites in E+group were significantly decreased by 1.15-fold, 1.17-fold, and 1.22-fold, and fructose, sedoheptulose, raffinose, galactitol, gluconic acid and mannose were significantly accumulated with 0.82-fold, 2.72-fold, 0.4-fold, 1.25-fold, 1.39-fold and 0.51-fold. Among the amino acid metabolites, lysine, glycine, tyrosine, asparagine, and 5-aminovaleric acid were significantly increased by 2.20-fold, 1.08-fold, 1.14-fold, 0.9-fold, and 2.70-fold, respectively, and oxoproline, glutamate, 3-aminoisobutyric acid, and aspartate were significantly decreased by 0.85-fold, 1.13-fold, 0.6-fold, and 0.43-fold, respectively. The top 5 up- and down-regulated metabolites are presented in *Figure 6*. The main components are carbohydrates and amino acids.

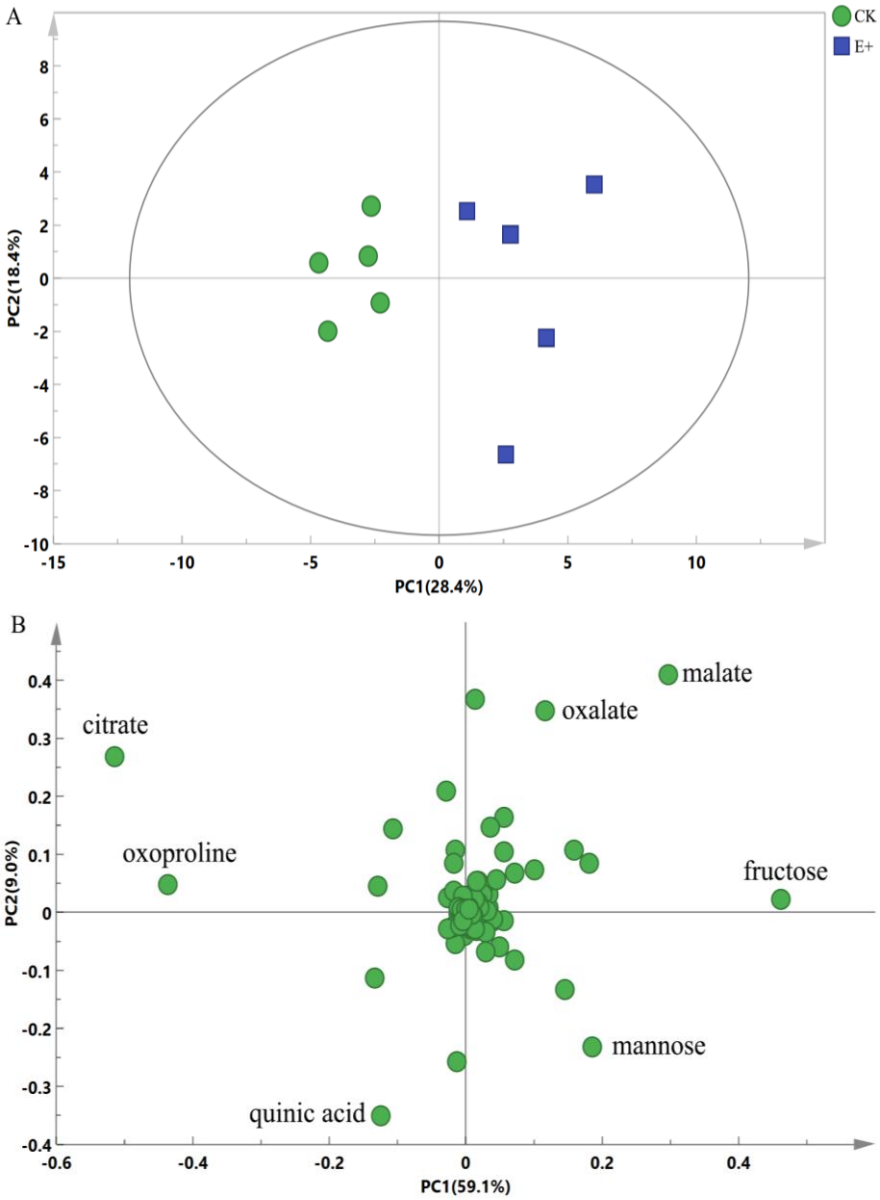

Figure 4. *Principal component analysis (PCA) of metabolite profiles in endophyte-uninfected (CK) vs -infected (E+) rice seedlings (A). The loading of metabolites to the PC1 and PC2 (B). CK: control group; E+: endophyte-infected group. PC1: the first principal component; PC2: the second principal component*

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Table 3. *Difference of metabolite profiles in endophyte-uninfected (CK) vs -infected (E+) rice seedlings*

Metabolite name		CK	E+	FC
Glycolysis and TCA cycle	Malate	164.96	274.43	0.73**
	Citrate	992.79	662.08	-0.58**
	Aconitate	0.27	0.15	-0.85**
	Fructose-6-phosphate	2.60	4.24	0.71**
	Glucose-6-phosphate	0.95	1.52	0.68**
	Succinate	36.63	72.75	0.99*
Organic acid	Mucic acid	0.49	2.13	2.12**
	4-hydroxycinnamic acid	0.11	0.36	1.70**
	Glutaric acid	0.00	0.10	5.39**
	Oxalate	517.36	552.53	0.09
Amino acid	Oxoproline	522.02	289.49	-0.85**
	Lysine	173.61	8.00	2.20**
	Glutamate	39.70	18.13	-1.13**
	3-Aminoisobutanoic acid	0.52	0.34	-0.60**
	Glycine	6.44	13.60	1.08**
	Aspartate	100.49	74.47	-0.43**
	Tyrosine	3.90	8.62	1.14**
	Asparagine	3.72	6.92	0.90*
	5-Aminovaleric acid	0.06	0.40	2.70*
Carbohydrate and sugar alcohol	Quinic acid	259.17	217.06	-0.26
	Fructose	334.52	589.41	0.82**
	Sedoheptulose	7.11	46.99	2.72**
	Isomaltose	0.87	0.39	-1.15**
	Raffinose	6.78	8.92	0.40**
	Sucrose	29.27	12.60	-1.22**
	Galactitol	3.86	9.24	1.25*
	Maltose	1.04	0.46	-1.17*
	Gluconic acid	0.27	0.70	1.39*
	Mannose	146.51	209.32	0.51
Fatty acid	Stearic acid	31.45	45.47	0.53**
	Palmitic acid	46.98	86.76	0.89*
Organic compound	Gentisin	0.15	0.06	-1.20*
	Inosine	0.00	0.03	4.68*
	Sphinganine	0.62	0.78	0.33*
	O-phosphorylethanolamine	1.06	0.69	-0.62**
	Putrescine	1.83	2.72	0.57**

Relative concentration values were increased 100 times. The * and ** indicate significant ($P < 0.05$) and extremely significant ($P < 0.01$), respectively. FC, fold change that relative concentrations is represented by $\log_2^{(E+/CK)}$

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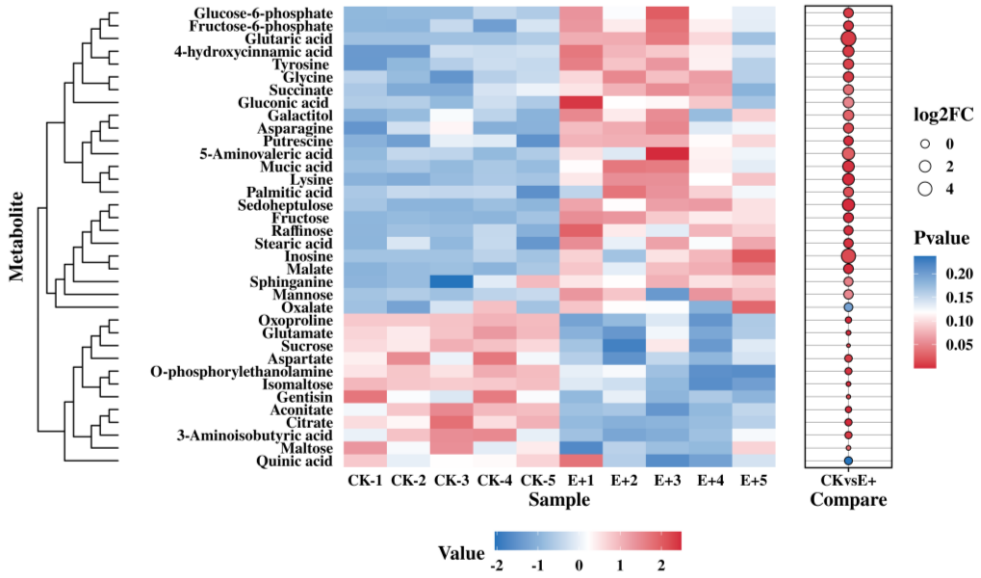

Figure 5. *Clustered heat map revealing DAMs in endophyte-uninfected (CK) vs -infected (E+) rice seedlings*

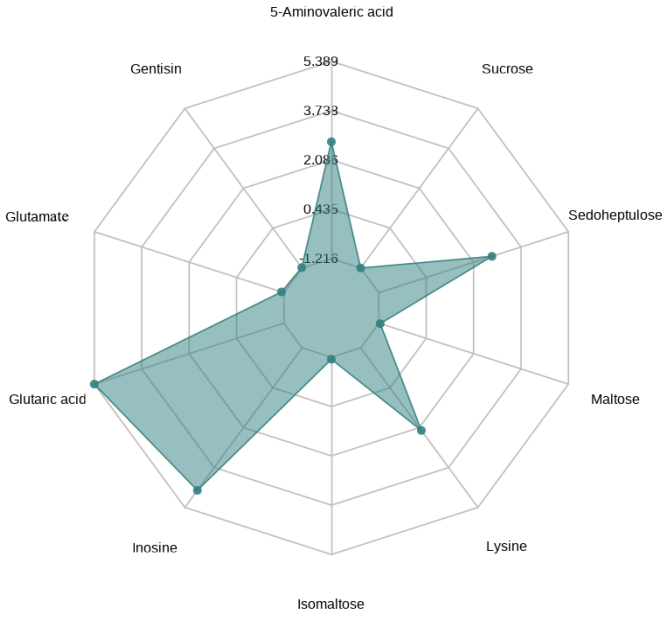

Figure 6. *Top 5 up-regulated and down-regulated DAMs in endophyte-uninfected (CK) vs -infected (E+) rice seedlings*

Metabolic pathway analysis

The MetaboAnalyst website was used to analyze the pathway enrichment between CK and E+ group. The results showed that 36 DAMs were enriched in 44 metabolic pathways. The top 20 metabolic pathways mainly involved glyoxylate and dicarboxylate metabolism, starch and sucrose metabolism, citrate cycle, and so on (*Fig. 7*). Based on $P < 0.05$ and Impact > 0.1, we obtained 8 significantly enriched

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metabolic pathways, including glyoxylate and dicarboxylate metabolism, starch and sucrose metabolism, citrate cycle, galactose metabolism, fructose and mannose metabolism pentose phosphate pathway and alanine, aspartate and glutamate metabolism (*Fig. 8*). According to the enrichment results, 8 metabolic pathways were mainly enriched in carbohydrate metabolism, amino acid metabolism and energy metabolism pathways.

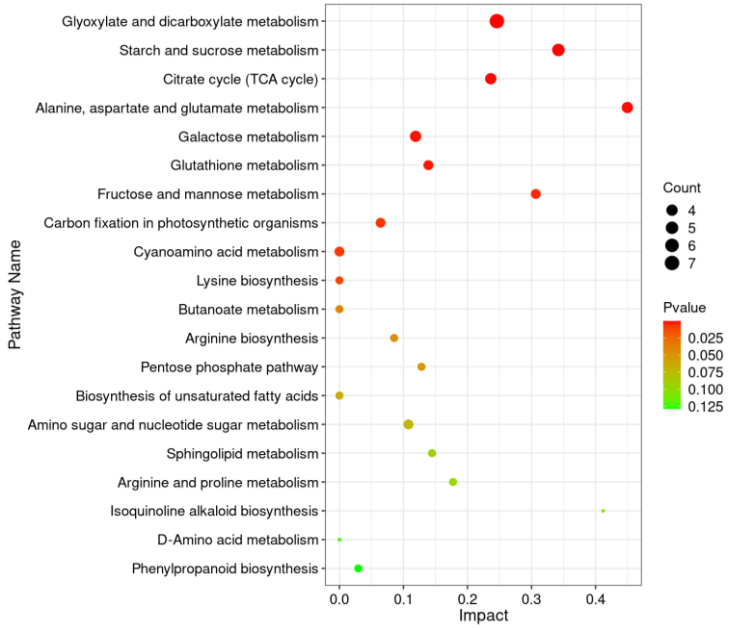

Figure 7. *TOP 20 pathway enrichment of DAMs in endophyte-uninfected (CK) vs -infected (E+) rice seedlings*

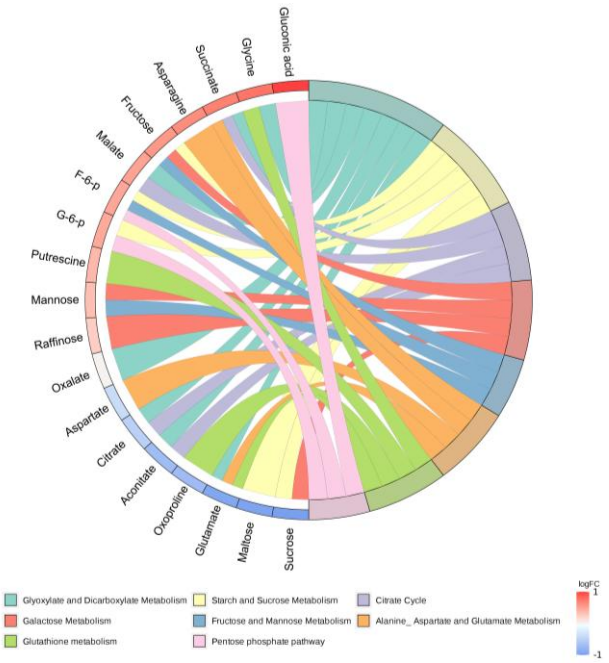

Figure 8. *KEGG pathway extremely enrichment of DAMs in endophyte-uninfected (CK) vs -infected (E+) rice seedlings*

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Discussion

Endophytes can alter the physiological responses of plants and have either harmful or beneficial effects on their hosts. In addition, endophytes have a variety of mechanisms for regulating plant growth, including phytostimulation, biocontrol, and biofertilization, and they can regulate plant growth by directly synthesizing or promoting the synthesis of growth hormones, gibberellins, and cytokinins in the host plant (Faeth and Sullivan, 2003; Shi et al., 2009; Hassan, 2017). This demonstrates the diversity of interactions between endophyte and host plants, and this plant-endophyte interaction has been described as a continuum from antagonism to mutual benefit (Eid et al., 2019; Hashim et al., 2020). In this study, endophyte infection increased plant height, aboveground dry weight, and underground dry weight of rice seedlings, but decreased root length. Shukla et al. (2012) reported that the inoculation of endophytic fungus *T. harzianum* can significantly increase rice plant height and above-ground dry weight. In contrast, root length reduction in rice was attributed to the endophytes changing the rhizosphere microenvironment, thereby promoting the growth of lateral and adventitious roots, so longer roots are not needed to absorb water (Ban et al., 2017). In addition, mycorrhizae of endophytic fungi can form extensive mycelial networks that enhance the plant's ability to probe for water and mineral nutrients, which greatly increase the range of plant root uptake (Li et al., 2013). Therefore, endophytes play an important role in plant growth-promoting (PGP).

Metabolomics methods are widely used in plant science to assess the metabolite content of individual plant species (Zhang et al., 2022). Metabolomics-based studies can provide a comprehensive understanding of the molecular responses of rice with endophyte infection. In this study, GC-MS metabolomics was utilized to obtain 36 DAMs. By KEGG analysis, 8 metabolism pathways were significantly enriched. The main differentially accumulated metabolites enriched included: amino acids, carbohydrates, glycolysis and TCA cycle intermediates.

Carbohydrates provide energy and carbon skeletons for plants and are important signaling molecules that regulate plant growth and development (Li et al., 2022). In plants, carbohydrate metabolism provides energy and numerous essential cofactors and substrates for other metabolic processes (Li et al., 2020). Carbohydrate metabolism pathways were significantly enriched in rice seedlings under endophyte infection, mainly including glyoxalate and dicarboxylic acid metabolism, fructose and mannose metabolism, starch and sucrose metabolism, and galactose metabolism. Five identified DAMs are involved in the carbohydrate metabolism pathway, including sucrose, fructose, maltose, mannose and raffinose. Among them, sucrose, fructose and maltose, as soluble sugars, play important roles in promoting plant growth and development, regulating osmotic homeostasis, and resisting abiotic stresses (Zhang et al., 2020). Sucrose is the main product of plant photosynthesis and provides the basic carbon skeleton and energy needed for seed formation and development (Du et al., 2020). Raffinose exists in the chloroplast matrix and can stabilize specific photosystem II and protect plants from oxidative damage (Guo et al., 2021). Mannose is a constituent of hemicellulose in plant cell walls and plays a role in the composition and morphological changes of plant cell walls (Huberman et al., 2021). It has been found that colonization of endophytes also leads to an increase in the expression of extensions, which strengthens the plant cell wall and improves its defense against the outside world (Chlebek et al., 2020). In the present study, carbohydrate metabolite relative concentrations up-regulate expression, and among them, sucrose and maltose were

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down-regulated, as well as fructose, raffinose, and mannose were up-regulated. During nutrient transport, sucrose in rice seedlings is hydrolyzed to fructose and glucose at the growth site for rice growth and development. In this study, we found that SPS, SS, and INV activities and soluble sugar contents were significantly elevated in rice with endophyte infection. SPS mainly catalyzes the formation of sucrose phosphate by the combination of uridine diphosphate glucose and fructose 6-phosphate, while SS and INV are the main enzymes in the process of sucrose catabolism and they hydrolyze sucrose into fructose and glucose (Wang et al., 2019; Duan et al., 2021). Qin et al. (2022) showed that sugarcane inoculated with endophyte DX120E promotes carbohydrate accumulation in leaves, and the SS and SPS activities and soluble sugar contents were significantly higher than the control group. This suggests that rice infected with endophyte can promote the related carbohydrate metabolism pathway and provide sufficient material basis for growth and development.

Glycolysis, TCA cycle and pentose phosphate pathway (PPP) are significant energy metabolic pathways in plants, providing energy and carbon skeletons for other metabolic pathways (Xin et al., 2019). TCA cycle and PPP are significantly enriched in E+ rice. The main DAMs involved in TCA cycle include citrate, malate, succinate, and aconitate, in which citrate and aconitate were down-regulated and succinate and malate were up-regulated. Li et al. (2019) reported that endophyte significantly increased succinic acid content in peanut root tips compared to the control. This suggests that endophyte infection promoted citrate and aconitate converting to succinate and malate, which enhanced TCA cycle and provided energy for E+ group rice. PPP was the main pathway for reducing power production (Xin et al., 2019). KEGG pathway analysis showed a significant accumulation of gluconic acid, G-6-p, and F-6-p in PPP. Studies have found that under endophyte infection, HXK activity increases in rice, which is an essential enzyme in the glucose and fructose phosphorylation process, and catalyzes the conversion of fructose and glucose to fructose 6-phosphate and glucose 6-phosphate (Granot et al., 2014). Wang et al. (2023) found that PPP was promoted in maize inoculated with dark septate endophytes. This indicated that PPP in E+ rice was enhanced, and thus produced more reducing power.

Amino acids are the main macromolecular materials for building organisms and play important roles in plants, including the maintenance of cellular osmotic homeostasis, structural integrity of proteins, and the improvement of plant stress tolerance (Wang et al., 2024). Amino acid metabolism is closely related to energy and carbohydrate metabolism, protein synthesis and secondary metabolism (Hu et al., 2021). Glutathione metabolism and alanine, aspartate and glutamate metabolism were significantly enriched in endophyte-infected rice, which differential metabolites included glycine, asparagine, aspartic acid, pyroglutamic acid and glutamic acid. Glycine is an indispensable amino acid in plants, which participates in the synthesis of glutathione in plants, promotes plant phosphorus uptake, scavenges reactive oxygen species (ROS), maintains cellular homeostasis, improves plant stress tolerance, and promotes plant growth (Yang et al., 2019; Gahir et al., 2021). Jha and Mohamed (2023) showed that maize inoculated with *Lysinibacillus fusiformis* strain YJ4 and *Lysinibacillus sphaericus* strain YJ5 resulted in a significant accumulation of glycine. Asparagine is an α-amino acid, and one of the most important amino acids for the long-distance transportation of plant nitrogen (N), mainly found in rice xylem and phloem sap (Luo et al., 2018). Aspartate is a common nitrogen carrier in plants and it plays a major role in nitrogen cycling, storage, and transportation in germinating seeds, nutrient organs and senescent

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organs. In addition, it participates in the synthesis of other amino acids such as asparagine and arginine (Lam et al., 1994; Gaufichon et al., 2016). Glutamate is a precursor substance for the synthesis of aspartate, asparagine, and other essential amino acids in plants (Xie et al., 2020). Oxoproline as a circulating amino acid, is a common precursor substance for the synthesis of glutamate and proline (Merewitz et al., 2012). In the present study, asparagine was found to be up-regulated while aspartate, glutamate and pyroglutamate were down-regulated. It indicates that aspartate, glutamate and pyroglutamate are converted to asparagine under endophyte infection, thus resulting in significant accumulation of asparagine. Hodge et al. (2001) found that inoculation with AMF promoted asparagine uptake in plants. It suggests that endophyte infection improved the efficiency of amino acid translocation and reutilization in rice seedlings.

So, it was found that endophytes affected carbohydrate, amino acid, and energy metabolism and improved rice seedling growth. A total of 36 DAMs and 8 significantly enriched metabolic pathways identified based on GC-MS analysis and KEGG enrichment analysis can be used as markers to study the effects of endophyte on rice seedlings and contribute to the understanding of potential molecular mechanisms of endophyte-plant interactions.

Conclusion

The present study emphasized the growth-promoting effect of infection with endophyte EF0801 on rice seedlings. Changes in metabolites of E+ rice seedlings were identified based on GC-MS analysis. The results showed that 36 metabolites were significantly altered and significantly enriched in carbohydrate metabolism, amino acid metabolism, and energy metabolism pathways, with fructose and mannose metabolism and pentose phosphate pathways being the most significantly up-regulated. It was found that the infection of endophyte EF0801 affected the intricate chemical exchanges of carbon, nitrogen, and sugar networks in rice seedlings, which further promoted growth and development. This study deepens our understanding of endophytes and provides new insights into the promotion of rice growth and development through endophyte infection.

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