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Effects of phosphorus-solubilizing bacteria and biochar application on phosphorus availability and tomato growth under phosphorus stress

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Abstract

Background Phosphorus-solubilizing bacteria (PSB) are vital in converting insoluble phosphorus into a soluble form that plants can readily absorb and utilize in soil. While previous studies have mainly focused on the extracellular secretion of microorganisms, few have explored the intricate intracellular metabolic processes involved in PSB-mediated phosphorus solubilization.

Results Here, we uncovered that $Ca_3(PO_4)_2$ could serve as a source of insoluble phosphorus for the PSB, *Pseudomonas* sp. NK2. High-performance liquid chromatography (HPLC) results indicated higher levels of organic acids released from insoluble phosphorus compared to a soluble phosphorus source (KH_2PO_4), with acetic acid released exclusively under insoluble phosphorus condition. Moreover, non-target metabolomics was employed to delve into the intracellular metabolic profile. It unveiled that insoluble phosphorus significantly enhanced the tricarboxylic acid cycle, glycolysis, glyoxylic acid metabolism, and other pathways, leading to the production of acetic acid, gluconic acid, oxalic acid, and citric acid for insoluble phosphorus solubilization. In our quest to identify suitable biochar carriers, we assessed seven types of biochar through the conjoint analysis of NBRIP medium culture and application to soil for 30 days, with cotton straw-immobilized NK2 emerging as the most potent phosphorus content provider. Lastly, NK2 after cotton straw immobilization demonstrated the ability to enhance biomass, plant height, and root development of *Solanum lycopersicum* L. cv. Micro Tom.

Conclusions *Pseudomonas* sp. NK2 with cotton straw biochar could enhance phosphorus availability and tomato growth. These findings bear significant implications for the practical application of phosphorus-solubilizing bacteria in agricultural production and the promotion of environmentally sustainable farming practices.

Keywords Phosphorus-solubilizing bacteria, Biochar, Organic acid, Non-target metabolomics, Tomato growth

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Background

Since phosphorus is a restrictive and non-renewable resource, how to use phosphorus efficiently is of great importance [1]. Total phosphorus content of soil is 0.02–0.2% which is mainly divided into inorganic phosphorus and organic phosphorus, and microbial activity is a key factor in redistributing phosphorus into different forms in the soil [2]. The majority of phosphorus is found in the soil in the form of insoluble phosphorus compounds, rendering it inaccessible for direct utilization by plants. Consequently, over 74% of arable soil in China and more than 40% of arable soil worldwide face shortages of available phosphorus [3–5]. The lack of available phosphorus in soil has become a key factor restricting crop production, leading to a substantial rise in the demand for chemical phosphate fertilizers [6]. However, the utilization rate of chemical phosphate fertilizer is as low as 10–25% [7]. This inefficiency arises from the tendency of most released phosphorus to immediately combine with or precipitate into insoluble forms, such as aluminum and iron phosphate in acidic soil and calcium phosphate in alkaline soil. This renders the phosphorus inaccessible for plant absorption [8]. Excessive application of phosphate fertilizer not only does not improve the state of soil phosphorus deficiency but also causes problems such as soil compaction, soil nutrient imbalance, heavy metal accumulation, water eutrophication, environmental pollution, etc. [9–11].

As an inherent and potent biological resource in nature, phosphate-solubilizing microorganisms (PSMs) represent a subset of plant rhizosphere growth-promoting microorganisms, playing a vital role in the soil phosphorus cycle [12]. PSMs have the ability to release organic ions or protons, converting insoluble phosphorus into a soluble form. They create microenvironments with ample phosphorus supply in the plant rhizosphere or soil, thereby enhancing the efficiency of phosphorus resource utilization [13]. Among PSMs, phosphorus-solubilizing bacteria (PSB) make up 50% [14–16], with *Pseudomonas* and *Bacillus* identified as the primary genera [17].

Since 1950, phosphorus-solubilizing bacteria (PSB) have served as biofertilizers, fostering plant growth and development by converting insoluble phosphate in the soil into a utilizable form [18]. However, challenges arise from the intricate composition of soil and the competition and antagonism posed by indigenous microorganisms. When applied to soil alone, PSB may struggle to colonize and thrive due to these factors [19], impeding the widespread adoption of PSB in sustainable agriculture. To address this, the use of suitable carrier materials becomes imperative. These materials provide a protective environment for the inoculated strains, enhancing their survival rate in soil and enabling them to effectively carry out their growth-promoting biological functions in the field [20].

The application of biochar in agriculture has garnered widespread attention from researchers and emerged as a research hotspot in recent years. Biochar is a solid product resulting from the high-temperature pyrolysis of organic materials, such as agricultural and forestry wastes, organic matter, and crop residues, under conditions of limited or no oxygen [21]. Notably, biochar is non-toxic, cost-effective, environmentally friendly, and easy to produce. Its inherent porosity and adsorption capacity create a conducive microenvironment for the growth and colonization of beneficial bacteria. This feature holds significant importance in enhancing the survival rate of PSB and promoting their phosphorus solubilization performance [22]. Besides, biochar itself can enhance plant-available phosphorus in agricultural soil [23].

While various mechanisms of phosphorus solubilization by PSB have been investigated, many current studies on phosphorus-solubilizing mechanisms predominantly concentrated on the extracellular secretion of PSB. Intracellular metabolic pathways of PSB have received comparatively less attention. Our previous data showed that *Pseudomonas* sp. NK2 could dissolve insoluble phosphorus [24]. In this study, a combination of HPLC and non-targeted metabolomics techniques was employed to analyze both the extracellular acid production and intracellular metabolic pathways of the phosphorus-solubilizing bacterium *Pseudomonas* sp. NK2 under phosphorus stress. The efficacy of seven different biochar as carriers for NK2 was assessed, and ultimately, the growth-promoting effect of appropriately immobilized bacteria on dwarf tomato of *Solanum lycopersicum* L. cv. Micro Tom, a model plant species, was verified. This study will provide a basis for enhancing soil phosphorus utilization efficiency and promote the development of environmentally friendly agriculture.

Results

Pseudomonas sp. NK2 had the ability to solubilize insoluble phosphorus $Ca_3(PO_4)_2$

Over a 5-day period, the available phosphorus content and pH values in the fermentation broth containing insoluble phosphorus sources, namely $Ca_3(PO_4)_2$, $AlPO_4$, and $FePO_4$, were measured every 24 h. The results revealed that the available phosphorus content and pH values in the $FePO_4$ and $AlPO_4$ treatment groups did not significantly differ from the CK control group (Fig. 1A and B). This suggested that *Pseudomonas* sp. NK2, a phosphorus-solubilizing bacterium, exhibited limited capacity to dissolve the insoluble phosphorus sources $AlPO_4$ and $FePO_4$.

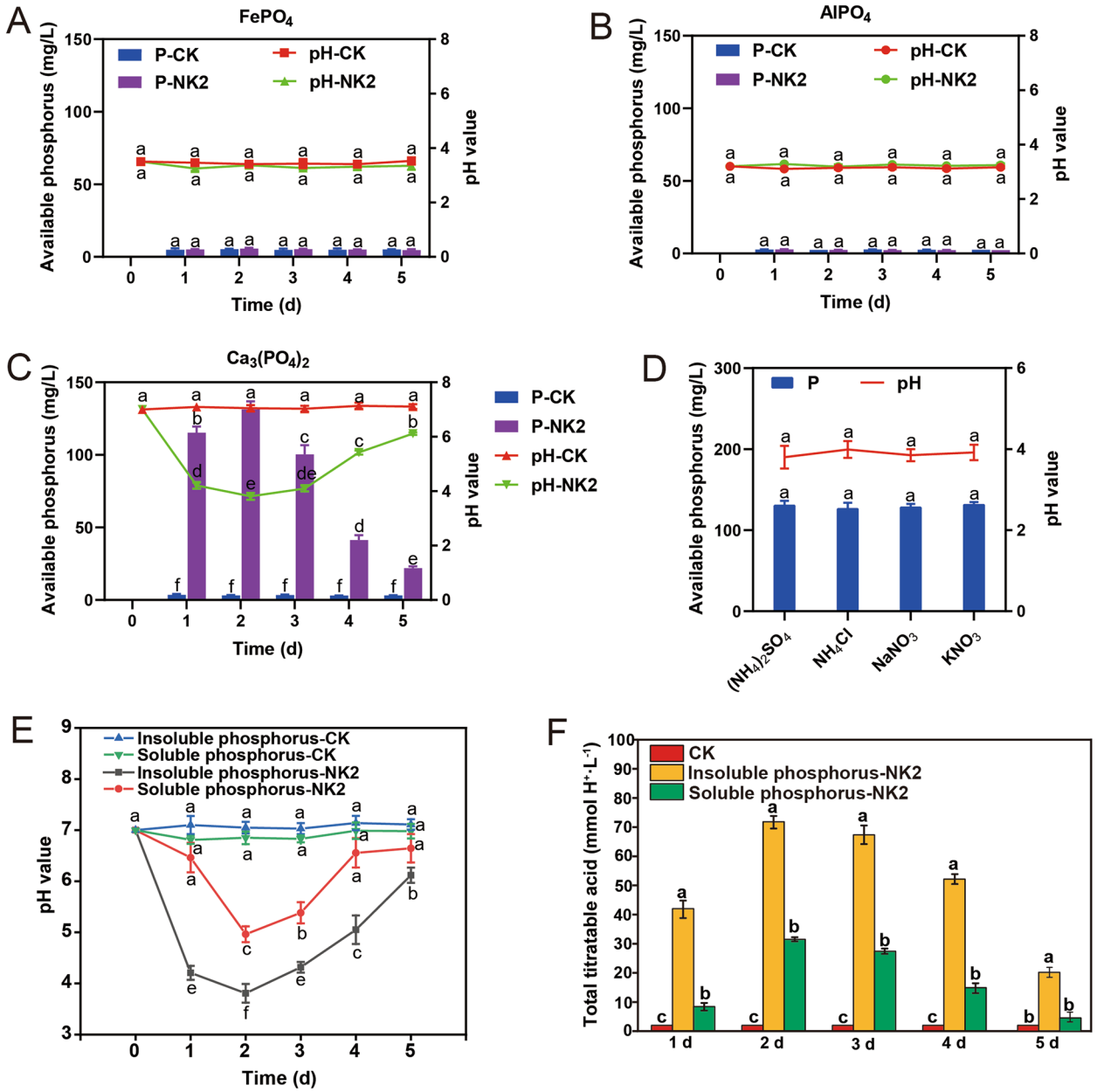

Fig. 1 Phosphorus-solubilizing bacterium, *Pseudomonas* sp. NK2, effectively solubilized phosphorus by releasing organic acids to lower pH. The available phosphorus content and pH values of NK2 in insoluble phosphorus sources $FePO_4$ (**A**), $AlPO_4$ (**B**), and $Ca_3(PO_4)_2$ (**C**) were measured at day 0, day 1, day 2, day 3, day 4, and day 5. **D** The available phosphorus content and pH values of NK2 in $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, and KNO_3 solutions were determined after 2 days of culture. **E** The pH values of CK (uninoculated bacteria) and NK2 were monitored under soluble phosphorus (KH_2PO_4) and insoluble phosphorus ($Ca_3(PO_4)_2$) conditions for 0, 1, 2, 3, 4, and 5 days. Bars correspond to one standard deviation (SD) from the mean ($n = 3$), while different letters above columns indicate significant differences ($P < 0.05$) among samples at different time points for available phosphorus contents and pH values according to a one-way ANOVA in conjunction with Tukey's multiple-comparison test. **F** The total titratable acids in NK2 fermentation broth under soluble phosphorus and insoluble phosphorus culture were quantified for 1, 2, 3, 4, and 5 days. CK designates the control with NK2 inoculated into LB medium. P represents available phosphorus contents and pH indicates pH values. Bars correspond to one standard deviation (SD) from the mean ($n = 3$), while different letters above columns indicate significant differences ($P< 0.05$) among samples at the same time points under different treatments according to a one-way ANOVA in conjunction with Tukey's multiple-comparison test

In contrast, the $Ca_3(PO_4)_2$ treatment group demonstrated a noteworthy increase in available phosphorus content, reaching a maximum of 131.45 mg/L on day 2, accompanied by a concurrent decrease in pH (Fig. 1C). These results indicated that NK2 displayed a robust ability to dissolve $Ca_3(PO_4)_2$. Consequently, $Ca_3(PO_4)_2$ was selected as the insoluble phosphorus for further exploration of the phosphorus-solubilizing mechanism of NK2.

Pseudomonas sp. NK2 solubilized phosphate through the release of organic acids

It has been previously reported that the production of organic acids and the assimilation of NH_4^+ both generate hydrogen ions, resulting in a decline in pH value [25]. Here, NH_4Cl, $NaNO_3$, KNO_3, and $(NH_4)_2SO_4$ were utilized as different nitrogen sources to investigate their impacts on the phosphate solubility of NK2 and the pH of the fermentation solution. After 48 h, there were no significant differences in available phosphorus content and pH among groups treated with different nitrogen source (Fig. 1D), indicating that the phosphate solubility of NK2 was unrelated to NH_4^+ assimilation.

The pH values of the supernatant from the fermentation broth of NK2, under treatment with soluble phosphorus (KH_2PO_4) and insoluble phosphorus ($Ca_3(PO_4)_2$) conditions, were monitored every 24 h for 1–5 days. During the initial 0–2 days, the pH of the insoluble phosphorus group exhibited a rapid decrease, significantly lower than that of the soluble phosphorus group (Fig. 1E). This indicated that phosphorus stress intensified the acidification of NK2, leading to a reduction in the pH of the medium.

To a certain extent, titratable acidity served as an indicator of the ability of strain to produce organic acid. The titratable acidity in the fermentation broth of strain NK2 under insoluble phosphorus and soluble phosphorus cultures was significantly higher than that of the CK strain (uninoculated bacteria), except on the fifth day (Fig. 1F). Notably, the concentrations of titratable acids secreted by NK2 in the insoluble phosphorus culture were significantly higher than those in the soluble phosphorus culture. Consequently, after accounting for the assimilation effect of NK2 on NH_4^+, it was preliminarily hypothesized that the phosphorus-solubilizing bacterium NK2 produced a substantial amount of organic acids in response to phosphorus stress, aiming to achieve the acid solution of insoluble phosphorus.

Extracellular changes in organic acids in the fermentation solution of *Pseudomonas* sp. NK2 under phosphorus stress

HPLC analysis was conducted to determine the organic acids in the NK2 fermentation broth cultured under insoluble phosphorus and soluble phosphorus conditions after 2 days. The results revealed that the types of organic acids produced by NK2 extracellularly under insoluble phosphorus condition were consistent with those under soluble phosphorus, except for acetic acid (Fig. 2A and C). This suggested that oxalic acid, gluconic acid, citric acid, and acetic acid were the primary organic acids responsible for dissolving insoluble phosphorus sources. In terms of organic acid production, NK2 significantly increased the secretion of oxalic acid (27.5%, 271.83 mg/L), gluconic acid (20%, 197.84 mg/L), citric acid (31.5%, 310.73 mg/L), and acetic acid (21%, 271.83 mg/L) under insoluble phosphorus treatment compared to soluble phosphorus condition (Fig. 2B and D). This suggested that oxalic acid, gluconic acid, citric acid, and acetic acid were the primary organic acids responsible for dissolving insoluble phosphorus sources. These results indicated variations in the composition and content of organic acids secreted by NK2 in the two conditions, implying a potential alteration in the intracellular metabolic pathway of NK2 induced by phosphorus stress.

Intracellular metabolic profiling of *Pseudomonas* sp. NK2 under phosphorus stress

The intracellular metabolic profile of *Pseudomonas* sp. NK2 under insoluble and soluble phosphorus conditions was analyzed through untargeted metabolomics, revealing the identification of 1348 metabolites. Annotation using the KEGG database showed that the lipids category exhibited the highest enrichment of metabolites (40 metabolites). Specifically, compounds of phospholipids (29 metabolites), monosaccharides (17 metabolites), carboxylic acids (16 metabolites), nucleotides (16 metabolites), and amino acids (15 metabolites) were the most abundant (Additional file 1: Fig. S1A). In the KEGG pathway, the metabolism pathway had the highest number of enriched metabolites, with amino acid metabolism, carbohydrate metabolism, and lipid metabolism-related compounds being the most prevalent (Additional file 1: Fig. S1B).

Differentially accumulated metabolites (DAMs) were identified under insoluble phosphorus condition compared to cells under soluble phosphorus condition. Cluster analysis was conducted on 416 DAMs, and the heat map results revealed distinct clustering between the soluble phosphorus and insoluble phosphorus conditions (Fig. 3A). Phosphorus stress significantly impacted the physiological activities and metabolites of NK2. Analyzing DAMs in the KEGG database, it was observed that monosaccharides (ten metabolites) in carbohydrates were the most abundant. Metabolites of carboxylic acids (five metabolites) in organic acids and fatty acids (five metabolites) in lipids were the second most prevalent (Fig. 3B). In the KEGG pathway, amino

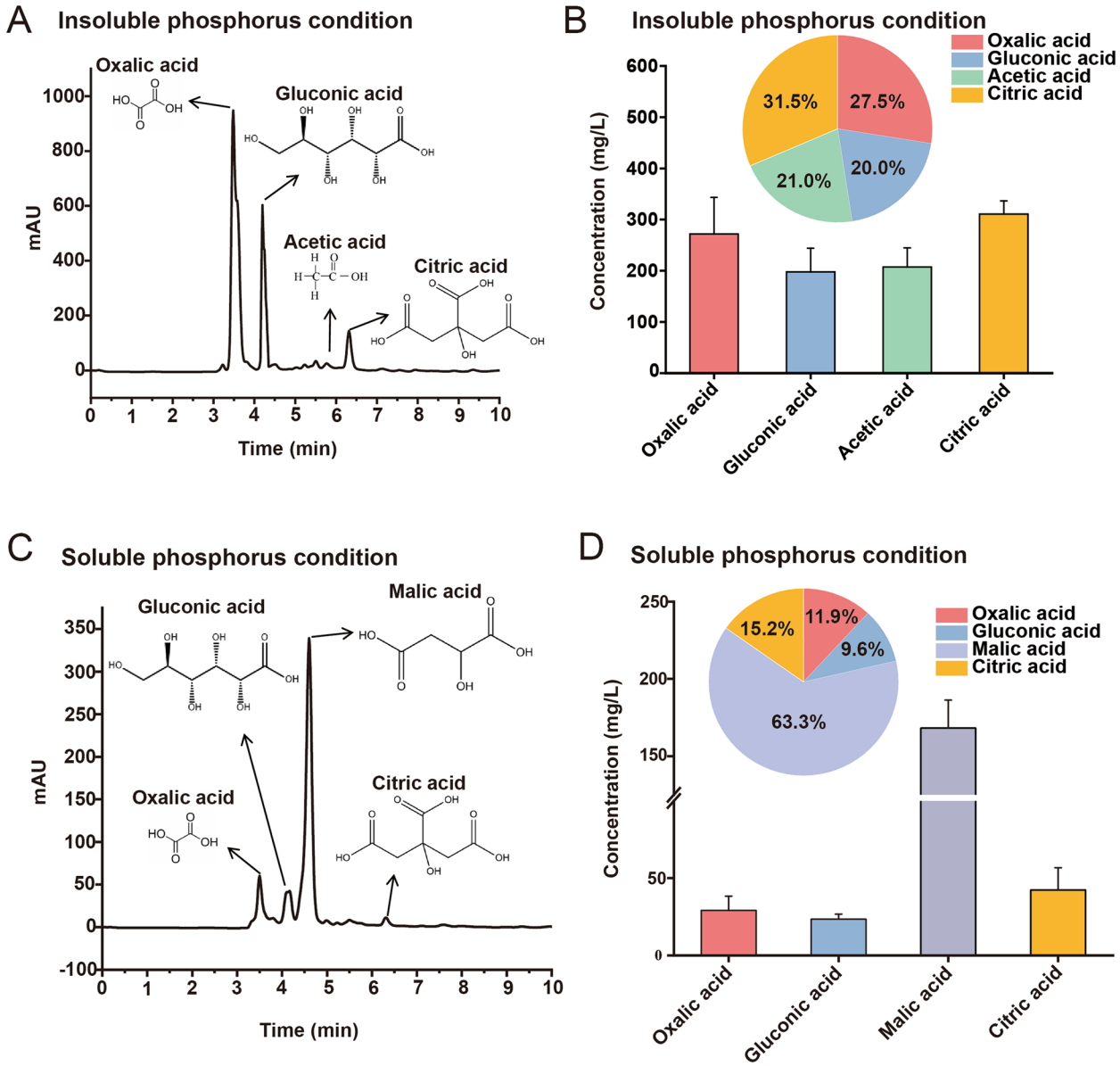

Fig. 2 Extracellular organic acids under soluble and insoluble phosphorus conditions. **A** HPLC chromatograms depict organic acids under insoluble phosphorus ($Ca_3(PO_4)_2$) condition. Arrows indicate the peak position of detected organic acids and molecular formulas are marked. **B** Types and concentrations of organic acids under insoluble phosphorus condition. Pie charts illustrate the proportion of each organic acid, while bar charts below show the concentration of organic acids. **C** HPLC chromatograms depict organic acids under soluble phosphorus (KH_2PO_4) condition. Arrows indicate the peak position of detected organic acids and molecular formulas are marked. **D** Types and concentrations of organic acids under soluble phosphorus condition. Pie charts illustrate the proportion of each organic acid, while bar charts below show the concentration of organic acids

acid metabolism and carbohydrate metabolism pathways were the most enriched (39 metabolites and 23 metabolites, respectively) (Fig. 3C). KEGG topology analysis indicated that DAMs were primarily enriched in alanine, aspartic acid and glutamate metabolism, lysine degradation and biosynthesis, amino sugar and nucleotide sugar metabolism, tricarboxylic acid cycle, glyoxalic acid and dicarboxylic acid metabolism, purine metabolism, carbohydrate metabolism, ABC transporters, and CoA biosynthesis (Fig. 3D). Analysis of 28 representative DAMs in these pathways, including carboxylic acids, amino acids, monosaccharides, and nucleotides, revealed mostly significant upregulation, particularly in carboxylic acids and amino acids (Table 1). The expressions of citrate, acetate, and oxalate, related to extracellular organic acids, were

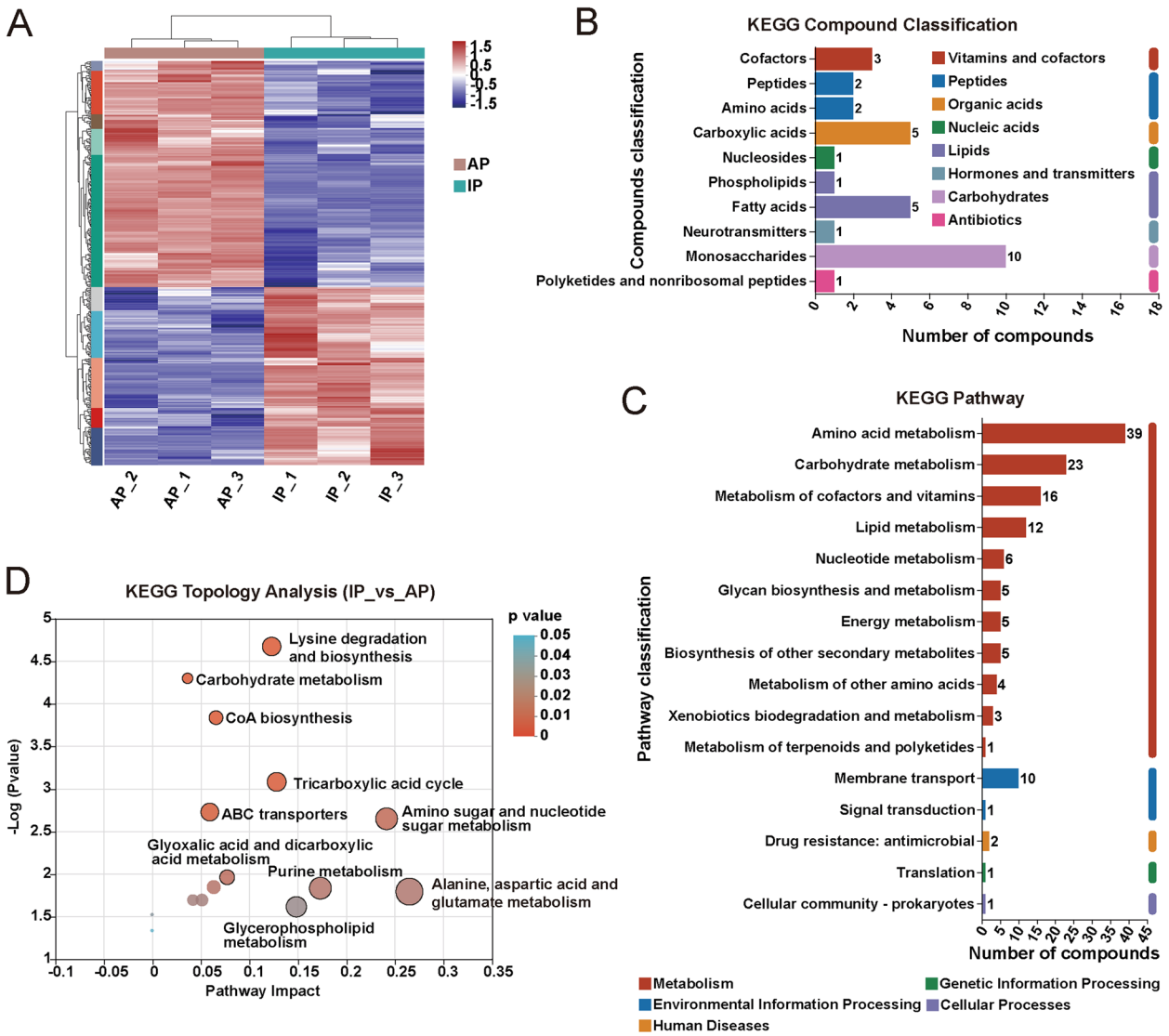

Fig. 3 Metabolic profiling of intracellular metabolites under soluble and insoluble phosphorus conditions using non-target metabolomics. **A** Heat map clustering analysis of 416 differentially accumulated metabolites (DAMs). AP and IP indicate soluble phosphorus (KH_2PO_4) and insoluble phosphorus ($Ca_3(PO_4)_2$), respectively. The numbers 1, 2, and 3 represent three different samples. **B** Enrichment of DAMs in KEGG compound classification. **C** Enrichment of DAMs in KEGG pathway. **D** KEGG topology analysis of enrichment to metabolic pathways of DAMs

upregulated, consistent with the detection of acetic acid and oxalic acid in the extracellular environment. Malate exhibited downregulated expression in the intracellular metabolism, aligning with the absence of extracellular malic acid detection under insoluble phosphorus condition.

To investigate the phosphorus-solubilizing mechanism of NK2 under phosphorus stress, non-target metabolomics was employed to study intracellular metabolism. Phosphorus stress induced the upregulation of numerous intracellular metabolites, impacting essential metabolic pathways such as the tricarboxylic acid (TCA) cycle, amino acid synthesis, and glycolysis, which were summarized in a pattern diagram (Fig. 4).

Cotton straw was proved to be the optimal biochar carrier for *Pseudomonas* sp. NK2

In order to facilitate effective isolation of NK2 in the subsequent steps, a GFP fluorescently labeled strain, NK2-GFPuv, was constructed. The available phosphorus content in the fermentation broth of the inoculated strains was monitored before and after GFP labeling, revealing no significant change (Additional file 1: Fig. S2A). This indicated that the introduction of

Table 1 The expression of representative differentially accumulated metabolites

Classification	Metabolites	Fold change (IP/AP)	Regulation
Carboxylic acids	Citrate	3.9	Up
	Succinate	3.81	Up
	Oxalate	3.5	Up
	3-Phosphoglyceric acid	3.31	Up
	Acetate	2.9	Up
	Propanoic acid	2.84	Up
	Pantothenic acid	2.71	Up
	Malate	0.18	Down
Animo acids	Glutamine	3.05	Up
	Glutamic acid	2.9	Up
	L-proline	2.37	Up
	Leucine	2	Up
	Lysine	1.98	Up
	Valyl-proline	1.91	Up
	L-Histidine	1.63	Up
Monosaccharides	Mannitol	3.1	Up
	Saccharin	3.02	Up
	6-Phosphoglucono-1,5-lactone	2.52	Up
	5-Ribulose phosphate	2.23	Up
	N-acetyl-D-glucosamine	2.2	Up
	D-Fructose	0.43	Down
	D-Glucose	0.31	Down
	D-Mannose	0.23	Down
Nucleotides	Adenosine monophosphate	3.29	Up
	Flavin adenine dinucleotide	2.59	Up
	Uracil	0.33	Down
	Adenine	0.22	Down
	Adenosine	0.21	Down

AP and IP indicate soluble phosphorus (KH_2PO_4) and insoluble phosphorus ($Ca_3(PO_4)_2$), respectively

the exogenous plasmid had no notable effect on the phosphorus solubilization ability of NK2. Organic acid secretion capacity of both strains, NK2 and NK2-GFPuv, was qualitatively and quantitatively measured by HPLC every 24 h within a 1- to 5-day period. The results showed no significant differences in the types and contents of organic acids secreted by these strains at different time points (Additional file 1: Fig. S2B, C, and D). These findings suggested that NK2-GFPuv exhibited similar biological characteristics to NK2, allowing its use as a replacement for subsequent experiments.

Numerous studies have consistently highlighted pinewood biochar as a preferable carrier for promoting the rhizosphere growth of bacteria with positive effects [26, 27]. Here, pinewood biochar was utilized as a positive control. The results revealed that PSB could be immobilized by biochar (Additional file 1: Fig. S3) and various types of biochar significantly influenced the phosphorus solubilization efficiency of PSB (Fig. 5A). There were no substantial differences observed between treatment groups using peanut shell, rice husk, and coconut shell biochar and those using pinewood biochar at different time points. The immobilized PSB exhibited low phosphorus solubilization effectiveness and a short duration of action, rendering these biochars unsuitable as carriers for NK2-GFPuv. While the maize straw treatment group showed significantly higher phosphorus solubilization efficiency than the pine biochar treatment group only on the fourth day, it exhibited a subsequent declining trend. The peak value of the wheat straw and cotton straw treatment groups at the 48th hour was significantly higher than that of the pinewood treatment group, with the cotton straw treatment group demonstrating an extremely significant superiority over other treatment groups. Despite a subsequent decrease after reaching the peak value, the phosphorus solubilization efficiency of the cotton straw treatment group remained significantly higher than that of pinewood biochar. Considering the phosphorus solubilization effect, cotton straw emerged as a more suitable immobilizing material.

In order to investigate the effect of biochar on the acid-producing capacity of phosphorus-solubilizing bacteria, considering that the available phosphorus content reached its peak at 48 h, the supernatant of all treatment groups at 48 h was taken, and the types and contents of organic acids in the supernatant were determined by HPLC. Except for the corn straw and pine straw treatment groups, the types of organic acids detected in each treatment group were the same as those in the no-carrier treatment group, which were oxalic acid, gluconic acid, acetic acid, and citric acid (Fig. 5B). The organic acids were divided into monocarboxylic acids, dicarboxylic acids, and tricarboxylic acids according to the composition and structure. The more carboxylic groups contained in the structure of each unit of organic acids, the stronger the dissolution ability of the acids. The proportion of carboxylic acids in the organic acids secreted by different treatment groups was different. Our results showed that the highest available phosphorus content in the cotton straw treatment group was significantly higher than that in other treatment groups, whereas the total organic acid concentration was significantly lower than that in the wheat straw treatment group and coconut shell treatment group. It was speculated that citric acid with a tricarboxylic acid structure accounted for 36.8%, which was higher than that of wheat straw and coconut

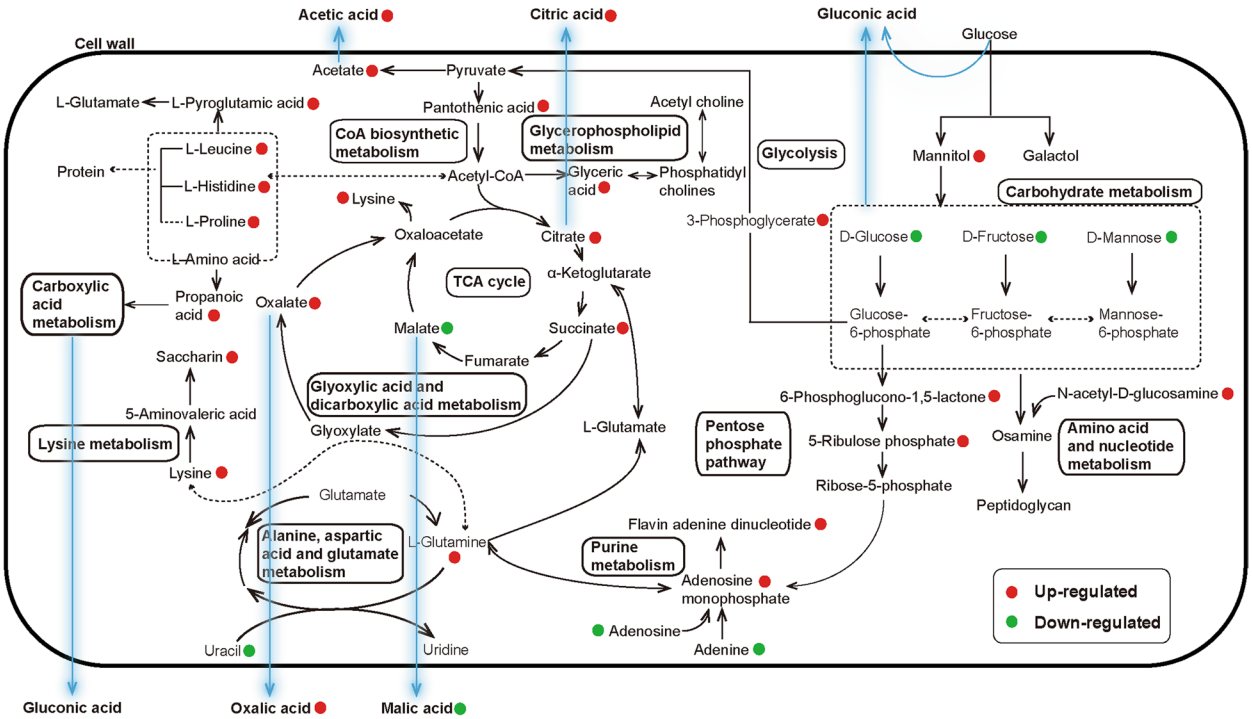

Fig. 4 Schematic of intracellular metabolite expression and model of extracellular organic acid release. Red circles represent upregulated expression, while green circles represent downregulated expression in the insoluble phosphorus condition compared to soluble phosphorus condition. Arrows indicate intracellular metabolites associated with the release of extracellular organic acids

shell. Moreover, the proportion of acetic acid with a monocarboxylic acid structure was 23.5%, which was the lowest among these treatment groups (Fig. 5B). Taken together, the cotton straw biochar treatment group had the most significant effect on the phosphorus solubilizing ability of phosphorus-solubilizing bacteria.

Different biochar carriers were used to immobilize NK2-GFPuv and applied to soil for 30 days to further test their efficacy. Bacterial population were detected for immobilized NK2-GFPuv with different biochars after 1 day and 30 days of culture in soil. The results showed that the bacterial population of cotton straw and wheat straw immobilized bacteria were higher than that of the no carrier group (Fig. 6A). After culture in soil for 30 days, the survival rate of all treatment groups significantly decreased, ranging from 87.09% to 94.00%. Compared with the no carrier group, the survival rate of the inoculant in the cotton straw, wheat straw, corn straw, and coconut husk treatment groups was significantly improved, while the survival rate in the rice husk, peanut husk, and pine treatment groups was not significantly different (Fig. 6B). According to the bacterial population and survival rate after 30 days, phosphorus-solubilizing bacteria immobilized by cotton straw biochar had the best colonization effect in soil.

The phosphorus solubilizing ability of different kinds of immobilized phosphorus-solubilizing bacteria in biochar-treated soil was assessed. Our results revealed that

(See figure on next page.)

Fig. 5 Available phosphorus content and extracellular organic acid detection of different biochars immobilizing phosphorus-solubilizing bacteria *Pseudomonas* sp. NK2-GFPuv in NBRIP medium. **A** The available phosphorus content of immobilized NK2-GFPuv by no carrier, pinewood, cotton straw, wheat straw, maize straw, coconut shell, rice husk, and peanut shell in the culture of 0 h, 12 h, 24 h, 36 h, 48 h, 72 h, 96 h, and 120 h. No carrier indicates that NK2-GFPuv is unimmobilized by any biochars. Bars indicate one standard deviation (SD) from the mean ($n = 3$), while asterisks indicate significant differences ($P < 0.05$) derived from a two-way ANOVA in conjunction with Dunnett's multiple-comparison test in comparison with the available phosphorus content of different biochar treatments at the same time points. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; no asterisks indicate no significant differences. **B** Types and concentrations of organic acids and the proportion of carboxylic acids at 48 h. The bottom bars show the type and concentration of extracellular organic acids, while the top pie chart illustrates the proportion of monocarboxylic, dicarboxylic, and tricarboxylic acids in organic acids. No carrier indicates that NK2-GFPuv is unimmobilized by any biochars

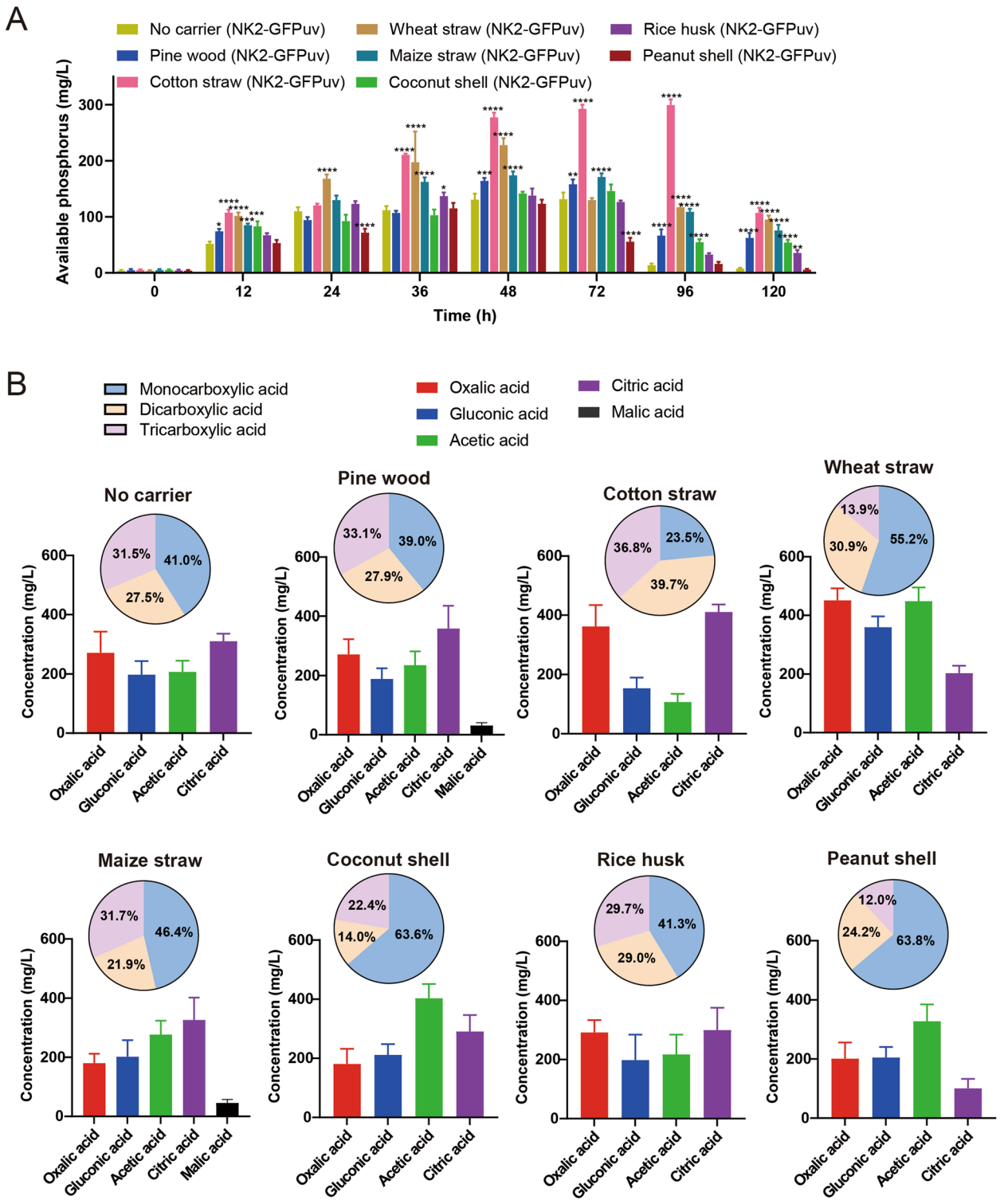

Fig. 5 (See legend on previous page.)

the biochar immobilization treatment group initially had no significant difference in available phosphorus content in the soil compared to the CK group. However, after 30 days of culture, a significant increase in soil available phosphorus content was observed in the biochar immobilization treatment group (Fig. 6C). After 30 days

of culture, the net increase in phosphorus in the soil of the cotton straw treatment group was the highest, reaching 38.13 mg/kg, which was 3.47 times that of the non-carrier treatment group (Fig. 6D). This indicated that the NK2-GFPuv immobilized by cotton straw biochar had the most effective phosphorus solubilization effect in the soil. Phosphate solubility was significantly positively correlated with phosphorus-solubilizing bacteria survival (Pearson's $r = 0.806$, $P < 0.05$), and higher phosphorus-solubilizing bacteria survival supported a greater net increase in available phosphorus (Fig. 6E).

Taken together, this study evaluated the impact of various biochars on phosphorus solubilization and organic acid production in the NBRIP medium. Additionally, the colonization survival of phosphorus-solubilizing bacteria and their ability to release available phosphorus after application to soil for 30 days were investigated. Among the seven types of biochar tested, cotton straw biochar emerged as a suitable immobilization material for the carrier of *Pseudomonas* sp. NK2-GFPuv, a strain of phosphate solubilizing bacteria. Subsequently, NK2-GFPuv immobilized by cotton straw biochar was chosen for further experiments.

The immobilized *Pseudomonas* sp. NK2-GFP enhanced the growth of *Solanum lycopersicum*

Cotton straw biochar was utilized to immobilize NK2-GFP and applied to *Solanum lycopersicum* L. cv. Micro Tom. The colonization amount of biochar-immobilized phosphorus-solubilizing bacteria in Micro Tom rhizosphere soil was examined at 0 day, 15 days, and 30 days. Our findings revealed that the rhizosphere soil colonization amount of immobilized phosphorus-solubilizing bacteria with biochar (IPB) was significantly higher than that of unimmobilized phosphorus-solubilizing bacteria (NK2-GFPuv, UPB). Moreover, the NK2-GFPuv colonization amount in rhizosphere soil in the IPB group was 9.2×10^7 CFU/g soil at 0 day, with a survival rate of 58.9%, representing a 1.83-fold increase compared to the UPB treatment group. At 30 days, the NK2-GFPuv colonization amount of IPB was 4.3×10^7 CFU/g soil, and the survival rate was 27.56%, indicating a 2.15-fold increase compared to the UPB group (Fig. 7A).

The introduction of phosphorus-solubilizing bacteria influenced the soil pH. With the addition of IPB, the rhizosphere soil pH decreased by 0.36 and 0.39 compared to the control group (untreated soil, CK) at 15 and 30 days, respectively. In the UPB group, the soil pH value also decreased by 0.19 compared to CK at 30 days. Notably, the pH reduction in the IPB group was 2.05 times greater than that in the UPB group (Fig. 7B).

The available phosphorus content in the rhizosphere soil was assessed, and a standard curve was constructed to quantify the available phosphorus content in the soil (Additional file 1: Fig. S4). In comparison to untreated soil (CK), the rhizosphere soil treated with cotton straw biochar alone (CBC) showed no significant change in available phosphorus content. However, the UPB group exhibited a 2.14-fold increase, and the IPB group demonstrated a remarkable 4.42-fold increase at 30 days. The application of UPB and IPB significantly elevated the available phosphorus content of soil, with IPB showing particularly pronounced effects (Fig. 7C).

Total chlorophyll in Micro Tom was assessed using a Soil and Plant Analyzer Development (SPAD) after the application of phosphorus-solubilizing bacteria. The total chlorophyll content in the leaves of the three treatment groups was significantly higher than that in the control group CK at 15 and 30 days. The impact of IPB on plant leaves was particularly pronounced in the treatment group, with the total chlorophyll content increasing by 63% compared to that in the CK treatment group at 30 days (Fig. 7D).

At day 15, there was no significant difference in the plant height of Micro Tom between the CK group and those subjected to single applications of biochar (CBC) or phosphorus-solubilizing bacteria solution (UPB). However, the plant height of Micro Tom growing in the

(See figure on next page.)

Fig. 6 The survival and available phosphorus content of different biochars-immobilized phosphorus-solubilizing bacteria *Pseudomonas* sp. NK2-GFPuv applied to soil for 1 and 30 days. **A** Bacterial population of NK2-GFPuv in soil after 1 and 30 days. Bars correspond to one standard deviation (SD) from the mean ($n = 3$). **B** The survival rate of NK2-GFPuv after 30 days of soil application, calculated as the ratio of bacteria population at 30 days to that at 1 day. Bars correspond to one standard deviation (SD) from the mean ($n = 3$), while different letters above columns indicate significant differences ($P < 0.05$) among samples under different treatments according to a one-way ANOVA in conjunction with Tukey's multiple-comparison test. **C** Content of available phosphorus of biochar-immobilized NK2-GFPuv applied to soil after 1 and 30 days. CK indicates untreated soil. Bars indicate one standard deviation (SD) form the mean ($n = 3$), while asterisks indicate significant differences ($P < 0.05$) derived from a two-way ANOVA in conjunction with Dunnett's multiple-comparison test in comparison with available phosphorus of CK at the same time points. ***$P < 0.001$; ****$P < 0.0001$; ns no significant differences. **D** Net increase of available phosphorus content at 30 days compared with 1 day. Bars indicate one standard deviation (SD) from the mean ($n = 3$), while asterisks indicate significant differences ($P < 0.05$) derived from a one-way ANOVA in conjunction with Dunnett's multiple-comparison test in comparison with the net phosphorus increase of NK2-GFPuv. ***$P < 0.001$; ****$P < 0.0001$; ns no significant differences. **E** Correlation between net increase of available phosphorus and survival rate of strains in soil

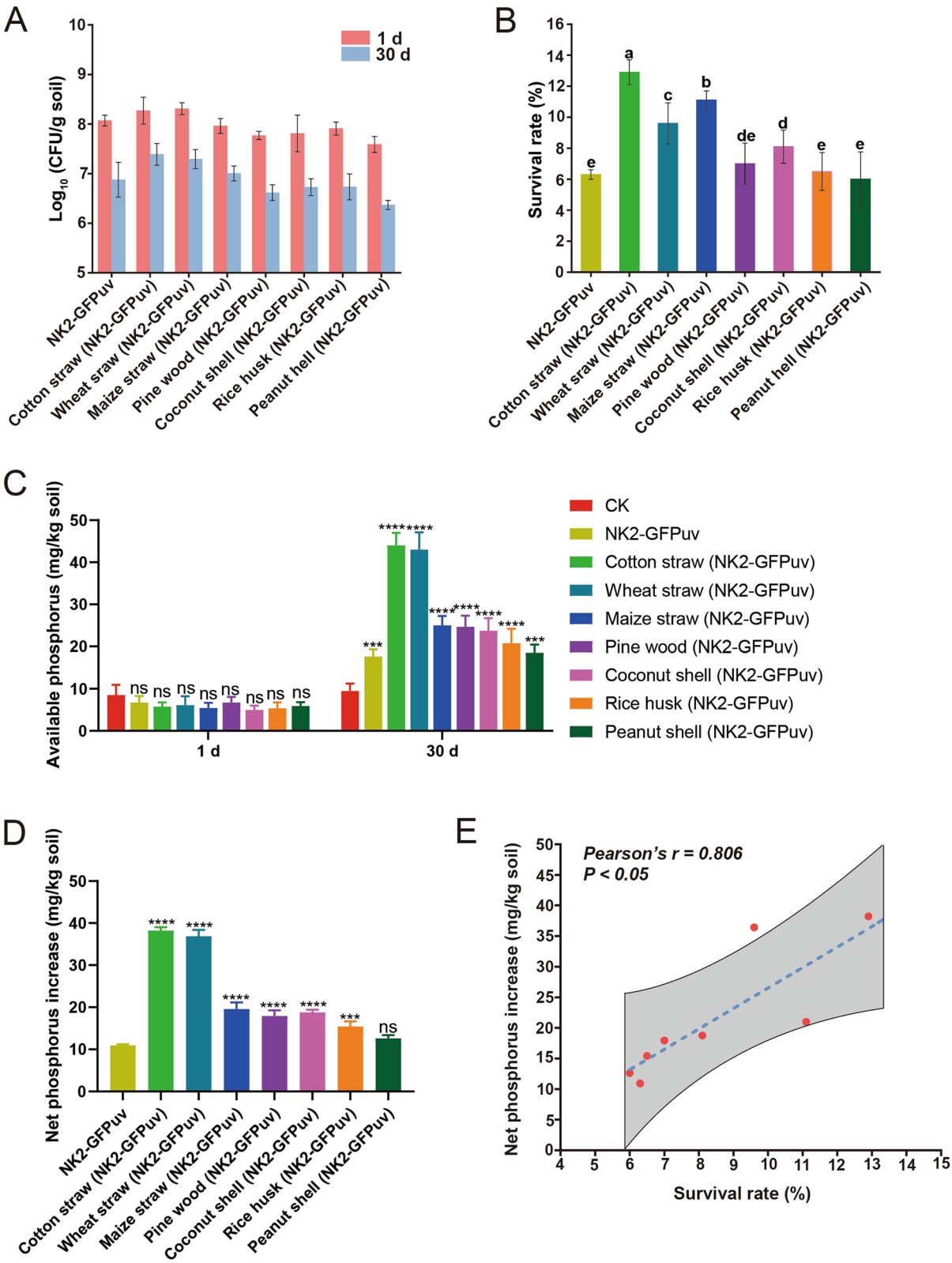

Fig. 6 (See legend on previous page.)

soil with immobilized phosphorus-solubilizing bacteria (IPB) was significantly higher than that of the CK group. At 30 days, the plant height of the UPB treatment group was significantly higher than that of the CK group, and the IPB treatment showed extremely significant improvement (Fig. 7E).

At 15 d, the fresh weight and dry weight of the above-ground part were significantly increased compared to the CK group, with the most pronounced effect observed in the IPB group. Compared to CK, both the fresh and dry weights of the underground parts in the three treatment groups were significantly increased, with the IPB treatment group showing an extremely significant increase. After 30 d, the above-ground biomass in the IPB group was significantly higher than in CK. Concerning underground biomass, except for the dry weight, the fresh weight index significantly increased in the CBC and IPB treatment groups compared to the CK group, with the IPB group showing a more significant effect (Fig. 7F, G, H, and I).

The root indices of the biochar-immobilized phosphorus-solubilizing bacteria (IPB) treatment exhibited a significant increase compared to other groups. At 30 days, the IPB treatment group had the most pronounced impact on plant root indices: the number of Micro Tom root tips in the IPB group increased by 2.875 times, the root surface area expanded by 3.85 times, and the root length extended by 3.75 times compared to CK, respectively (Fig. 7J, K, and L).

Taken together, the application of immobilized *Pseudomonas* sp. NK2 using cotton straw biochar in the soil led to an increase in rhizosphere-colonizing bacteria, a decrease in rhizosphere soil pH, and an increase in available phosphorus content. These changes contributed to the promotion of Micro Tom leaf chlorophyll content, plant height, biomass, and root development.

Discussion

The secretion of organic acids played a crucial role in phosphorus solubilization mechanisms within Gram-negative phosphorus-solubilizing bacteria [28]. Measurement of NK2 extracellular organic acid secretion revealed that the insoluble phosphorus group exhibited a substantial release of organic acids, with concentrations significantly higher than those observed in the soluble phosphorus group (Fig. 2). This disparity also contributed to the lower pH value in the insoluble phosphorus group compared to the soluble phosphorus group (Fig. 1E). Thus, a deficiency in soluble phosphorus promoted the secretion of organic acids by PSB, aligning with previous research findings [29]. It was noteworthy that under normal growth conditions, bacteria inherently produced some organic acids due to their metabolism. In the soluble phosphorus group, in addition to secreting oxalic acid, gluconic acid, and citric acid like the insoluble phosphorus group, a significant amount of malic acid was produced. Conversely, malic acid secretion was not detected in the insoluble phosphorus group, whereas acetic acid production was evident. This suggested that phosphorus stress altered the organic acid metabolic pathway of NK2, activating the acetic acid pathway, enhancing the secretion of oxalic acid, gluconic acid, and citric acid into the environment, while inhibiting the malic acid metabolic pathway. It was interesting to note that different phosphorus-solubilizing bacteria exhibit distinct patterns of organic acid secretion. For instance, previous studies showed that *Burkholderia multivorans* WS-FJ9 primarily secreted pyruvate when dissolving insoluble phosphorus [26]. Similarly, *Enterobacter cloacae* RW8 secreted lactic acid, succinic acid, and citric acid as its main organic acids [30]. These findings collectively highlighted the diversity among PSB in terms of the types and concentrations of organic acids they secrete to dissolve insoluble phosphorus.

The introduction of insoluble phosphorus sources stimulated the TCA cycle, leading to the regulation of the metabolic pathway from succinate to glyoxylic acid and dicarboxylic acid. This resulted in an increase in citrate and oxalate content, accompanied by a decrease in malate. These changes were consistent with the significant elevation of extracellular oxalic acid content, and notably, no malic acid was detected (Fig. 2A and B).

(See figure on next page.)

Fig. 7 Promotion of *Solanum lycopersicum* L. cv. Micro Tom growth by biochar-immobilized phosphorus-solubilizing bacteria. **A** The survival rate of the NK2-GFPuv strain in the soil was assessed after 0, 15, and 30 days of applying phosphorus-solubilizing bacteria immobilized by cotton straw biochar. Soil pH values (**B**) and available phosphorus contents (**C**) were measured after 0, 15, and 30 days. Chlorophyll content (**D**), plant height (**E**), above-ground fresh weight (**F**), above-ground dry weight (**G**), underground fresh weight (**H**), underground dry weight (**I**), root tips (**J**), root length (**K**), and root surface area (**L**) of tomato plants were recorded after 15 and 30 days of growth. CK indicates untreated soil. CBC indicates the rhizosphere soil treated with cotton straw biochar alone. UPB indicates the rhizosphere soil treated with unimmobilized phosphorus-solubilizing bacteria (NK2-GFPuv alone) and IPB indicates the rhizosphere soil treated with immobilized phosphorus-solubilizing bacteria by cotton straw biochar. Bars correspond to one standard deviation (SD) from the mean ($n = 3$), while different letters above columns indicate significant differences ($P < 0.05$) among samples at the same time points under different treatments according to a one-way ANOVA in conjunction with Tukey's multiple-comparison test

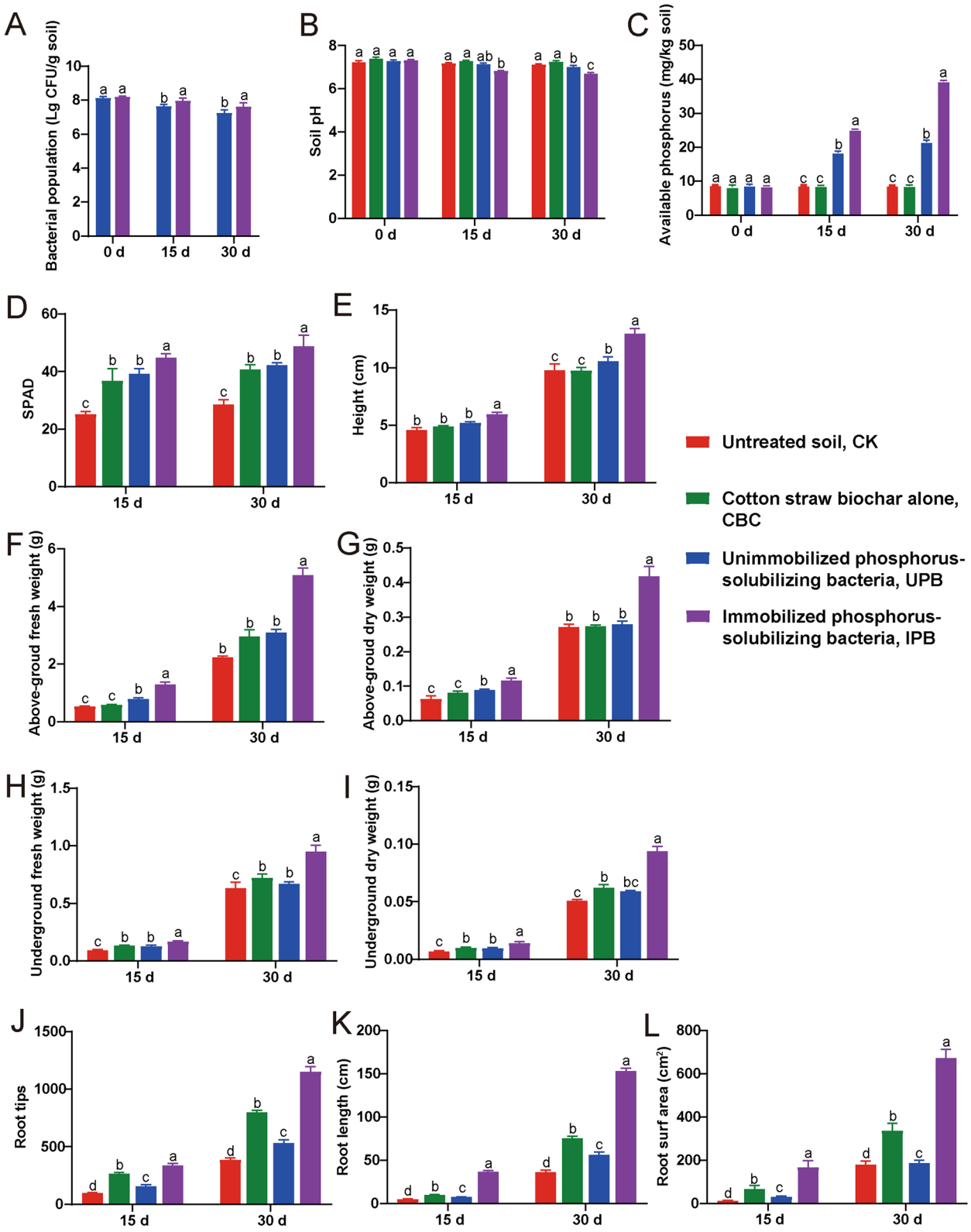

Fig. 7 (See legend on previous page.)

Furthermore, the upregulation of the pyruvate metabolic pathway leading to acetate production was observed, aligning with the detection of substantial acetic acid in the extracellular metabolites (Fig. 2A and B). Propanoic acid, known to regulate organic acid production under phosphorus deficiency stress [31], exhibited an upregulation specifically in the treatment with insoluble phosphorus (Table 1). Bacteria have the ability to convert propionic acid to propionyl-CoA for the production of carboxylic acids, including gluconic acid, galactoic acid, and glucuronic acid [32]. The observed upregulation of propionic acid might contribute to the conversion of gluconic acid, potentially explaining the significantly higher content of gluconic acid in the insoluble phosphorus treatment group compared to the soluble phosphorus treatment group in the extracellular organic acid data.

Amino acids, synthesized from amino sugars, glutamic acid, and lysine through exogenous glucose metabolism or protein decomposition [33], were analyzed in the context of insoluble phosphorus sources treatment (Table 1). High levels of glycogenic amino acids, such as glutamic acid and glutamine, were notably detected. The elevation in glycogenic amino acids could be attributed to gluconeogenesis converting to glucose, inducing glucose production, and the release of significant amounts of gluconic acid [34]. The observed increase in amino acid levels, particularly glycogenic ones, might contribute to accelerating the degradation metabolism of proteins to synthesize new proteins, ultimately facilitating the survival of organisms under environmental stress [35]. Specific amino acids, like histidine, contain imidazolyl structures with a strong affinity for divalent metal ions, forming coordination compounds with them [36]. Additionally, active groups in proline, such as amino, carboxyl, and hydroxyl groups, could combine with metal ions to form non-toxic complexes. Simultaneously, they release soluble phosphate ions, achieving passivation, detoxification, and phosphorus dissolution effects [37]. In this study, the contents of L-histidine, L-proline, and valylproline in NK2 cells were upregulated when phospholytic bacteria were exposed to Ca^{2+} in insoluble phosphorus sources (Table 1). These findings suggested that proline and histidine played pivotal roles in the phosphorus dissolution process. The functional groups in these amino acids released phosphate ions in the insoluble phosphorus source by binding with Ca^{2+}, thereby achieving the dissolution of phosphate.

Glucose serves as a direct energy source for organisms [38]. Under phosphorus stress, the content of D-glucose was downregulated (fold change was 0.31) (Table 1). This indicated that when faced with $Ca_3(PO_4)_2$, on the one hand, NK2 degraded glucose to supply energy for cell metabolism was degraded and, on the other hand, transformed intracellular glucose into gluconic acid, which was then excreted from the cell. This finding aligned with the results of extracellular organic acid detection. Moreover, the content of 3-phosphoglyceric acid was upregulated (fold change was 3.31) (Table 1), indicating that under the conditions of an insoluble phosphorus source, phosphorus stress activated the intracellular glycolysis pathway to provide energy for cells. Simultaneously, acetic acid was generated to alleviate the toxic effects of metal ions, resulting in a significant increase in extracellular acetic acid production.

Collectively, this dynamic interplay between intracellular metabolomics and extracellular organic acid production contributed to the dissolution of inorganic phosphorus sources in response to environmental phosphorus stress. These insights pave the way for novel approaches to explore the intracellular metabolomics of phosphorus-solubilizing bacteria NK2, with the goal of modulating extracellular organic acid production to enhance phosphorus solubilization capacity. This study opens up new avenues for optimizing the agricultural application potential of NK2 in soil phosphorus regulation.

Biochar frequently serves as a carrier for phosphorus-solubilizing bacteria, and this study aimed to assess the impact of different biochar types on the phosphorus solubilization ability of the phosphorus-solubilizing bacterium NK2. Additionally, the study examined the type and content of organic acid production through HPLC to elucidate their effects on the functional activity of phosphorus solubilizers. The findings revealed that NK2 immobilized by cotton straw exhibited the most effective phosphorus solubilization capacity. The mechanism underlying the increased dissolved amount of insoluble phosphorus in the phosphorus-solubilizing bacteria (PSB) fermentation solution when biochar was introduced involved several phenomena. The addition of a neutralizer was observed to enhance the acid-producing capacity of certain acid-producing microorganisms. The buffer capacity of the medium played a role in the complex process of insoluble phosphates' solubilization, where the microenvironment in a buffered state could either inhibit or stimulate the release of organic acids by microorganisms [39]. Biochar played a crucial buffering role in the phosphorus dissolution process of PSB, thereby augmenting the acid-producing capacity of PSB. Furthermore, biochar, with its hydroxyl and carboxyl functional groups, contributed to the dissolution of phosphorus [40]. Additionally, the increased carbon sources in the medium following the addition of biochar were speculated to promote the metabolic processes of PSB, thereby enhancing the solubility of phosphorus [41].

To assess the promoting effect of phosphorus-solubilizing bacteria immobilized by cotton straw biochar (IPB) on *Solanum lycopersicum* L. cv. Micro Tom, various parameters such as SPAD value of plant leaves, plant height, and plant biomass were measured in different soil treatments over 15 and 30 days (Fig. 7). The results demonstrated that IPB had a more significant promoting effect on Micro Tom, aligning with previous findings that *Bacillus subtilis* SL-13 immobilized by cotton straw significantly enhanced the growth of pepper plants [22]. The root system played a pivotal role in nutrient absorption, and crop yield was often contingent on robust root development [42]. Here, the root indexes of IPB were notably higher than those of other treatment groups. However, it was noteworthy that the number of root tips in the UPB treatment group was significantly lower than that in the CBC treatment group, while the root surface area and root length were significantly higher. This suggested that the single application of biochar might loosen the soil structure, facilitating lateral root differentiation and promoting an increase in root tip number while shortening root length. On the other hand, the single application of phosphorus-solubilizing bacteria significantly promoted the elongation of underground roots, leading to increased root length and surface area. The IPB treatment group, benefiting from both phosphorus-solubilizing bacteria and biochar, showcased a harmonized enhancement in root development, consistent with prior reports [43, 44].

Phosphorus-solubilizing bacteria played a crucial role in releasing organic acids, increasing available phosphorus in soil, and promoting plant growth. The effective colonization of these bacteria in the plant rhizosphere was essential for their optimal functioning [21]. Biochar, with its rich pore structure and high charge density, possesses strong adsorption capacity and a large ion exchange capacity, influencing the growth and activity of microorganisms [4]. In this study, the impact of cotton straw biochar-immobilized phosphorus solubilizers on the rhizosphere of tomato was investigated, focusing on colonization amount, available phosphorus content, and soil pH. The results revealed that the colonization amount and available phosphorus content of phosphorus solubilizers immobilized in cotton straw biochar were 1.83–2.15 and 1.5–1.73 times higher, respectively, while the pH value decreased by 1.8–2.05 times compared to the single application of phosphorus-solubilizing bacteria (Fig. 7A, B, and C). This suggested that phosphorus solubilizer NK2 was effectively pre-settled in biochar. Furthermore, biochar significantly enhanced the survival rate and activity of phosphorus solubilizers by providing an optimal microenvironment and nutrients, mitigating the harsh conditions. The released organic acids by phosphorus solubilizers contributed to a reduction in soil pH, promoting phosphorus transformation in the soil and fostering plant root development. This enhanced nutrient absorption by plants, leading to increased biomass. Previous studies have also reported similar findings, indicating that biochar could indirectly elevate available phosphorus in soil by increasing the abundance of phosphorus-solubilizing bacteria and the combined application of biochar and phosphorus-solubilizing bacteria demonstrated the most effective growth promotion effect [45]. Therefore, the immobilization of phosphorus-solubilizing bacteria in cotton straw biochar enhanced their colonization ability in soil, resulting in improved phosphorus solubilization efficiency and subsequent plant growth promotion. These findings suggest that cotton straw biochar can serve as an effective carrier for phosphorus-solubilizing microorganisms, contributing to the advancement of sustainable agriculture practices.

Conclusions

In this study, our findings revealed the intracellular metabolic profile of phosphorus-solubilizing bacterium, *Pseudomonas* sp. NK2, changed under phosphorus stress, including tricarboxylic acid cycle, amino acid synthesis, and glycolysis. This alteration led to the release of higher concentrations of organic acids in vitro and also resulted in the production of acetic acid, gluconic acid, oxalic acid, and citric acid for insoluble phosphorus solubilization. Following the conjoint analysis of NBRIP medium culture and application to soil for 30 days, we discovered that NK2 immobilized by cotton straw exhibited stronger phosphorus-dissolving ability than NK2 immobilized by other biochars. Additionally, we observed that NK2 immobilized by cotton straw improved the content of available phosphorus in the soil, ultimately promoting tomato growth. These findings can contribute to the advancement of sustainable agricultural practices.

Methods

Bacterial strains, plasmids, and culture conditions

The bacterial strain *Pseudomonas* sp. NK2, exhibiting efficient inorganic phosphorus solubilization, was routinely cultured in Luria-Bertani (LB) broth (5 g/L yeast extract, 5 g/L NaCl, and 10 g/L tryptone) at 28°C with shaking (120 rpm) for 12 h, while the *Escherichia coli* isolates were cultured in LB broth at 37°C with shaking (200 rpm). The plasmid pDSK-GFPuv, labeled with GFPuv (Green Fluorescent Protein), was obtained from the MiaoLing Plasmid Platform. NBRIP medium (10 g/L glucose, 0.3 g/L NaCl, 0.3 g/L KCl, 0.5 g/L $(NH4)_2SO_4$, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.03 g/L $MnSO_4 \cdot H_2O$, 0.03 g/L $FeSO_4 \cdot 7H_2O$, 5.0 g/L $Ca_3(PO_4)_2$) served as the phosphorus solubilizing medium. $Ca_3(PO_4)_2$ could be substituted with $FePO_4$, $AlPO_4$, and KH_2PO_4 as necessary, while

$(NH4)_2SO_4$ could be replaced with NH_4Cl, $NaNO_3$, and KNO_3 as required. When required, solid media was prepared by the addition of 12.8 g/L agar and 50 μg/mL kanamycin was supplemented.

Construction of *Pseudomonas* sp. NK2-GFPuv

The plasmid of pDSK-GFPuv was introduced to competent cells of *Pseudomonas* sp. NK2 though electroporation. A total of 1 μg of denatured pDSK-GFPuv plasmid was transformed into 80 μL of competent cells of *Pseudomonas* sp. NK2 using 1.8 kV/cm voltage, 25 μF, and 600 Ω. The transformed cells were resuspended in 1 mL of LB broth and incubated at 28°C for 2 h before being plating onto LB agar supplemented with 50 μg/mL kanamycin. The resulting transformants were verified by fluorescence microscope at excitation wavelength of 488 nm and PCR using the GFPuv-F (5'-CAGTCACGACGTTGTAAAACGACG-3') and GFPuv-R (5-GAGCGGATAACAATTTCACACAGG-3') primer sets.

Measurement of pH and titratable acid in the supernatant of NK2 fermentation broth

Phosphorus-solubilizing bacterium NK2 was inoculated in 100 mL LB medium and cultured at 28°C with shaking (180 rpm) until $OD_{600} = 1.0$, which was as the seed solution to inoculate into soluble phosphorus source medium and insoluble phosphorus source medium according to 1% inoculation amount. The medium inoculated with the same amount of LB was used as the blank control and cultured at 28°C with shaking (180 rpm) for 5 days. The samples were taken every 24 h, centrifuged at 6000 rpm for 10 min, and then the pH value of the supernatant was determined. The standard NaOH solution of 0.1 mmol/L was used to titrate the fermentation supernatant (with 1–2 drops of phenolphthalein reagent) to titrate the consumption of NaOH solution when the pH value of supernatant was 8.4 to calculate the titratable acidity

Determination of phosphorus solubilization ability

The standard phosphorus curve was constructed according to previous report [46]. The 0, 2, 4, 6, 8, 10, and 12 mL of standard phosphorus solution (5 mg/L) was pipetted into a 50-mL volumetric bottle, which was diluted to 20 mL with water. A few drops of 2, 4-dinitrophenol were added as the indicator solution and then sulfuric acid solution was added to adjust the pH to make the solution just yellow. The 5 mL of molybdenum-antimony anticolor development agent was taken into the solution. The volume was adjusted to 50 mL with water and the mass concentration of phosphorus in this standard series solution was 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mg/L in turn. After placing them at 25°C for 30 min, the absorbance of other phosphorus solution was measured at 720 nm and the 0 mg/L phosphorus standard solution was used as the control. The standard phosphorus curve was constructed based on the absorbance values and corresponding phosphorus concentrations.

For the determination of phosphorus solubilization ability of different phosphorus sources, the fermentation solution was centrifuged at 6000 rpm for 10 min, and 2.5 mL of supernatant was taken into a 50-mL volumetric bottle. The steps for color development and phosphorus content measurement were carried out as described in the construction of the phosphorus standard curve. Finally, the phosphorus concentration in the sample was calculated based on the standard phosphorus curve.

Assessment of NH_4^+ assimilation capacity in phosphorus-solubilizing bacteria NK2

In order to evaluate the assimilation effect of NK2 on NH_4^+, equal amounts of NH_4Cl, $NaNO_3$, and KNO_3 were used to replace $(NH_4)_2SO_4$ in NBRIP medium [25]. The seed solution was pipetted into the medium of insoluble phosphorus source with different nitrogen sources at 1% inoculation rate, and the medium inoculated with the same amount of LB was used as the blank control. After 48 h of shaking (180 rpm) at 28°C, the samples were taken, and the supernatant was collected by centrifugation at 6000 rpm for 10 min. The pH value was determined, and the content of soluble phosphorus in different treatment groups was determined using molybdenum-antimony anticolorimetry [46].

Quantification of organic acids using HPLC method

The organic acids were determined by HPLC method using a 250 mm × 4.6 mm Zorbax Eclipse Plus C18 column (5 μm particle size). Mobile phase A consisted of 3% methanol and 97% buffer (5 mM, pH 2.5). Each 10-μL sample was injected at a flow rate of 0.7 mL/min. Signals were detected by UV absorbance at 210 nm. The standard organic acid mixture (oxalic acid, tartaric acid, malic acid, lactic acid, acetic acid, citric acid, succinic acid, formic acid and gluconic acid) was diluted 2 times, 5 times, 10 times, 20 times, and 200 times to establish the standard curves. The standard curve was made by plotting the peak area against its corresponding concentration. The total run time was 15 min for each sample.

Non-targeted metabolomic analysis of intracellular metabolites

The bacterial solution was inoculated into both a soluble phosphorus source medium and an insoluble phosphorus source medium separately, then cultured at 28°C with shaking (180 rpm) for 48 h. Subsequently, the bacterial solution was centrifuged at 8000 rpm and 4°C for 5 min. The resulting precipitate was collected, washed with PBS

buffer three times, and, after discarding the supernatant, the bacteria were frozen in liquid nitrogen and stored at −80°C until further use.

Metabolites were extracted using a 400 μL extraction solution (methanol: water = 4:1, v/v) containing 0.02 mg/mL internal standard (L-2-chlorophenylalanine). The sample solution was ground in a frozen tissue grinder for 6 min at −10°C and 50 Hz, followed by extraction through sonication at 5°C for 30 min (40 kHz). After the samples were placed at −20°C for 30 min and centrifuged for 15 min at 4°C (13000 rpm), the supernatant was transferred into a vial with an internal cannula and injected into the HPLC-MS/MS system.

The 3 μL of the sample was separated using the ACQUITY UPLC® HSS T3 column (100 mm × 2.1 mm, particle size 1.8 μm) and then directed into mass spectrometry detection. The separation was achieved through gradient elution with 95% water and 5% acetonitrile with 0.1% formic acid as solvent A, and 47.5% acetonitrile, 47.5% isopropanol, and 5% water as solvent B. The gradient program was set as follows: 0–3 min 0% B to 20% B, 3–4.5 min 20% B to 35% B, 4.5–5 min 35% B to 100% B, 5–6.3 min 100% B, 6.3–6.4 min 100% B to 0% B, and 6.4–8 min 0% B. The flow rate was maintained at 0.4 mL/min, and the column temperature was set at 40°C.

The mass spectrometric data were acquired using a UHPLC-Q Exactive HF-X system equipped with an electrospray ionization source operating in either positive or negative ion mode. The mass scanning range of mass spectrum conditions was set to 70–1050 m/z. The sheath gas and auxiliary gas flow rates were 50 arb and 13 arb, respectively. The positive and negative mode ion spray voltages were configured at 3500 V and −3500 V, respectively. The heater and capillary temperatures were set to 425°C and 325°C, respectively. Normalized collision energy was applied at 20-40-60 V cyclic collision energy. Full MS resolution was set to 60,000, and MS/MS resolution was adjusted to 7500. The data acquisition was carried out using the Data Dependent Acquisition (DDA) mode.

The intracellular metabolites of bacteria in different treatment groups were detected using the mainstream public database and the metabolome database MJDB established by Shanghai Meiji Technology Co., Ltd. The original data were imported into the metabolomics processing software Progenesis QI (Waters Corporation, Milford, USA) for baseline filtering, peak identification, integration, retention time correction, peak alignment, etc. Subsequently, a data matrix containing retention time, mass–charge ratio, peak intensity, and other information was generated. The software was then utilized to identify characteristic peaks, and mass spectrum information (MS and MS/MS) was matched with the metabolic database. A mass error of less than 10 ppm was set for MS, and metabolites were identified based on the secondary mass spectrum matching score.

The data underwent comprehensive preprocessing to enhance its quality. Initially, missing values were eliminated utilizing the 80% rule in the data matrix. To mitigate errors originating from sample preparation and instrument instability, the response intensity of essential spectral peaks in the samples was normalized through the sum normalization method. Variables exhibiting a relative standard deviation (RSD) exceeding 30% of quality control (QC) samples were removed, followed by $\log_{10}$ logarithmic processing. The resulting refined data matrix was prepared for subsequent analyses. Multidimensional statistical analyses, encompassing supervised partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA), were conducted. Differentially accumulated metabolites (DAMs) were identified by calculating the fold change (FC) based on the mean metabolite expression ratio between the insoluble phosphorus and soluble phosphorus groups. Additionally, variable importance in the projection (VIP) of OPLS-DA > 1 and P value < 0.05 were set as thresholds for DAMs screening. The volcano map illustrating multiple changes and the logarithmic transformation of each metabolite were generated using R. Hierarchical clustering was performed using cluster tree view and MetaboAnalyst 3.0, while intracellular metabolic pathway analysis was conducted with the KEGG database. The data were analyzed on the online platform of Majorbio Cloud Platform (www.majorbio.com) [47].

Biochar preparation procedure

The fresh raw materials of pinewood, cotton straw, wheat straw, maize straw, coconut shell, rice husk, and peanut shell were placed in the oven at 180°C for 20 min and then placed in the oven at 55°C for 48 h of dehydration. The materials treated were placed at room temperature for 24 h. After grinding through 1-mm sieve, the sifted raw material was wrapped tightly with aluminum foil, squeezed out the air in the middle, and sealed. They were placed in Muffle furnace and heated to 300℃ (heating rate of 10℃/min) under anaerobic conditions to prepare biochar. For the biochar raw materials that have been dried, they were directly crushed. After sifting through 1-mm sieve, the materials were placed in the Muffle furnace for firing.

Immobilization of phosphorus-solubilizing bacteria (NK2-GFPuv) onto biochar

The bacterial suspension (10^9 CFU/mL) was added to the biochar at a ratio of 100: 5 (v/w), and the mixture was immobilized in a constant temperature of 30°C with

shaking at 150 rpm for 24 h [26]. For the control group without biochar, only the bacterial suspension was cultured under the same conditions.

Isolation of GFP-labeled immobilized bacterial strain (NK2-GFP) from soil

Rhizosphere soil was collected from the root surface using a sterile brush. The 3 g of soil was added into a conical bottle containing 30 mL of sterilized water and shaken at 200 rpm and 28°C for 30 min. The soil suspension was continuously diluted and coated on LB solid plates containing kanamycin. After 3 days of culture at 28°C, only the colonies that showed green fluorescence under UV excitation were counted, and the colonization amount of GFPUV-labeled phosphorus-solubilizing bacteria NK2 in the soil of different treatment groups (CFU/g soil) was calculated. Survival was assessed by the ratio of the number of bacteria after 30 days treatment to the initial inoculation.

Evaluation of the growth of *Solanum lycopersicum* L. cv. Micro Tom

The pot experiment was carried out in a pot of 20 cm × 15 cm. Soil sterilized twice was used. The main properties of soil were shown in the Additional file 1: Table S1. The soil was added with 100 ml of 0.9% NaCl solution, 0.9% NaCl solution with 5 g of cotton straw biochar, NK2-GFPuv suspension (0.9% NaCl), and NK2-GFPuv suspension (0.9% NaCl) immobilized by 5 g of cotton straw biochar, respectively. The *Solanum lycopersicum* L. cv. Micro Tom seeds were planted in the variously treated soil mentioned above. The experiment was conducted in a light incubator with the parameters set at 28°C for 16 h of light and 18°C for 8 h of darkness.

After growing for 15–30 days, the Micro Tom plants were sampled, and the measurements for plant height and Soil Plant Analyzer Development (SPAD) were recorded. The plant was separated into above-ground and underground (root) portions using scissors to measure the fresh weight. Subsequently, both above-ground and underground parts were defoliated at 105°C for 30 min and ultimately dried at 75°C to obtain the dry weight. After gently washing the roots with water, they were subjected to scanning using a root scanner to obtain indicators related to root development.

Abbreviations

PSB	Phosphorus-solubilizing bacteria
HPLC	High-performance liquid chromatography
PSMs	Phosphorus-solubilizing microorganisms
DAMs	Differentially accumulated metabolites
IPB	Immobilized phosphorus-solubilizing bacteria
UPB	Unimmobilized phosphorus-solubilizing bacteria
CBC	Cotton straw biochar alone
SPAD	Soil and Plant Analyzer Development
TCA	Tricarboxylic acid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12915-024-02011-y.

Additional file 1: Table S1 and Figures S1-S4. Table S1. Soil characteristics in the pot experiment with *Solanum lycopersicum* L. cv. Micro Tom in this study. Fig. S1. Enrichment of all metabolites identified under non-target metabolome under soluble phosphorus (KH_2PO_4) and insoluble phosphorus ($Ca_3(PO_4)_2$) conditions in the KEGG database. Fig. S2. Comparison of biological characteristics of *Pseudomonas* sp. NK2 and GFP labeled NK2 strain (NK2-GFPuv). Fig. S3. NK2-GFPuv was immobilized by biochar. Fig. S4. Standard curve of available phosphorus content in the soil.

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Authors' contributions

KB and WW performed the experiments, analyzed and interpreted the data, and wrote the manuscript. JZ conceived and designed the research. PY, CC and ZX helped analyze the data. LL and TL revised the manuscript. ZW designed the research. All authors read and approved the final manuscript.

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Availability of data and materials

All data needed to evaluate the conclusions in the paper are included in this published article and its supplementary information flies. Additional data related to this paper are available from the corresponding authors upon reasonable request.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

This article does not contain any experiments with animals or human participants that were performed by the contributing authors.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

1. Scholz RW, Ulrich AE, Eilittä M, Roy A. Sustainable use of phosphorus: a finite resource. Sci Total Environ. 2013;461–462:799–803.
2. Hedley MJ, Stewart JWB, Chauhan BSC. Changes in inorganic and organic soil phosphorus fractions induced by cultivation practices and by laboratory incubations. Soil Sci Soc Am J. 1982;46:970–6.

3. Daneshgar S, Callegari A, Capodaglio A, Vaccari D. The potential phosphorus crisis: resource conservation and possible escape technologies: a review. Resources. 2018;7(2):37.
4. Yu H, Zou W, Chen J, Chen H, Yu Z, Huang J, Tang H, Wei X, Gao B. Biochar amendment improves crop production in problem soils: a review. J Environ Manag. 2019;232:8–21.
5. Li H, Liu J, Li G, Shen J, Bergström L, Zhang F. Past, present, and future use of phosphorus in Chinese agriculture and its influence on phosphorus losses. Ambio. 2015;44(S2):274–85.
6. Alewell C, Ringeval B, Ballabio C, Robinson DA, Panagos P, Borrelli P. Global phosphorus shortage will be aggravated by soil erosion. Nat Commun. 2020;11(1):4546.
7. Etesami H. Enhanced phosphorus fertilizer use efficiency with microorganisms. In: Dynamics for Sustainable Crop Production. 2019.
8. Aboulfazli F, Forghani A, Norouzi M. Effects of phosphorus and organic fertilizers on phosphorus fractions in submerged soil. J Soil Sci Plant Nutr. 2012;12:349–62.
9. Bisht N, Chauhan P. Excessive and disproportionate use of chemicals cause soil contamination and nutritional stress. 2020.
10. Rashmi I, Roy T, Kartika KS, Pal R, Coumar V, Kala S, Shinoji KC. Organic and inorganic fertilizer contaminants in agriculture: impact on soil and water resources. In: Naeem M, Ansari AA, Gill SS, editors. Contaminants in agriculture: sources, impacts and management. Cham: Springer International Publishing; 2020. p. 3–41.
11. Chien SH, Prochnow LI, Tu S, Snyder CS. Agronomic and environmental aspects of phosphate fertilizers varying in source and solubility: an update review. Nutr Cycl Agroecosyst. 2010;89(2):229–55.
12. Rawat P, Das S, Shankhdhar D, Shankhdhar SC. Phosphate-solubilizing microorganisms: mechanism and their role in phosphate solubilization and uptake. J Soil Sci Plant Nutr. 2020;21(1):49–68.
13. Sahu A, Bhattacharjya S, Mandal A, Thakur JK, Atoliya N, Sahu N, Manna MC, Patra AK. Microbes: a sustainable approach for enhancing nutrient availability in agricultural soils. In: Meena VS, editor. Role of rhizospheric microbes in soil: volume 2: nutrient management and crop improvement. Singapore: Springer Singapore; 2018. p. 47–75.
14. Kishore N, Pindi PK, Ram Reddy S. Phosphate-solubilizing microorganisms: a critical review. In: Bahadur B, VenkatRajam M, Sahijram L, Krishnamurthy KV, editors. Plant biology and biotechnology: volume I: plant diversity, organization, function and improvement. New Delhi: Springer India; 2015. p. 307–33.
15. Bandeppa S, Kumar K, Latha PC, Manjusha PGS, Phule A, Chandrakala C. 19 - Phosphate-solubilizing microbial inoculants for sustainable agriculture. In: Soni R, Suyal DC, Yadav AN, editors. Trends of applied microbiology for sustainable economy. Goel R: Academic Press; 2022. p. 501–24.
16. Ameen F, AlYahya SA, AlNadhari S, Alasmari H, Alhoshani F, Wainwright M. Phosphate solubilizing bacteria and fungi in desert soils: species, limitations and mechanisms. Arch Agron Soil Sci. 2019;65(10):1446–59.
17. Khan MS, Rizvi A, Saif S, Zaidi A. Phosphate-solubilizing microorganisms in sustainable production of wheat: current perspective. In: Kumar V, Kumar M, Sharma S, Prasad R, editors. Probiotics in agroecosystem. Singapore: Springer Singapore; 2017. p. 51–81.
18. Anand K, Kumari B, Mallick M. Phosphate solubilizing microbes: an effective and alternative approach as biofertilizers. Int J Pharm Pharm Sci. 2016;8:37–40.
19. Khan MS, Zaidi A, Wani PA. Role of phosphate-solubilizing microorganisms in sustainable agriculture — a review. Agron Sustain Dev. 2007;27(1):29–43.
20. Bashan Y, de-Bashan LE, Prabhu SR, Hernandez J-P. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). Plant Soil. 2013;378(1–2):1–33.
21. Nartey OD, Zhao B. Biochar preparation, characterization, and adsorptive capacity and its effect on bioavailability of contaminants: an overview. Adv Mater Sci Eng. 2014;2014:1–12.
22. Ajeng AA, Abdullah R, Ling TC, Ismail S, Lau BF, Ong HC, Chew KW, Show PL, Chang J-S. Bioformulation of biochar as a potential inoculant carrier for sustainable agriculture. Environ Technol Innov. 2020;20:101167.
23. Glaser B, Lehr V-I. Biochar effects on phosphorus availability in agricultural soils: a meta-analysis. Sci Rep. 2019;9:9338.
24. Zhang W, Wu S, Guo H, Wang W, Li Y, Zhang J, Zhang S. Effects of phosphate-solubilizing bacteria on Micro-Tom and soil of Micro-Tom rhizosphere. In: Proceedings of the 11th International Conference on Biomedical Engineering and Bioinformatics. 2022.
25. Xie J, Yan Z, Wang G, Xue W, Li C, Chen X, Chen D. A bacterium isolated from soil in a karst rocky desertification region has efficient phosphate-solubilizing and plant growth-promoting ability. Front Microbiol. 2021;11:625450.
26. Wang Z, Chen H, Zhu Z, Xing S, Wang S, Chen B. Low-temperature straw biochar: sustainable approach for sustaining higher survival of B. megaterium and managing phosphorus deficiency in the soil. Sci Total Environ. 2022;830:154790.
27. Hale L, Luth M, Kenney R, Crowley D. Evaluation of pinewood biochar as a carrier of bacterial strain Enterobacter cloacae UW5 for soil inoculation. Appl Soil Ecol. 2014;84:192–9.
28. Ludueña LM, Anzuay MS, Angelini JG, McIntosh M, Becker A, Rupp O, Goesmann A, Blom J, Fabra A, Taurian T. Strain Serratia sp. S119: a potential biofertilizer for peanut and maize and a model bacterium to study phosphate solubilization mechanisms. Appl Soil Ecol. 2018;126:107–12.
29. Zeng Q, Wu X, Wang J, Ding X. Phosphate solubilization and gene expression of phosphate-solubilizing bacterium *Burkholderia multivorans* WS-FJ9 under different levels of soluble phosphate. J Microbiol Biotechnol. 2017;27(4):844–55.
30. Li XD, Wang XL, Chen X, Cai L, Zeng QF, Shu JH, Cai YM. Transcriptome profiling analysis of the phosphate-solubilizing mechanism of the white clover rhizosphere strain RW8. Acta Prataculturae Sin. 2017;26:168–79.
31. Chen W, Cui Y, Yang Y, Huangfu Q, Sun C. The mechanical of organic acids secreted by roots of tartary buckwheat under the effects of low nitrogen stress. 2018.
32. Sousa-Silva M, Casal M, Soares-Silva I. Identification and functional characterization of novel plasma membrane carboxylate transporters. 2017.
33. Patel M, Fatnani D, Parida AK. Silicon-induced mitigation of drought stress in peanut genotypes (Arachis hypogaea L.) through ion homeostasis, modulations of antioxidative defense system, and metabolic regulations. Plant Physiol Biochem. 2021;166:290–313.
34. Jiang Y, Ge F, Li F, Zhang D, Deng S, Tian J. Intracellular metabolomics switching alters extracellular acid production and insoluble phosphate solubilization behavior in *Penicillium oxalicum*. Metabolites. 2020;10(11):441.
35. Dai Z-L, Wu G, Zhu W-Y. Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. Front Biosci. 2011;16:1768–86.
36. Remelli M, Nurchi VM, Lachowicz JI, Medici S, Zoroddu MA, Peana M. Competition between Cd(II) and other divalent transition metal ions during complex formation with amino acids, peptides, and chelating agents. Coord Chem Rev. 2016;327–328:55–69.
37. Nobahar A, Carlier JD, Miguel MG, Costa MC. A review of plant metabolites with metal interaction capacity: a green approach for industrial applications. BioMetals. 2021;34(4):761–93.
38. Hui S, Ghergurovich JM, Morscher RJ, Jang C, Teng X, Lu W, Esparza LA, Reya T, Le Z, Yanxiang Guo J, et al. Glucose feeds the TCA cycle via circulating lactate. Nature. 2017;551(7678):115–8.
39. Takeda M, Knight JD. Enhanced solubilization of rock phosphate by Penicillium bilaiae in pH-buffered solution culture. Can J Microbiol. 2006;52(11):1121–9.
40. Chen H, Zhang J, Tang L, Su M, Tian D, Zhang L, Li Z, Hu S. Enhanced Pb immobilization via the combination of biochar and phosphate solubilizing bacteria. Environ Int. 2019;127:395–401.
41. Dai Z, Xiong X, Zhu H, Xu H, Leng P, Li J, Tang C, Xu J. Association of biochar properties with changes in soil bacterial, fungal and fauna communities and nutrient cycling processes. Biochar. 2021;3(3):239–54.
42. Jafri N, Wong WY, Doshi V, Yoon LW, Cheah KH. A review on production and characterization of biochars for application in direct carbon fuel cells. Process Saf Environ Prot. 2018;118:152–66.
43. Li J. Preparation of biochar-PGPR microbial agent and facilitation of rice development in seedling stage. China: Zhengjiang University, Master's thesis; 2018.
44. Liu S, Du H, Huang Y, Lan Y, Lu J, Wang J. Effects of biochar application and phosphorus-solubilizing bacteria on rice seedling growth and rhizosphere phosphorus availability under phosphorus stress. Chin J Ecol. 2022;41(8):1560–9.

45. Zhou C, Heal K, Tigabu M, Xia L, Hu H, Yin D, Ma X. Biochar addition to forest plantation soil enhances phosphorus availability and soil bacterial community diversity. For Ecol Manag. 2020;455:117635.
46. Zhang X. Analysis of the factors affecting the available P content in the fermentation liquid of P bacteria determined by Mb-Sb colorimetry. J Anhui Agric Sci. 2008;36(12):4822–3.
47. Ren Y, Yu G, Shi C, Liu L, Guo Q, Han C, Zhang D, Zhang L, Liu B, Gao H, et al. Majorbio Cloud: a one-stop, comprehensive bioinformatic platform for multiomics analyses. iMeta. 2022;1(2):e12.

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