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Phosphate-Solubilizing Microorganisms Stimulate Physiological Responses of Perennial Ryegrass to Phosphorus Deficiency with Assistance of Straw Compost

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Abstract: Biofertilizers with phosphate-solubilizing microorganism (PSM) inoculations have been suggested to diminish the limitation of phosphorus (P) deficiency in plants. However, their applications in agriculture are restricted due to the inconstant effects of various PSMs. Proper carriers for the inoculations may overcome this shortcoming and improve PSMs' effectiveness. The objective of this study was to investigate whether straw compost, a type of organic material, can act as a carrier for improving the efficiencies of phosphate-solubilizing bacteria and fungi named *Acinetobacter* sp. and *Aspergillus niger*, respectively, in soils. We monitored the growth and cellular physiological responses of one type of model plants, named perennial ryegrass (*Lolium perenne* L.), under four soil treatments, including non-fertilization, PSM inoculation alone, straw compost addition alone, and the combined applications of both PSMs and straw compost. We found the combined treatments significantly improved the growth by 14.7% for shoot height and 79.7% for shoot weight, respectively, on average. P and potassium (K) uptakes of ryegrass were also increased by 102.5% and 65.3%, respectively, after the application of both PSMs and straw compost. Furthermore, physiological properties, such as photosynthetic efficiency and P-transportation capacity, of ryegrass were also significantly improved under combined treatments when compared to other treatments, regardless of the types of PSM included. The piecewise structural equation model further indicated that PSM inoculation and straw compost input are synergistically contributing to the nutrient uptake of ryegrass through many direct and indirect ways. We propose that straw compost is a good carrier material for PSMs' survival and would improve their plant growth promotion ability in soil. Our results provide valuable insights into the exploitation and utilization of P-biofertilizers in agriculture.

Keywords: plant growth promotion; plant photosynthesis; plant P-transportation capacity; bacteria and fungi; biofertilizers

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1. Introduction

Phosphorus (P) is one of the most important macronutrients for plants; it is involved in multiple plant physiological processes, e.g., controlling enzyme reactions and regulation of metabolic pathways [1]. However, P, especially in available forms, is widely limited in soil, making P deficiency an important abiotic stress for plant growth [2]. To diminish the limitation of P deficiency on plants, chemical P fertilizers are frequently applied. While

chemical fertilizers negatively affect soil biota, generally have low use efficiency, and may cause multiple environmental problems (e.g., water pollution and accumulation of toxic elements). More environmentally friendly and sustainable approaches, such as biofertilizers with phosphate-solubilizing microorganisms (PSMs), are proposed to improve soil P availability by mobilizing insoluble P and breaking down organophosphorus compounds, as well as to promote plant growth by facilitating biosynthesis of phytohormones such as indole-3-acetic acid and gibberellins nowadays [3–6]. Although these studies have demonstrated the feasibility and effectiveness of PSM application, the processes and mechanisms of plant-soil-microbe interactions therein are still not fully understood.

PSMs are a group of a significant number of beneficial bacteria and fungi capable of transforming insoluble P, e.g., calcium phosphates and inositol hexaphosphates, into soluble forms in soils [7]. They apply various approaches, such as the production of organic acids and phosphatases, to release available P from insoluble phosphates and organophosphorus compounds for plant use [8]. It has been well documented that phosphate-solubilizing bacteria and fungi are able to significantly solubilize insoluble P in vitro within several days as they are fed sufficient energy and nutrients except for P [9,10]. However, there are still several challenges in PSM application in situ; for example, the survival rates are relatively low when PSMs are applied alone in soils with lower fertility [11], and the plant growth promotion effects of PSMs vary according to soil conditions [12]. Remarkably, very few studies have clearly measured the abilities of both phosphate-solubilizing bacteria and fungi to solubilize insoluble P from soils and promote plant growth [13,14]. Thus, it is urgent to overcome these challenges by exploring an inoculum carrier suitable for both phosphate-solubilizing bacteria and fungi.

Straw residues, as the byproducts of crop production, are an important source of organic matter and multiple essential nutrients, such as P, carbon (C), nitrogen (N), potassium (K), and magnesium [15]. Composting is one of the most common approaches for treating straw residues as it can break down bio-macromolecules into more simple molecules that are easily available to plants and microorganisms [16]. Compost derived from straw residues has been widely recognized as a favorable organic fertilizer in agriculture according to its positive effects on soil fertility and crop yields [16–18]. It was also proposed to be an efficient carrier material for exogenous beneficial microorganisms for the appropriate environments and the abundant nutrients it provides [19]. Priming effects caused by organic matter input can elevate P availability by stimulating the growth of native soil PSMs such as *Bacillus asahii*, as well as by increasing soil microbial biomass [20–22]. The larger soil microbial communities may occupy more vacant resource niches and thus resist the survival of exogenous microorganisms [23]. Yet, whether straw compost improves exogenous PSMs' effectiveness in minimizing the negative effects of soil P deficiency on plants is still a knowledge gap.

Plant growth and productivity depend on various P-related physiological processes [24,25]. For example, as an essential component of key molecules like ATP, nucleic acids, and phospholipids, P plays a key role in plant photosynthesis [1,26]. It is suggested that P deficiency decreases photosynthetic efficiency by disrupting the photosynthetic machinery and the electron transport chain, thus limiting plant productivity [27,28]. In addition, P use efficiency is largely determined by the efficiency of P acquisition and internal transportation. The corresponding physiological processes, like the liberation of inorganic orthophosphate (Pi) from organic P components and the uptake and distribution of Pi in plants, are mainly medicated by several intracellular enzymes like acid phosphatase, phytase, and H^+-ATPase. Results from previous studies suggest the activities of these enzymes will be increased when P is deficient [29–31]. Although it is well known that P plays a critical role in these physiological processes, the potential direct or indirect effects of PSMs on these processes have not been fully discussed.

In the current study, we conducted a pot experiment using P-deficient soil (fluvo-aquic soil) and cultivated perennial ryegrass (*Lolium perenne* L.) as a model system to assess the interactions between PSMs and plants. By doing so, we aimed to test to what extent straw

composts are boosting the positive effects of phosphate-solubilizing bacteria and fungi inoculations on plant growth, photosynthesis, and P-use efficiency. This study will not only figure out how plants respond to abiotic stress (i.e., P deficiency) with the help of plant growth-promoting microorganisms (i.e., PSMs) but also expand the accessibility of microbial P-fertilizers in the future.

2. Materials and Methods

2.1. Soil Sampling and Materials Collections

Fluvo-aquic soil (Calcareous Cambisol, FAO) was sampled from a long-term crop rotation system consisting of wheat (*Triticum aestivum*) and maize (*Zea mays*) in 2017 at Fengqiu State Key Agro-Ecological Experimental Station (35°00′ N, 114°24′ E), which is affiliated to Chinese Academy of Sciences. Ten soil samples were randomly collected from upper 20 cm of soil surface using an 8-cm core catcher and subsequently mixed for further soil pot experiments. Before pot experiment, plant debris and stones in soil were removed. Soil analyses found 7.65 g organic C kg^{-1}, 1.25 g total N kg^{-1}, 0.67 g total P $kg^{-1,}$ and 29.90 g total K kg^{-1}, with a pH of 8.51. Additionally, straw compost and phosphate-solubilizing microorganisms were used as soil amendments in this study. Straw compost was purchased from Mingzhu Organic Fertilizer Co., Ltd., Jiangsu province. The nutrient contents of straw compost include 276.0 g organic C kg^{-1}, 9.8 g total N kg^{-1}, 7.9 g P $kg^{-1,}$ and 28.3 g K kg^{-1}. Phosphate-solubilizing bacteria and fungi were named *Acinetobacter* sp. (No. CGMCC_13078; isolating from cattle manure) and *Aspergillus niger* (No. CGMCC_15994; isolating from fluvo-aquic soil), respectively, of which were able to release soluble P from varied Pikovskaya's media modifying with different inorganic phosphates and organic phosphorus compounds (Figure S1; Table S1).

For conducting pot experiment, we first designed a soil incubation experiment to test the survival dynamics of both *Acinetobacter* sp. and *Aspergillus niger*. We inoculated the two PSMs in γ-irradiated (52 kGy) sterilized and non-sterilized fluvo-aquic soils, respectively, with an inoculation dose of 10^9 cells g^{-1}. We sampled soils nondestructively at days 7, 14, 21, 30, and 45 and subsequently counted the colonies of *Acinetobacter* sp. and *Aspergillus niger* according to a series dilution-coated plate method. To clearly identify the colonies, we introduced red fluorescent protein (RFP) MCS5 plasmid (Sangon Biotech., Shanghai) into *Acinetobacter* sp. using electroporation transformation [32]. The colonies of *Aspergillus niger* were identified according to its morphology [33]. The unit of survival dynamics of the two PSMs in soils was represented by log CFU g^{-1}. We found that these two PSMs successfully colonized into fluvo-aquic soil, and the numbers of their colonies were stabilized between 10^6 and 10^7 cells g^{-1} of soil after day 45 of incubation (Table S2).

2.2. Pot Experimental Design

Soil pot experiment was arranged from June to August 2017. Moreover, 900 g of experimental soils were placed in 13 cm × 12 cm plastic pots. Six treatments were designed in this study, including (1) unfertilized control (S), (2) soil mixed with *Acinetobacter* sp. (S + *Aci*.), (3) soil mixed with *Aspergillus niger* (S + *Asp*.), (4) soil mixed with straw compost (S + C), (5) soil mixed with straw compost and *Acinetobacter* sp. (S + C + *Aci*.) and (6) soil mixed with straw compost and *Aspergillus niger* (S + C + *Asp*.). The volume of straw compost in fertilization treatments was 5 g kg^{-1} soil, and 10 milliliters of bacterial and sporular suspension were mixed with soil to form inoculation treatments with approximately 1×10^7 cells g^{-1} soil (representing the numbers of colonies successfully surviving into soils), respectively. All of the above treatments include four replicates.

In the present study, perennial ryegrass was chosen as a model plant species to compare its physiological responses to different PSMs treatments in a P-deficient soil (i.e., fluvo-aquic soil). Before sowing, ryegrass seeds were immersed in 95% ethanol for 1 min, followed by 1% sodium hypochlorite for 10 min, and finally washed eight times with sterile distilled water. Sterilized seeds were sown in plastic pots of the above treatments under a greenhouse condition (25 ± 2 °C) and a long-day photoperiod (18 h light/6 h

dark). After germination, one hundred germinated seeds with the same shoot height were retained. During the growth period of ryegrass, soils were irrigated regularly to maintain the soil moisture at approximately 60% of water hold capacity. To maintain the plant's metabolic processes and improve productivity, mowing is used to reduce the extra components during ryegrass growth. Ryegrass shoots were mowed on days 15, 30, and 45 after germinating, respectively, and left stubble height at 5 cm for tillering. All of the plant samples were frozen in liquid nitrogen and stored at −80 °C immediately, which were used to determine the physiological parameters. Soil samples were also nondestructively collected at the same time for further analyses. One portion of the soil was kept at 4 °C for biological properties analyses, and the remaining soil was dried for 5–7 days at room temperature condition.

2.3. Plant Intracellular Enzymes Extraction and Assay

To prepare intracellular enzyme extracts, 1 g of shoot tissues was frozen in liquid nitrogen and grounded with a mortar and pestle with 2 mL of buffer. MES buffer (0.5 mM $CaCl_2$ and 1 mM EDTA, pH 5.5) was used to extract acid phosphatase [34]. Sodium acetate buffer (0.22 M, pH 5.5) was used to extract phytase [35]. The extraction buffer of H+-ATPase contains 250 mM sucrose, 10% (*v*/*v*) glycerol, 10 mM glycerol-1-phosphate, 2 mM $MgSO_4$, 2 mM EDTA, 2 mM ATP, 2 mM DTT, 5.7% (*w*/*v*) choline chloride and 25 mM BTP (pH 7.5) (Keming Co., Ltd., Suzhou, China) [36]. After extraction, the mixed solutions were centrifuged at 4 °C for 15 min at 8000 rpm to obtain the supernatants, which supernatants were used to further determine the activities of plant extracellular enzymes.

Acid phosphatase was assayed according to the method described by Sharma and Sahi in 2005 [37]. Moreover, 10 mM of p-nitrophenyl phosphate (pNPP) (Keming Co., Ltd., Suzhou, China) was used as a substrate. The reaction was started by adding the substrates and stopped by adding an equal volume of 0.25 M NaOH. The total incubation period was 15 min at 37 °C. One unit of acid phosphatase activity was measured from the release of p-nitrophenol (pNP) and defined as μmol g^{-1} min^{-1}. pNP concentration was determined spectrophotometrically using a UV spectrophotometer at 510 nm.

The activity of phytase was assayed according to the method described by Nielsen et al. in 2006 [38]. Moreover, 6 mM of sodium phytate (Keming Co., Ltd., Suzhou, China) was used as a substrate. The equal volume of substrates was mixed with supernatants and incubated at 37 °C for 30 min. Finally, 2 mL 10% TCA was added to terminate the reactions. One unit of the activity of phytase was defined as the amount of phytase required to release 1 μmol of inorganic phosphate per gram of fresh tissues in one hour. The concentration of inorganic phosphate was measured at 700 nm at a microtiter plate reader by the molybdenum blue method. The activity of phytase was expressed as μmol g^{-1} min^{-1}.

The activity of H^+-ATPase was determined by antibody-sandwich enzyme-linked immunosorbent assay (ELISA) [39]. Firstly, solid phase antibody (Keming Co., Ltd., Suzhou, China) was prepared using microplate coated by purified antibody of plant H^+-ATPase. Secondly, the extracts of plant tissue were added to react for 30 min at 37 °C and subsequently combined with HRP labeled antibody of H^+-ATPase for 30 min at 37 °C to form compounds that consist of antibodies, antigens, and enzyme-labeled antibodies. Thirdly, microplate was washed five times and then TMB substrates for chromogenic reaction were added. Finally, the reaction was terminated in acidic conditions. The ATPase concentrations were determined spectrophotometrically at 450 nm, and the activity of H^+-ATPase was expressed as U mL^{-1}.

2.4. Determination of Photosynthetic Efficiency

The photosynthetic efficiency of ryegrass was represented by the concentration of total chlorophyll in shoot tissues. The total chlorophyll was constituted by both chlorophyll a and chlorophyll b, which were determined according to the method described by Hendry and Price in 1993 [40]. Briefly, 2 g liquid nitrogen frozen tissues were homogenized in 5 mL of 80% acetone and then centrifuged at 8000 rpm for 5 min at 4 °C. The chlorophyll

concentrations were determined by a spectrophotometer. The absorbances of supernatant at 663, 645, and 652 nm were used to calculate the concentrations of chlorophyll a, chlorophyll b, and total chlorophyll. The calculation method was described by Lichtenthaler in 1987 [41].

2.5. Determination of Nutrients in the Plant Tissues

The fresh tissues of ryegrass shoots were dried at approximately 70 °C for 12 h and then grounded to power for analyzing contents of plant nutrients, including total N, P, and K. The power of plant tissues was digested at 360 °C with 5 mL H_2SO_4 and multiple additions of H_2O_2 until the color in the extracts disappeared. Total N and P contents in the extracts were determined by Kjeldahl's method [42] and vanadomolybdate yellow colorimetric method [43], respectively. Total K content in the extracts was determined by the flame photometric method.

2.6. Soil Analysis

Since PSMs release available nutrients in relation to the growth of plants mainly by secreting organic acids in fluvo-aquic soil, soil pH, available P, and available K were measured in this study. In detail, soil pH was measured by a digital pH meter after shaking a soil water suspension (1:5 w/v) for 30 min. Available P was extracted by 0.5 M $NaHCO_3$ (pH 8.5) and measured by the molybdenum blue method [44]. Available K was extracted by 1M NH_4OAc (pH 7.0) and measured by the flame spectrophotometry method [45].

2.7. Statistical Analysis

Multiple-way ANOVA was used to evaluate the effect of PSM inoculations, straw compost addition, and sampling times on ryegrass growth, plant intracellular enzyme activities, nutrient concentrations, and soil properties. Pairwise comparisons between different treatments at the same time were evaluated using Tukey's HSD test with a significance level of 0.05. Pearson's correlation analysis was used to evaluate the potential relationships among variables that are associated with the properties of both ryegrass and soil. Structural equation model (SEM) was conducted to identify the direct and indirect contributions of PSM inoculations and straw compost addition to the nutrient uptake of ryegrass shoots using the *piecewiseSEM* package (version 2.3.0) [46]. Before modeling, we normalized all variables except for soil pH using log transformation and then reduced the number of variables for soil nutrients (i.e., available P and K), intracellular enzyme activities (i.e., activities of acid phosphatase, phytase, and H^+-ATPase) and nutrients (total N and K in the shoots) in ryegrass shoots through Principal Component Analysis (PCA), respectively. The first component (PC1) for each was used in the SEM analysis (Figure S2). The goodness-of-fit of SEM was assessed by a maximum likelihood χ^2-test, and the model was accepted when $p > 0.05$. All the above analyses were conducted in R software version 3.5.2 [47].

3. Results

3.1. Plant Growth and Nutrient Uptake

Firstly, the successful colonization of the two PSMs, i.e., *Acinetobacter* sp. and *Aspergillus niger*, in fluvo-aquic soil has provided solid evidence for the subsequent study testing the effects of combined applications of PSMs and straw compost on the growth and nutrients uptake of ryegrass (Table S2). In the pot experiment, combined applications of PSMs and straw compost increased plant growth and nutrient concentrations (Figures 1 and 2). Compared with control, the average height and fresh weight of ryegrass shoots under the treatments inoculating PSMs alone (S + *Aci.* and S + *Asp.*) did not show significant increases until day 30, while the combined applications of PSMs and straw compost (S + C + *Aci.* and S + C + *Asp.*) both significantly increased the average height and the weight of ryegrass shoots at days 15 and 30, respectively (Figure 1). Overall, the average height and the weight of ryegrass shoots were both significantly higher for the treatments with straw compost application for those inoculating *Acinetobacter* sp. than for

those inoculating *Aspergillus niger* during the whole incubation period (ANOVA, $F = 30.86$, $p < 0.001$ for the averaged shoot height; $F = 9.26$, $p < 0.001$ for the shoot weight) (Table S3).

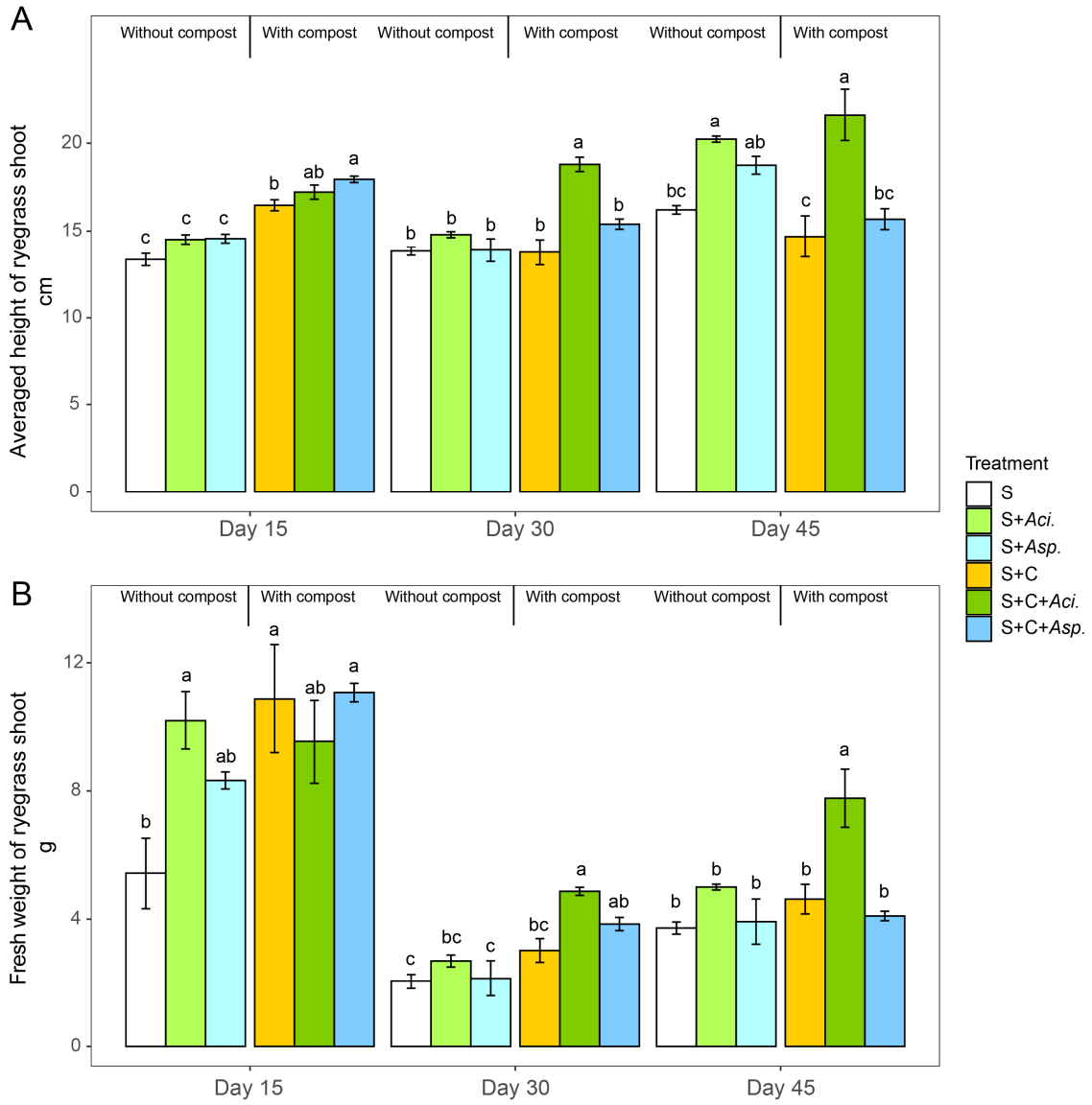

Figure 1. The average height (**A**) and fresh weight (**B**) of ryegrass shoots at three sampling time points (day 15, day 30, and day 45) across the six treatments. S: soil without fertilization, S + *Aci*.: soil inoculated with *Acinetobacter* sp., S + *Asp*.: soil inoculated with *Aspergillus niger*, S + C: soil mixed with straw compost, S + C + *Aci*.: soil mixed with straw compost and *Acinetobacter* sp., and S + C + *Asp*.: soil mixed with straw compost and *Aspergillus niger*. Data are shown as mean ± S.E. ($n = 4$). Different lowercase letters (a–c) indicate significant differences among different treatments at the same time point (Tukey's HSD test at $p < 0.05$).

Compared with control, treatment inoculating *Aspergillus niger* alone performed better than that inoculating *Acinetobacter* sp. alone in increasing the concentrations of total P and K in shoot significantly until day 30 (Figure 2); however, there were no significant differences in total N between these two treatments (Figure S3). Compared with the treatments of inoculating PSMs alone, the total P concentration in shoots was significantly increased by 96.43% at day 45 in the treatment S + C + *Aci*. and by 108.64% at day 30 in the treatment S + C + *Asp*., respectively (Figure 2); the total K concentration in shoots was significantly increased by 65.33% at day 30 in the treatment S + C + *Aci*. (Figure 2A). Overall, the inoculation of *Aspergillus niger* resulted in higher concentrations of total P and K in plant

shoots than for the treatments inoculating *Acinetobacter* sp. (ANOVA, $F = 17.68$, $p < 0.001$ for total P concentration; and $F = 12.14$, $p = 0.001$ for total K concentration) (Table S4). In addition, the concentrations of total P and K in plant shoots still increased significantly on day 45 under the combined applications of PSMs and straw compost but except for a decrease in total K for the treatment S + C + *Asp*., while no difference was observed between control and any treatment inoculating PSMs alone (Figure 2).

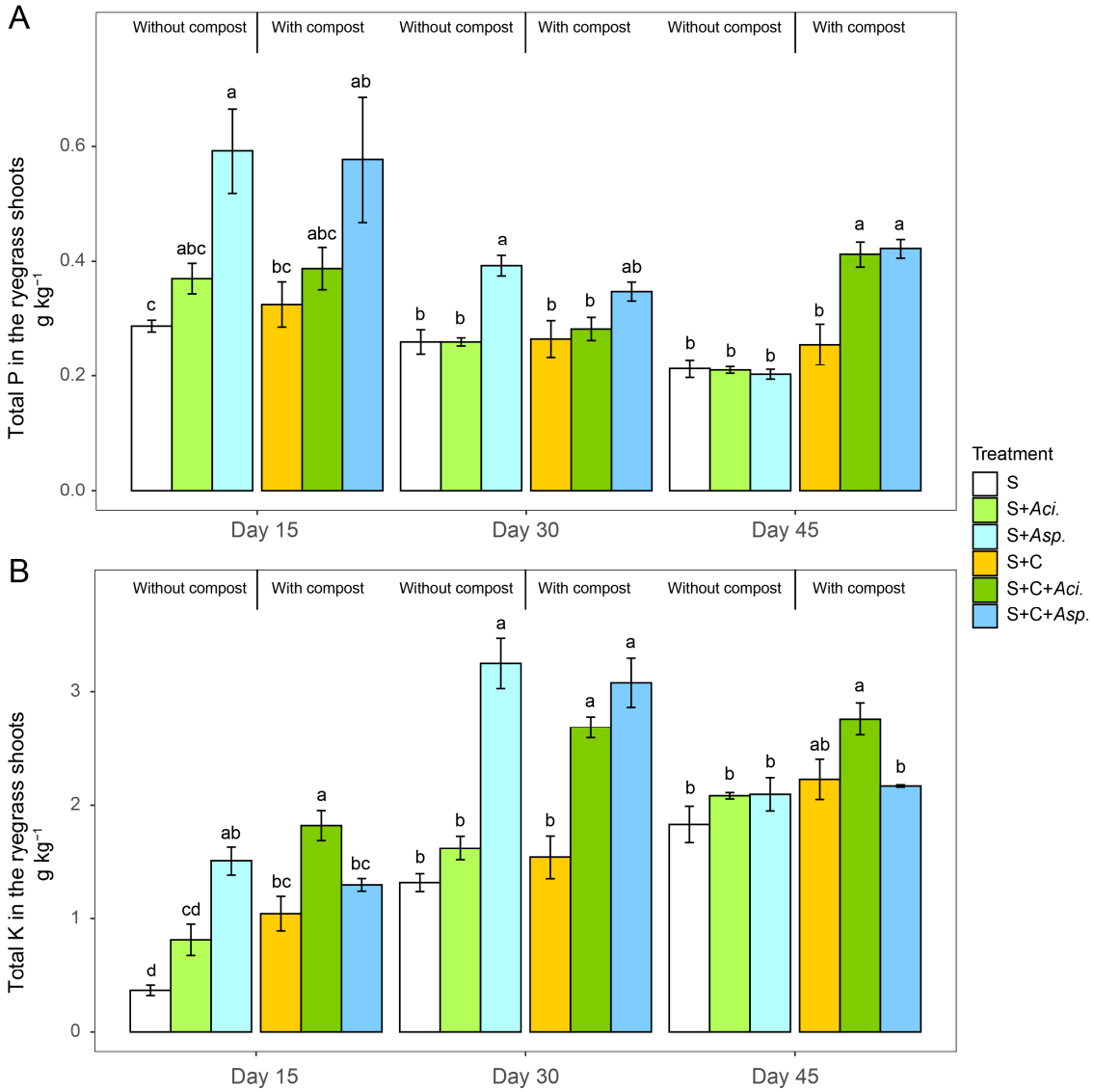

Figure 2. Concentrations of total P (**A**) and total K (**B**) in shoot tissues of ryegrass at three sampling time points (day 15, day 30, and day 45) across the six treatments. S: soil without fertilization, S + *Aci*.: soil inoculated with *Acinetobacter* sp., S + *Asp*.: soil inoculated with *Aspergillus niger*, S + C: soil mixed with straw compost, S + C + *Aci*.: soil mixed with straw compost and *Acinetobacter* sp., and S + C + *Asp*.: soil mixed with straw compost and *Aspergillus niger*. Data are shown as mean ± S.E. ($n = 4$). Different lowercase letters (a–d) indicate significant differences among different treatments at the same time point (Tukey's HSD test at $p < 0.05$).

3.2. Plant Intracellular Enzyme Activities and Photosynthetic Capacity

The responses of the three enzymes involved in intracellular P-transportation to different applications were different (Figure 3). Compared with the control, the activities of acid phosphatase were significantly decreased to a larger extent in the treatments inoculating PSMs than those adding straw compost alone during the incubation (Figure 3A;

Table S5). Compared with the treatment of adding straw compost alone, S + C + *Aci.* and S + C + *Asp.* decreased the activity of acid phosphatase by 36.01–60.33% and by 33.89–76.04%, respectively, during incubation (Figure 3A). In contrast, the activities of H^+-ATPase exhibited a significant decrease in the treatments S + C + *Aci.* and S + C + *Asp.* only at day 15 by 31.72% and by 33.10%, respectively, when compared with the treatment adding straw compost alone, while no clear pattern was observed for the activities of phytase at any time point (Figure 3B,C).

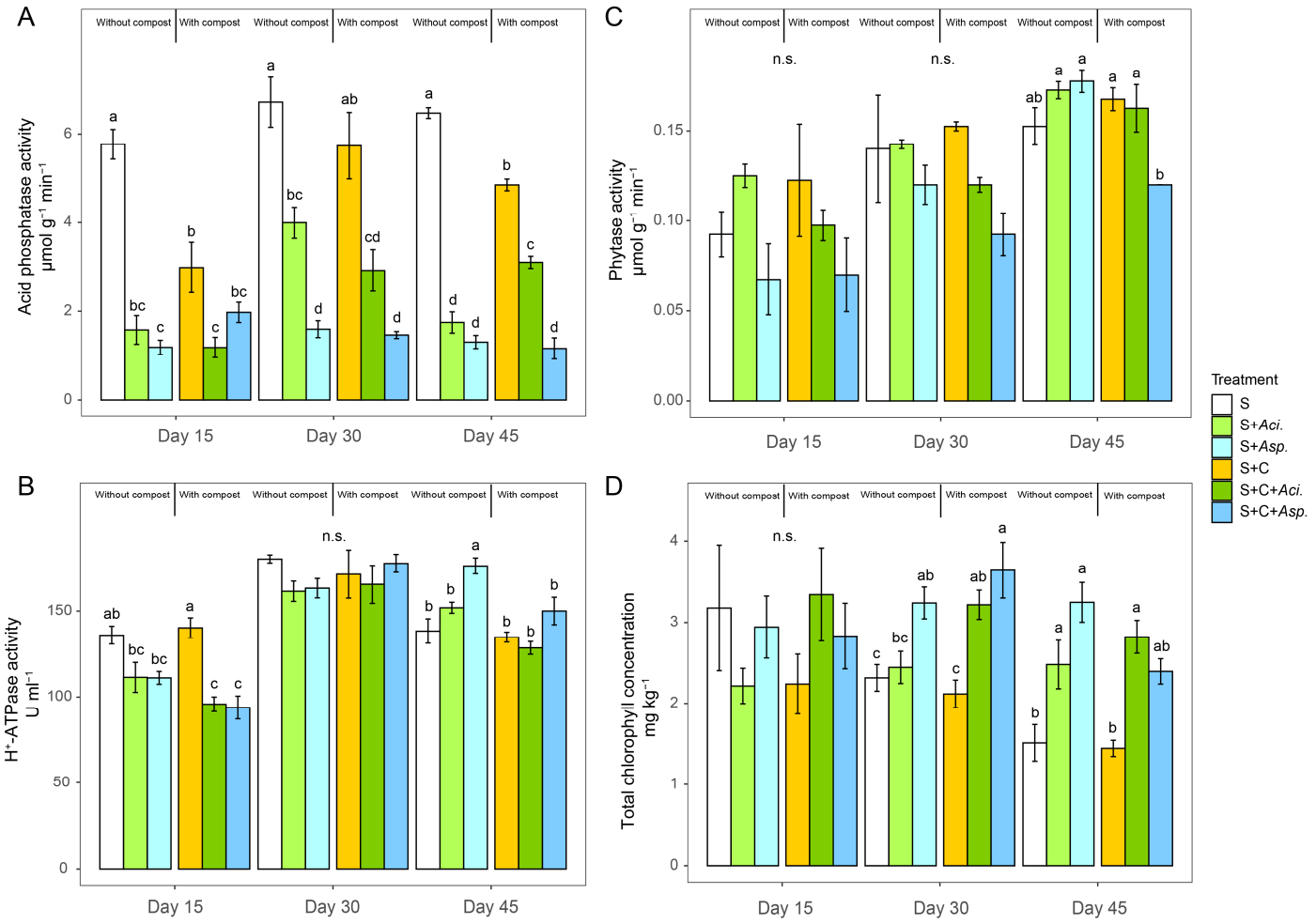

Figure 3. Activities of acid phosphatase (**A**), H^+-ATPase (**B**) and phytase (**C**), as well as the concentrations of total chlorophyll (**D**) in shoot tissues of ryegrass at three sampling time points (day 15, day 30, and day 45) across the six treatments. S: soil without fertilization, S + *Aci.*: soil inoculated with *Acinetobacter* sp., S + *Asp.*: soil inoculated with *Aspergillus niger*, S + C: soil mixed with straw compost, S + C + *Aci.*: soil mixed with straw compost and *Acinetobacter* sp., and S + C + *Asp.*: soil mixed with straw compost and *Aspergillus niger*. Data are shown as mean ± S.E. (n = 4). Different lowercase letters (a–d) indicate significant differences among different treatments at the same time point (Tukey's HSD test at $p < 0.05$).

Combined applications of PSMs and straw compost also improve plant photosynthetic efficiency but not exhibits significant differences between inoculations of *Acinetobacter* sp. and *Aspergillus niger* (Figure 3D; Table S5). Compared with control, S + *Aci.* and S + C + *Aci.* significantly increased total chlorophyll concentration in ryegrass shoots by 63.95% and 86.61% at day 45, while S + *Asp.* and S + C + *Asp.* also significantly increased total chlorophyll concentration in ryegrass shoots by 39.96% and 57.34% at day 30 (Figure 3D). Overall, total chlorophyll concentration in ryegrass shoots was only significantly affected

by the interaction of straw compost addition and PSMs inoculation (ANOVA, $F = 6.98$, $p = 0.01$) (Table S3).

3.3. Relationships between Soil Properties and Plant Physiological Parameters

Inoculation of PSMs significantly but weakly decreased soil pH but increased the soil's available P and K. Compared with control, soil pH was significantly decreased in the treatments adding straw compost and PSMs simultaneously (S + C + *Aci.* and S + C + *Asp.*) on day 15, but not until day 45 for those in the treatments inoculating PSMs alone (S + *Aci.* and S + *Asp.*) (Table 1). Combined applications of straw compost and PSMs (S + C + *Aci.* and S + C + *Asp.*) also significantly increased soil available P and K by ranging from 67.46% to 105.79% when compared with the treatments inoculating PSMs alone (S + *Aci.* and S + *Asp.*) during the incubation (Table 1). Pearson's correlation results showed that soil pH was not only negatively correlated with concentrations of soil available P and K as well as total P in the ryegrass shoots but positively correlated with the activities of acid phosphatase and H^+-ATPase in the ryegrass shoots (Figure S4).

Table 1. Soil physiochemical properties at three-time points across the six treatments. S: soil without fertilization, S + *Aci.*: soil inoculated with *Acinetobacter* sp., S + *Asp.*: soil inoculated with *Aspergillus niger*, S + C: soil mixed with straw compost, S + C + *Aci.*: soil mixed with straw compost and *Acinetobacter* sp., and S + C + *Asp.*: soil mixed with straw compost and *Aspergillus niger*.

Time	Treatment	Soil pH	Available P mg kg^{-1}	Available K mg kg^{-1}
Day 15	S	8.42 ± 0.02 [1] a [2]	13.14 ± 0.51 b	84.50 ± 8.78 b
	S + *Aci.*	8.30 ± 0.01 ab	13.95 ± 0.10 b	82.00 ± 1.08 b
	S + *Asp.*	8.35 ± 0.04 ab	14.45 ± 0.86 b	105.00 ± 7.88 b
	S + C	8.45 ± 0.01 a	14.99 ± 0.42 b	170.75 ± 15.51 a
	S + C + *Aci.*	8.22 ± 0.09 b	23.36 ± 1.82 a	168.75 ± 22.99 a
	S + C + *Asp.*	8.39 ± 0.03 ab	16.58 ± 0.88 b	162.50 ± 2.47 a
Day 30	S	8.46 ± 0.00 b	7.19 ± 0.68 c	64.50 ± 3.88 d
	S + *Aci.*	8.41 ± 0.00 b	11.81 ± 0.58 abc	70.00 ± 2.83 cd
	S + *Asp.*	8.45 ± 0.03 b	14.32 ± 2.62 ab	72.00 ± 0.71 cd
	S + C	8.55 ± 0.02 a	9.58 ± 0.80 bc	86.33 ± 3.47 ab
	S + C + *Aci.*	8.42 ± 0.03 b	16.24 ± 1.88 a	95.00 ± 1.22 a
	S + C + *Asp.*	8.57 ± 0.00 a	12.14 ± 0.84 abc	81.50 ± 3.59 bc
Day 45	S	8.49 ± 0.01 a	6.86 ± 0.80 b	60.50 ± 0.87 c
	S + *Aci.*	8.38 ± 0.02 b	11.01 ± 0.88 a	68.75 ± 0.25 bc
	S + *Asp.*	8.38 ± 0.02 b	10.10 ± 0.54 ab	74.00 ± 2.04 b
	S + C	8.52 ± 0.03 a	11.54 ± 0.84 a	102.50 ± 4.56 a
	S + C + *Aci.*	8.48 ± 0.00 a	13.03 ± 0.31 a	112.25 ± 2.06 a
	S + C+*Asp.*	8.46 ± 0.01 ab	13.35 ± 1.44 a	109.00 ± 0.71 a

[1] Data are means ± S.E. ($n = 4$). [2] Different lowercase letters (a–d) indicate significant differences among different treatments at the same time point (day 15, day 30, and day 45) (Tukey's HSD test at $p < 0.05$).

The piecewise SEM further described the interaction pathways among soil properties as well as the nutrient uptake, photosynthetic efficiency, and activities of P-transportation-related enzymes of ryegrass shoots in response to PSMs inoculation and straw compost addition. PSM inoculation improves nutrient uptake of ryegrass shoots not only directly but indirectly by decreasing soil pH and then releasing available nutrients in soils. The negative relationship between soil nutrients and total nutrients in ryegrass shoots confirms the nutrient uptake. Straw compost addition significantly boosted the positive effects of PSMs on nutrient uptake of ryegrass shoots directly or indirectly ($R^2 = 0.55$) (Figure 4). In addition, PSM inoculation also improves the photosynthetic efficiency of ryegrass shoots to a lesser extent ($R^2 = 0.21$), while the activities of P-transportation related enzymes both negatively related to photosynthetic efficiency and total nutrients in ryegrass shoots (Figure 4).

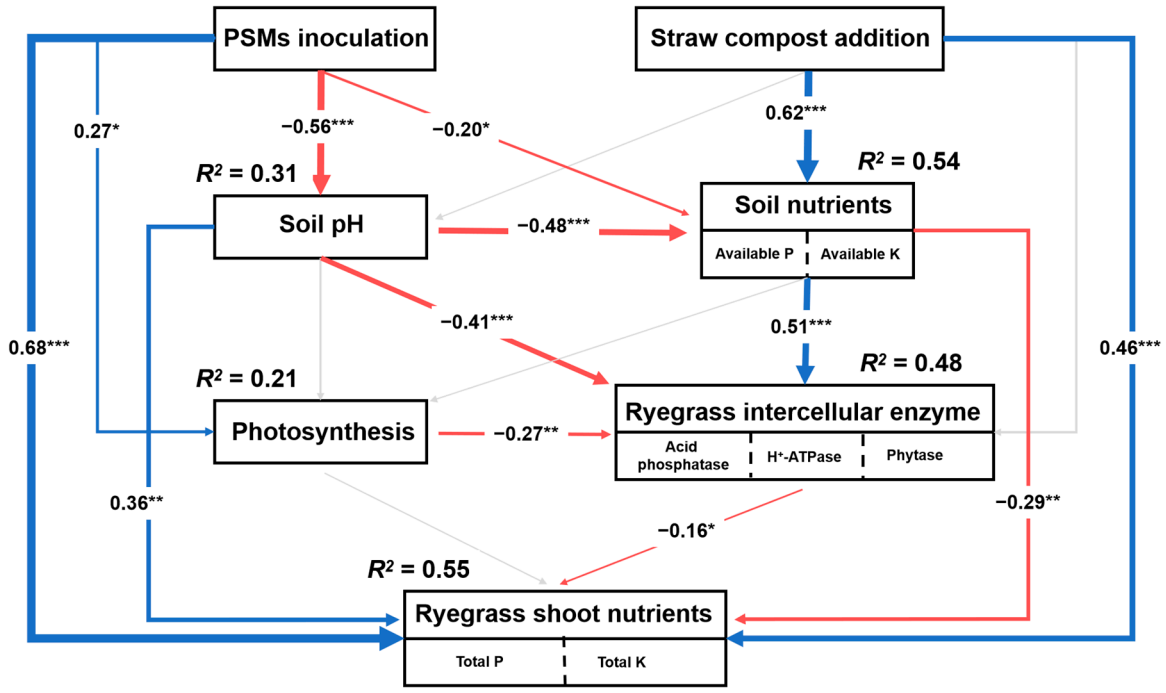

Figure 4. Piecewise structural equation model (SEM) showing effects of PSMs inoculation and straw compost addition on nutrient uptake of ryegrass shoots. In this model, soil properties (pH and nutrients) and physiological responses of ryegrass shoots (photosynthesis and activities of P-transportation related enzymes) were assigned as fixed factors, whereas replicate was assigned as random factors. Blue arrows indicate significant positive relationships ($p < 0.05$), and red arrows indicate significant negative relationships ($p > 0.05$). Non-significant effects were represented by the grey arrows. Numbers in the arrows are standardized path coefficients. * means $p < 0.05$, ** means $p < 0.01$ and *** means $p < 0.001$. R^2 values associated with fixed factors indicate the proportion of variation explained by relationships with other factors. Results of the optimal model fitting are Chi-square (χ^2) = 0.46, degree of freedom (df) = 4, and $p = 0.98$.

4. Discussion

Plant biomass and nutrient uptake ability are crucial parameters for assessing the effect of PSM inoculations on crop growth [48]. Plant intracellular P-transportation enzyme activities and photosynthetic efficiency are two determinant factors for crop growth and P uptake [25,26]. In the present study, we have confirmed that both inoculations of phosphate-solubilizing bacteria (*Acinetobacter* sp.) and fungi (*Aspergillus niger*) promote aboveground growth of ryegrass during the incubation, as well as changes in plant physiological parameters, especially photosynthetic efficiency and the activity of acid phosphatase. Meanwhile, the combined applications of straw compost and PSMs further improve the nutrient uptake and the corresponding physiological properties, suggesting that straw compost addition positively contributed to the plant growth-promoting ability of PSMs.

Compared with PSMs inoculation and straw compost application alone, the combined applications significantly improve the average height, fresh weight, and nutrient uptake of ryegrass shoots, thus supporting the hypothesis in our study. Consistently, many previous studies have also confirmed that combined uses of phosphate-solubilizing microorganisms and other organic amendments were all able to promote plant growth and nutrient uptake efficiently, and these positive effects were largely attributed to the existence of effective substrates in organic amendments [13,49]. Straw compost contains abundant nutrients and organic matters, especially lignocellulose-degraded products [50], which not only provide appropriate habitats for PSMs survival but also provide carbon resources for PSMs and thus create phosphatase and organic acids to release soluble P and K from soil for plant uptake [51,52]. As such, the limited nutrient uptake of ryegrass under *Acinetobacter* sp.

incubation alone may be attributed to the lack of environmental adaptability and nutrient competitiveness for bacteria when compared with the native soil microorganisms and plant roots [53]. However, we still found that *Acinetobacter* sp. inoculation alone significantly increased the average height of ryegrass shoots at the later stage of incubation. Mechanically, several studies have reported that many types of *Acinetobacter* are able to promote plant growth alternatively by using special exudates of plant root as precursors for producing phytohormones [6,54]. On the contrary, given that fungi can survive in soils alone with their stronger ability to exploit resource niches than bacteria [55], *Aspergillus niger* inoculation alone prefers to promote P and K uptake of ryegrass at the earlier stage of incubation, and thus largely explains the direct contribution of PSMs inoculation on the nutrient uptake of ryegrass shoots (Figure 4).

The inoculations of *Acinetobacter* sp. and *Aspergillus niger,* as well as their combined applications with straw compost, further improved the photosynthetic efficiency of ryegrass according to the increased concentration of photosynthetic pigment, e.g., total chlorophyll [56], in the ryegrass shoots when compared with straw compost application alone. Piecewise SEM also shows a direct improvement of PSMs inoculation on the photosynthesis of ryegrass shoots (Figure 4). Consistently, several previous studies also confirmed that several PSMs belonging to *Acinetobacter* and *Aspergillus* were beneficial to increase photosynthesis and growth of many crops, such as pepper, sugar beet, rice, etc. [57–59]. Since plants capture light and convert it into photochemical products and thus improve biomass accumulation, photosynthesis is also a key determinant of plant growth [60]. Several previous studies have demonstrated that the mitigation of nutrient deficiency, especially P deficiency, slows down the breakdown of chlorophyll and thus improves the accumulation of chlorophyll in plant tissues [61,62]. In the present study, we also found that total chlorophyll concentration was positively correlated with soil available P, suggesting that PSM inoculation improves photosynthesis and growth of plants by increasing the available P in soils. However, there were no significant differences observed in the positive effects of inoculating *Acinetobacter* sp. and *Aspergillus niger* on the accumulations of total chlorophyll in ryegrass shoots (Table S3). Likewise, the content of soil available P, which did not show any significant differences between the treatments inoculating *Acinetobacter* sp. and *Aspergillus niger* after day 30 of incubation (Table 1), again confirms that the abilities of phosphate-solubilizing bacteria and fungi to solubilize soil phosphate are similar in soils [63].

The activity of acid phosphatase is another proxy of growth promotion and P uptake of ryegrass according to the significant correlations between each other (Figure S4). Many previous studies also agreed that the change in the activity of intracellular acid phosphatase is better evidence for the physiological responses of plants to P deficiency [24,31]. Since the activity of intracellular acid phosphatase in plants is always induced by low available P [64], the significant decrease in the activity of intracellular acid phosphatase, as well as its negative correlations with plant total P contents in our study, both confirmed the mitigation of P deficiency in ryegrass shoots. However, although the activities of phytase and H^+-ATPase are also related to the P transportation of ryegrass, the changes in these activities were not always consistent with acid phosphatase for several reasons. Firstly, the substrate of phytase, inositol hexakisphosphate (InsP6), primarily presents in the soil environment instead of in plant cells [65]. Instead, the organic P compounds in plant cells mostly consisted of phospholipids [30]. Therefore, the constant intracellular phytase activities in the present study may largely be attributed to the lack of substrates in plant cells of ryegrass. Secondly, since H^+-ATPase is also a major ion pump of the plant plasma membrane and plays a crucial role in N transport. In addition to the P supply [66,67], the fewer variations of the activity of H^+-ATPase may be explained by the relatively invariable total N concentration in ryegrass shoots (Figure S3).

5. Conclusions

The significant improvement of ryegrass growth and associated physiological responses under the combined applications suggests that using straw compost as the inoculum carrier for both phosphate-solubilizing bacteria and fungi is a feasible practice to prepare bio-fertilizer and then improve plant growth. In addition to providing useful information for inoculum carriers' exploitation, our study also provides a strong incentive to improve the efficiency of biofertilizer applications by combining inoculants and organic amendments and further promoting the development of sustainable agriculture.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14051008/s1, Figure S1: Growth of PSMs in the modified Pikovskaya's agar media. (a) and (g) were $Ca_3(PO_4)_2$ modified media inoculating *Acinetobacter* sp. and *Aspergillus niger*, respectively; (b) and (h) were $FePO_4$ modified media inoculating *Acinetobacter* sp. and *Aspergillus niger*, respectively; (c) and (i) were $AlPO_4$ modified media inoculating *Acinetobacter* sp. and *Aspergillus niger*, respectively; (d) and (j) were fluorapatite modified media inoculating *Acinetobacter* sp. and *Aspergillus niger*, respectively; (e) and (k) were lecithin modified media inoculating *Acinetobacter* sp. and *Aspergillus niger*, respectively; (f) and (l) were phytate modified media inoculating *Acinetobacter* sp. and *Aspergillus niger*, respectively. All of the media were incubated for four days at 30 °C; Figure S2: Contribution of the different variables to the principal component analysis. Soil nutrients were represented by the concentrations of available P and K in the soils (a); Plant intracellular enzyme activities were represented by the activities of acid phosphatase, phytase, and H^+-ATPase in the plant shoots (b); and Plant nutrients were represented by the concentrations of total P and K in the plant shoots (c); Figure S3: The contents of total N in shoots of ryegrass at three-time points across six treatments. S: soil without fertilization, S + *Aci*.: soil inoculated with *Acinetobacter* sp., S + *Asp*.: soil inoculated with *Aspergillus niger*, S + C: soil mixed with straw compost, S + C + *Aci*.: soil mixed with straw compost and *Acinetobacter* sp., and S + C + *Asp*.: soil mixed with straw compost and *Aspergillus niger*. Data are shown as mean ± S.E. (n = 4). Different letters (a–d) indicate significant differences among different treatments at the same time point (Tukey's HSD test at $p < 0.05$); Figure S4: Pearson's correlation coefficients among soil physiochemical properties, ryegrass growth parameters, and physiological properties of plant shoots. Red box represents negative correlation, and blue box represents positive correlation. The proportions in the pie plots mean corresponding correlation coefficients (R). The inserted symbols mean significances, * means $p < 0.05$, ** means $p < 0.01$ and *** means $p < 0.001$; Table S1: Quantifying phosphate-solubilizing abilities of *Acinetobacter* sp. and *Aspergillus niger* in the Pikovskaya's liquid media modified by different insoluble phosphorus sources during a 5-day incubation; Table S2: Survival dynamics (lg CFU g^{-1} soil) of Acinetobacter sp. and *Aspergillus niger* on sterilized and non-sterilized fluvo-aquic soils during a 45-day incubation.; Table S3: Multi-way ANOVA testing the effects of sampling time point, straw compost input, and type of the inoculated PSMs, as well as their interactions on the average height and weight of ryegrass shoots; Table S4: Multi-way ANOVA testing the effects of sampling time point, straw compost input and type of the inoculated PSMs, as well as their interactions on the total N, P and K concentrations in the ryegrass shoots; Table S5: Multi-way ANOVA testing the effects of sampling time point, straw compost input and type of the inoculated PSMs, as well as their interactions on the plant intracellular enzyme activities and total chlorophyll concentration in the ryegrass shoots.

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