Current Microbiology (2021) 78:932–943
https://doi.org/10.1007/s00284-021-02354-7

Phosphorus Solubilizing and Mineralizing *Bacillus* spp. Contribute to Rice Growth Promotion Using Soil Amended with Rice Straw

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Received: 30 May 2020 / Accepted: 10 January 2021 / Published online: 12 February 2021

Abstract

Rice (*Oryza sativa* L.) is a staple food for more than two billion people worldwide. Its cultivation demands large amounts of nutrients, particularly nitrogen and phosphorus (P). Consequently, low availability of these nutrients in the soil has led to the use of chemical fertilizers, generating increases in production costs and environmental damage. Soil host microorganisms known as plant growth-promoting rhizobacteria (PGPR) colonize the rhizosphere and facilitate the uptake of nutrients by the plants. In this study, rice seeds inoculated with PGPR were grown for 30 days in an inert substrate and fertilized with modified Hoagland nutrient solution with phosphate rock as a source of P. Treatments were repeated over time, obtaining five isolates which significantly increased plant length by up to 56% and dry weight of stems and roots up to 45% and 169% respectively relative to an uninoculated control. Selected strains showed in vitro tri-calcium phosphate solubilizing activity, mineralizing phytate activity, and phosphate release from rice straw (RS). Based on the above criteria, three isolates (IBUN-02755, -02,704 and -02,724) that contained β propeller phytase (BPP) genes, were selected to evaluate their effect as PGPR in rice seedlings. These were planted in a soil amended with RS under greenhouse conditions. The results showed that selected *Bacillus* spp. strains significantly increased plant length and dry weight or increased plant phosphate uptake up to two times compared to an un-inoculated control. This suggests that selected strains may have a capacity as PGPR using RS as carbon and a P amendment.

Introduction

Rice is the world´s most important food crop in terms of global food security and the livelihood of millions of farmers [1]. It produced nearly 759 million tons in about 165 million hectares during 2017 [2] with an average yield per region oscillating between 1 and 10 ton/ha^{-1}, depending on the supply of water and industrialization of the crop [3]. Rice cultivation has huge nutritional requirements for growth, particularly nitrogen (N), phosphorus (P) and potassium (K), which are needed for the proper development of the plants [4]. Unfortunately, P fertilizer efficiency is less than 10% because after its application in the field, it forms insoluble complexes with aluminum (Al), iron (Fe), or calcium (Ca) that prevent its absorption by the plants [5, 6]. Organic phosphate is another important fraction of P in the soil that comes from organic matter of animal and plant decay [7]. Depending on the content of organic matter in the soils, it represents between 4 and 90% of the total P soil content and may play a very important role in the recycling of P in agricultural ecosystems [6, 8]. Inositol phosphate (phytic acid), is one of the most abundant organic sources of P in nature; but also it is one of the less available for plants, due to their highly reactive capacity with metallic ions, amino acids, carbohydrates and proteins [8, 9]. The situation described above leads to the use of P fertilizers at rates between 50–100 kg of P.ha^{-1} to achieve high-productivity levels in rice crops [10].

Rice straw (RS) incorporation has been suggested as an appropriate alternative to an increase in the nutritional status of soil, through the availability of nutrients like C and P with a content of approximately 380 kg and 1 kg of C and P respectively per ton of RS incorporated [11, 12]. This leads to a more sustainable fertilization strategy in rice crops. However, the burning of RS in open fields is a very common activity in most of the rice producing areas in the world. This activity represents a major constraint for rice producers due to air pollution [13] and nutrient loss

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[14, 15]. In terms of nutrient loss, authors like Dobermann and Fairhurst [15] have shown that around 40% of N, 85% of K, 35% of P and 50% of S assimilated by plants are lost when RS is burned.

Mineral and organic P trapped in the soil matrix becomes available to plants due to the action of a highly diverse microbial community present in the soil. Several mechanisms have been described as part of the strategies that microorganisms can use to improve the acquisition of such nutrients by the plants [16]. Between them, inorganic phosphate solubilization through the production of organic acids [17] and mineralization of organic sources of phosphate due to the action of phosphatases, such as phosphomonoesterases and phytases [18]. Even the production of plant growth promoter hormones such as indole acetic acid [19] through the increase of root biomass has been suggested.

Some bacteria of the genus *Bacillus* have been recognized as one of the most relevant groups of the so called plant growth-promoting rhizobacteria (PGPR), due to the expression of some of the mechanisms described above [20]. Some of them have been evaluated for their capacity to use pretreated RS as substrate for the production of fermentable sugars to be transformed in biochemical compounds of industrial interest (e.i β-hydroxibutirate or biosurfactants) [21, 22]. However, to the best of our knowledge the capacity of *Bacillus* strains for the growth and release of P from RS as the only source of P has not been evaluated. The aim of this work was to assess the capacity of rice growth-promoting rhizobacteria for releasing P from both mineral insoluble P and organic sources of P like phytic acid and RS. We hypothesize that selected *Bacillus* strains are able to release P from rice plant residues, and this may contribute to their plant growth-promoting activity under P-limiting conditions.

Materials and Methods

Bacterial Isolates

Microorganisms evaluated in the present work were isolated from rhizosphere soils from several plant species: 20 isolates were obtained from rice (*Oryza sativa*) rhizosphere, 22 isolates from maize (*Zea mays*), eight from a Colombian native palm tree (*Carludovica palmata*) and one isolate obtained from rhizospheres of the potato (*Solanum tuberosum*) (Table S1). These isolates were obtained from the collection of microbial strains of the Biotechnology Institute of the Universidad Nacional de Colombia in Bogotá. The strain IBUN 2755 is under the genetic access contract No 160 de 2017 from the Ministry of Environment of Colombia.

Plant Growth Promotion Using an Inert Substrate Under Greenhouse Conditions

In order to evaluate the effect of inoculating *Bacillus* strains on plant development, an experiment under greenhouse conditions was performed using rice seeds Fedearroz 733 cultivar (F733 cv). To carry out the experiment, bacterial isolates were plated on the sporulation *Bacillus* medium suggested by Posada-Uribe et al. [23] with some modifications: glucose 3 $g.L^{-1}$, $MgSO_4 7H_2O$ 0.12 $g.L^{-1}$, KH_2PO_4 6.8 $g.L^{-1}$, tryptone 7 $g.L^{-1}$, $FeSO_4.7H_2O$ 0,028 $g.L^{-1}$, $ZnSO_4 7H_2O$ 0.014 $g.L^{-1}$, $CaCl_2.2H_2O$ 0.088 $g.L^{-1}$, $MnCl_2 4H_2O$ 0.02 $g.L^{-1}$ and bacteriological-agar 1% w/v. The cultures were incubated for 10 days at 28° C, estimating subsequently the percentage of endospores through microscopic observations. All biomass was suspended in 20 mL of sterile distilled water (SDW) and heated at 80 °C for 10 min before being stored in amber vials of 50 mL at 4 °C previously to their use. The plant growth-promoting activity was evaluated in black polyethylene bags with 1 kg of a mixture of sand and gravel (1:1). Rice seeds, superficially disinfected [24] were germinated for 4 days in a petri dish with agar-water 1% w/v in darkness. Seedlings were inoculated for 30 min with bacterial suspensions at 10^7 spores.mL^{-1}. An un-inoculated treatment was used as negative control. Two seeds were sown in each bag and only one plantlet was maintained after 10 days of growth. Each treatment had three experimental replicates and three sampling units in a completely randomized design. The plants were left under greenhouse conditions for 30 days with a photoperiod of 12 h:12 h light:darkness at 28 ± 5 °C with a relative humidity of 40%. The plants were irrigated so that the substrate remained saturated during the period of the experiment. Nutritional requirements were satisfied with the application of a Hoagland solution at 20% without P [25]. Then 260 mg per bag of rock phosphate was applied at the time of sowing. After the growth period the seedlings were carefully removed from the substrate and stems, root lengths, and dry weight were evaluated (drying process in an oven at 60 °C for 5 days). The experiment was repeated once in time.

Phosphate Solubilization and Mineralization In Vitro

Quantitative evaluation was performed in 15 mL of NBRIP liquid medium supplemented with tricalcium phosphate and aluminum phosphate at 0.5% and 0.3% w/v respectively [26]. Afterwards, 150 µL of the bacterial suspension adjusted to 1×10^7 spores.mL^{-1} were inoculated and incubated for five days at 150 rpm and 28 ± 2 °C. Solubilizing activity was quantified following the manufacturer´s

instructions for Spectroquant® Phosphate Test (Merck®, Darmstadt, Germany) in a microplate reader at a wavelength of 405 $_{nm}$. A qualitative assessment of phytic acid mineralization was performed using solid NBRIP supplemented with phytic acid at 1% w/v phytic acid dodecasodium salt from Sigma Chemical Co, adjusting to pH 7 [27]. Following this, 10 µL of a bacterial suspension (10^7 spores.mL^{-1}) was inoculated onto the medium´s surface. After 5 days of incubation at 28° C, the halo´s production was evaluated, and mineralization efficiency (ME) was measured through the formula $ME = \frac{\text{Halo diameter}}{\text{Colony diameter}} * 100$. Four experimental replications were used per treatment with *Pseudomonas fluorescens* IBUN-00466 strain used as a positive control [28] while a negative control without microbial inoculation was used.

Production of Indole Acetic Acid Compounds

The capacity to produce indolic compounds was evaluated using 9 mL of LB broth supplemented with 1 mL of 0.3 mM L-tryptophan in amber bottles. 100 µL of each bacterial suspension adjusted to a concentration of 10^7 spores.mL^{-1} were inoculated, and each medium was incubated in agitation for three days at 150 rpm and at 28 ± 2 °C. An aliquot of the sample was mixed with the reagent of Salkowski in a 1:1 ratio [29]. Last, the amount of indolic-like compounds present in the sample reaction was quantified colorimetrically at 540 $_{nm}$. The evaluation of each isolate was run in triplicate. SDW, instead of the bacterial suspension, was used as a negative control.

Release of Phosphorus from Rice Straw

Flasks with 125 mL of capacity previously prepared with 27 mL of basal salt medium supplemented with one gram of RS fragments of around 2 cm of length (RS basal medium-RSBM), with a composition of cellulose, hemicellulose, lignin and P of 34.2%, 29.1%, 2.9% and 0.13% respectively as previously described [30], was sterilized at 121 °C and 15 psi during 20 min The flasks were then inoculated with 3 mL of a suspension of selected isolates at 1×10^7 spores.mL^{-1}. The media were incubated in agitation at 150 rpm and 28 ± 2° C for 20 days. Soluble P at 0, 3, 12 and 20 days of incubation was quantified after inoculation, following the manufacturer´s instructions of the Spectroquant® phosphate test previously described. Three replicates were used per treatment. RSBM without bacterial inoculation were used as a negative control. *Pseudomonas fluorescens* IBUN-00466 strain was used as a positive control.

Molecular Identification of Phytate Genes from the PGPR Strains

Fifteen *Bacillus* isolates were used to identify the presence of the phytase coding gene. DNA was extracted by using the alkaline lysis protocol previously described [31]. A set of primers were designed after finding conserved regions in *phy* genes, previously reported for the genus *Bacillus* at the NCBI database (http://www.ncbi.nlm.nih.gov/). Primers: AEFB*phy*F: 5′-GCTGATGATCCTGCGATTTG-3′ and AEFB*phy*R: 5′-GTCAGTTTTCTCGGGTCAAC-3′ were used to amplify a region of about 980 bp. The *phy* gene was amplified by PCR in a final volume of the reaction mixture of 50 µL, using a final concentration of dNTPs at 0.2 mM, 0.4 µM of each primer and 1 U of Biolase DNA Pol. The PCR conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 2 min and a final elongation cycle of 72 °C for 10 min. PCR products were purified using the Promega Wizard® PCR Clean-Up system and sequenced by Sanger capillary sequencing system at SIGMOL, Institute of Genetics at the Universidad Nacional de Colombia.

Plant Growth Promotion of Bacillus Strains Using Soil Amended with Rice Straw

The three best isolates of *Bacillus*, in terms of capacity to promote the growth of plants in inert substrates, solubilize/mineralize P, release P from RS, and be positive for the amplification of the *phy* gene were evaluated for their ability to promote plant growth from soil amended with RS as the sole source of P. Rice cv Fedearroz 733 seedlings with four days of germination were inoculated with a bacterial suspension adjusted to 5×10^7 spores.mL^{-1}. Two seeds were planted in black polyethylene bags (one plantlet was maintained after 10 days of growth) with 1 kg of a substrate prepared with soil mixed with gravel and a standard commercial conditioner (Terra-Green® Absorbent Granules) (70:25:5), plus 10 g of RS fragments of 5 cm of length. RS was previously fertilized with urea (equivalent to a dose of 150 kg.ha^{-1}) and incubated in darkness on the soil surface for 20 days to facilitate its degradation. It was then incorporated into the soil. The assay was conducted under greenhouse conditions at 28 ± 5 °C with a relative humidity of 20%–40%. Soil fertilization was done using a modified Hoagland nutrient solution (without P and N) [25] at 20%. Soil was watered maintaining field capacity. After 40 days of growth, shoot, root length, and dry weight of plants were measured (a graphical description of the set-up is in Fig S1). Additionally, the following variables were determined: P soluble in the soil [32], microbial P biomass [33], and P in plant tissue [34]. Five replications were used per treatment. A negative control without PGPR application, was used.

Statistical Analysis

Data were analyzed through an ANOVA test after confirmation of their normal distribution and homoscedasticity through the Shapiro–Wilk and Levene test, respectively. The Duncan test ($\alpha = 0.05$) for treatments means comparison, was used. The data were analyzed with the statistical package SAS 9.1.3.

Results

Plant Growth Promotion Using an Inert Substrate Under Greenhouse Conditions

Fifteen out of the 51 isolates evaluated in this study were highlighted due to their ability to promote growth in rice seedlings. These increased the agronomic variables up to 20.5% and 94.3% for stem and root length and up to 54.9% and 95.8% by dry weight of stem and root compared to the untreated control (Table S2). Selected strains were again evaluated under the same experimental conditions (Table 1). Isolates IBUN-02755, -02704, -02724, -00328 and -00248, were confirmed in terms of their activity as PGPR of rice plants, presenting statistically significant increases in terms of stem and root length up to 31.9% and 56.2% and increases of root dry weight up to 169.8%.

Production of IAA-Like Compounds

Eleven of the fifteen isolates evaluated in this study showed an IAA-like production of compounds, highlighting the IBUN-00270, -00362 and -00255 strains with values between 9.9 and 29.1 µg IAA-like compounds.mL^{-1}, while IBUN-02704, -02694, -02723 and -02711 isolates did not produce IAA-like compounds (Table 2). *Bacillus* strains that statistically promoted the growth of rice plants with any variable produced low levels of IAA-like compounds (between 0.0 and 2.3 µg IAA-like compounds.mL^{-1}) (Table 2). Besides, the three strains with the highest production of IAA-like compounds did not increased plant growth in relation to untreated controls (Table 2), suggesting a relation between low level of IAA-like compounds production and plant growth promotion.

Phosphate Solubilization In Vitro

The phosphate solubilizing capacity of selected strains using tricalcium phosphate in liquid NBRIP medium ranged from 142 mg of PO_4.L^{-1} of strain IBUN-02712 and 3.4 mg PO_4.L^{-1} for strain IBUN-02711. The highest value was 9.8% higher than the phosphate solubilization capacity of the positive control strain IBUN-00466 (129.3 mg PO_4.L^{-1}). Nine strains (IBUN-02694, -02712, -02704, -00255, -00263, -02755, -02724, -00270 and -00362) showed poor capacity to solubilize $AlPO_4$ but a middle to high capacity to solubilize tricalcium phosphate (Table 2).

Table 1 Effect of fifteen *Bacillus* strains in terms of length and dry weight of treated rice seedlings of F733cv after 30 days of growth under greenhouse conditions

Treatment	Stem length cm	Root length	Stem dry weight mg	Root dry weight
IBUN-02704	31.9 ± 5.9 (31.2%)^{a}	20.1 ± 4.6 (48.8%)^{ab}	64.1 ± 15.8 (41.5%)^{a}	24.0 ± 8.4 (126.4%)^{ab}
IBUN-02724	31.8 ± 3.6 (30.8%)^{a}	19.4 ± 4.2 (43.7%)^{ab}	61.7 ± 11.2 (36.2%)^{ab}	17.5 ± 4.6 (65.0%)^{abc}
IBUN-00328	31.5 ± 2.4 (29.6%)^{a}	19.1 ± 3.6 (41.4%)^{ab}	63.3 ± 5.0 (39.7%)^{a}	23.4 ± 6.3 (120.7%)^{ab}
IBUN-02755	31.3 ± 2.8 (28.8%)^{ab}	21.1 ± 4.5 (56.2%)^{a}	66.0 ± 8.7 (45.6%)^{a}	28.6 ± 4.0 (169.8%)^{a}
IBUN-02694	29.5 ± 4.1 (21.3%)^{ab}	16.8 ± 0.9 (24.4%)^{ab}	54.2 ± 10.0 (19.6%)^{ab}	17.5 ± 4.6 (65.0%)^{abc}
IBUN-00263	29.3 ± 1.8 (20.5%)^{ab}	14.7 ± 0.5 (8.8%)^{ab}	56.5 ± 5.1 (24.7%)^{ab}	18.3 ± 5.7 (72.6%)^{abc}
IBUN-00255	29.3 ± 1.1 (20.5%)^{ab}	19.3 ± 0.7 (42.9%)^{ab}	65.0 ± 1.4 (43.4%)^{a}	20.6 ± 4.4 (94.3%)^{abc}
IBUN-00248	28.7 ± 4.5 (18.1%)^{ab}	17.0 ± 4.2 (25.9%)^{ab}	54.5 ± 19.1 (20.3%)^{ab}	22.2 ± 12.1 (109.4%)^{ab}
IBUN-00270	28.4 ± 3.5 (16.8%)^{abc}	19.1 ± 2.7 (41.4%)^{ab}	50.2 ± 16.6 (10.8%)^{ab}	15.1 ± 4.2 (42.4%)^{bc}
IBUN-02711	28.3 ± 2.0 (16.4%)^{abc}	17.0 ± 0.7 (25.9%)^{ab}	59.4 ± 10.0 (31.1%)^{ab}	18.2 ± 1.8 (71.6%)^{abc}
IBUN-02712	27.7 ± 4.9 (13.9%)^{abc}	17.3 ± 4.4 (28.1%)^{ab}	47.4 ± 5.7 (4.6%)^{ab}	17.7 ± 5.0 (66.9%)^{abc}
IBUN-00362	26.8 ± 1.2 (10.2%)^{abc}	15.7 ± 2.0 (16.2%)^{ab}	50.7 ± 8.6 (11.9%)^{ab}	18.0 ± 1.5 (69.8%)^{abc}
IBUN-02723	26.8 ± 6.1 (10.2%)^{abc}	16.3 ± 2.8 (20.7%)^{ab}	54.5 ± 25.3 (20.3%)^{ab}	18.4 ± 8.3 (73.5%)^{abc}
IBUN-00175	26.1 ± 3.2 (7.3%)^{abc}	17.1 ± 3.6 (26.6%)^{ab}	49.5 ± 18.8 (9.2%)^{ab}	16.5 ± 5.5 (55.6%)^{bc}
SDW + P	24.3 ± 0.9 (0.0%)^{bc}	13.5 ± 5.3 (0.0%)^{b}	45.3 ± 12.1 (0.0%)^{ab}	10.6 ± 2.5 (0.0%)^{c}
IBUN-00241	21.6 ± 3.9 (−11.1%)^{c}	13.8 ± 2.7 (2.2%)^{b}	36.3 ± 15.3 (−19.8%)^{b}	9.7 ± 2.5 (−8.4%)^{c}

SDW + P control without bacterial inoculation. The data correspond to the average of three experimental units per treatment and three sampling units per experimental unit. In parentheses percentages increase over the control are displayed. Different letters indicate statistical differences with the multiple comparison test of Duncan ($\alpha = 0.05$)

Table 2 Functional characterization in terms of phosphate solubilization and mineralization capacity, IAA-like compounds production and the presence of phytase coding gene of fifteen selected *Bacillus* strains under in vitro conditions

Treatment	P solubilization (mg $PO_4.L^{-1}$)		P mineralization*	IAA Production	*phy* gene presence
	$Ca_3(PO_4)_2$	$AlPO_4$	ME	($\mu g.mL^{-1}$)	
IBUN-02712	142.0 ± 11.9^{a}	4.9 ± 2.0^{b}	130.5 ± 4.7^{e}	0.7 ± 0.2^{e}	–
P. fluorescens IBUN-00466	129.3 ± 49.1^{ab}	0.0 ± 0.0^{d}	300.0 ± 0.0^{b}	ND	ND
IBUN-02694	110.2 ± 3.4^{ab}	12.2 ± 1.3^{a}	355.2 ± 74.2^{a}	0.0 ± 0.0^{e}	–
IBUN-02724	95.4 ± 27.3^{b}	1.4 ± 0.3^{cd}	135.6 ± 6.6^{e}	1.9 ± 0.2^{de}	+
IBUN-00362	$95,0 \pm 40.2^{b}$	0.3 ± 0.1^{cd}	251.8 ± 33.1^{c}	20.7 ± 2.8^{b}	–
IBUN-00241	50.9 ± 5.9^{c}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	1.0 ± 0.2^{e}	–
IBUN-02704	39.1 ± 8.2^{cd}	2.6 ± 0.0^{c}	136.3 ± 19.0^{e}	0.0 ± 0.0^{e}	+
IBUN-00328	34.8 ± 11.1^{cd}	0.0 ± 0.0^{d}	199.5 ± 39.3^{d}	2.3 ± 0.2^{de}	–
IBUN-00270	34.5 ± 0.7^{cd}	1.2 ± 0.2^{cd}	161.5 ± 15.7^{e}	29.1 ± 3.7^{a}	+
IBUN-00175	25.7 ± 6.2^{cd}	ND	202.5 ± 30.7^{d}	2.3 ± 0.6^{de}	–
IBUN-00255	24.0 ± 12.7^{cd}	2.0 ± 2.6^{cd}	0.0 ± 0.0^{f}	9.9 ± 0.7^{c}	–
IBUN-00248	23.0 ± 6.7^{cd}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	1.8 ± 0.1^{de}	–
IBUN-02755	22.7 ± 2.0^{cd}	1.6 ± 0.3^{cd}	203.1 ± 31.3^{d}	0.2 ± 0.0^{e}	+
IBUN-00263	15.1 ± 1.9^{cd}	1.9 ± 1.0^{cd}	147.1 ± 5.8^{e}	3.8 ± 1.4^{d}	+
IBUN-02723	4.3 ± 11.4^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}	–
IBUN-02711	3.4 ± 0.4^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}	–
SDW	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}	–

ME mineralization efficiency (diameter of the halo/diameter colony × 100), *SDW* negative control applying sterile distilled water, without bacterial inoculation, *P. fluorescens* IBUN-00466: positive Control for tricalcium phosphate solubilizing and mineralizing of phytic acid activity in vitro. The data correspond to the mean of three experimental units per treatment. Different letters indicate statistical differences with the multiple comparison test of Duncan ($\alpha = 0.05$)

ND not determined

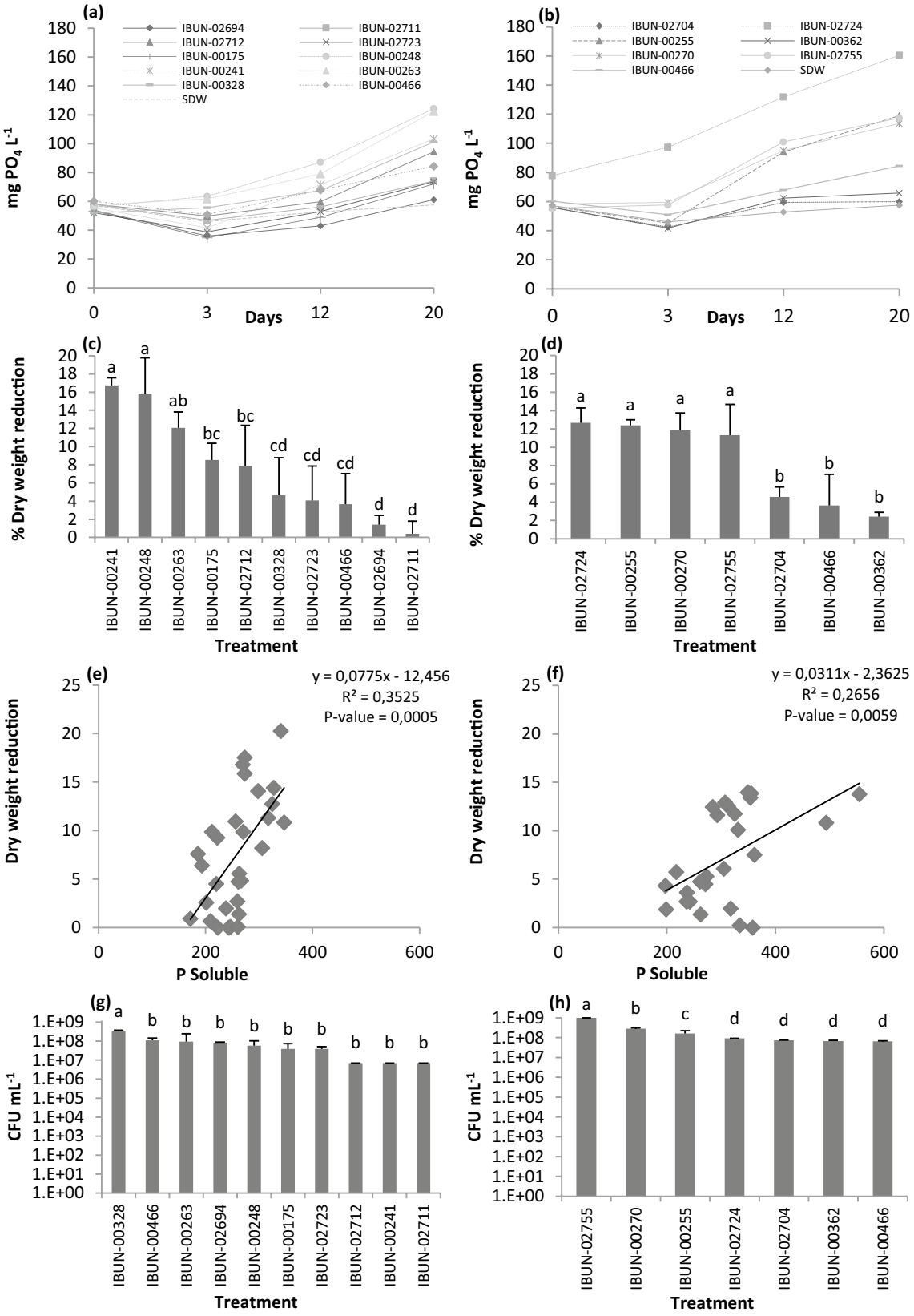
*Four experimental units per treatment

Ten strains had the ability to mineralize phytic acid, highlighting strain IBUN-02694 which showed a ME of 355%, overcoming the positive control IBUN-00466 that had a ME of 300%. The IBUN-02694 strain also showed a relatively good phosphate solubilization capacity with a release of orthophosphate of 110 mg $PO_4.L^{-1}$ and 12.2 mg $PO_4.L^{-1}$ for calcium and aluminium phosphate, respectively. Similarly, the strains IBUN-00362, -02712 and -02724 showed both relatively good solubilization (near or above 100 mg $PO_4.L^{-1}$ for tricalcium phosphate and between 0.3 and 4.9 mg $PO_4.L^{-1}$ for aluminum phosphate) and a mineralization capacity (ME at least 30% larger than the colony size). Other strains showed a middle to low solubilization capacity (between 3.4 and 39.0 mg $PO_4.L^{-1}$) and some showed a relatively good mineralizing capacity (with a clarification halo size above 36% of the colony size). However, it is worth mentioning that none of the strains presented both activities (solubilization and mineralization capacity) better than the control strain IBUN-00466 (Table 2).

Release of Phosphorus from Rice Straw

The IBUN-00248, -00263, -00241, -02724, -00255, -02755 and -00270 strains released 124, 122, 160, 119, 117, 113 and 103 mg $PO_4.L^{-1}$, respectively after 20 days of incubation in a liquid media with RS as the only source of P (Fig. 1a, b and Table S3), which was more than two fold that of the un-inoculated control (57.4 mg $PO_4.L^{-1}$). Similarly, the IBUN-00241, -00248, -00263, -02724, -00255, -00270 and -02755 isolates generated the highest percentages of dry weight reduction of RS (with values between 11.3% and 16.7%), and statistically significantly higher than the positive control *P. fl.* IBUN-00466 (3.65%) (Fig. 1c, d). It is worth mentioning that the correlation coefficient between the release of P and the reduced dry weight of RS showed a significantly positive correlation ($P \leq 0.05$) with a determination coefficient (R^2) of 0.35 and 0.26 for the first and second fermentation (Fig. 1e, f respectively). This suggested that between 26 and 35% of the variation of RS dry weight, could be explained by the release of P.

All bacterial isolates were inoculated at a starting concentration of 1×10^6 spores.mL^{-1} and showed an increase of biomass after 20 h of incubation (Fig. 1g, h). This indicated an active growth of the microorganisms using RS, not only as a source of P but also as a source of carbon, because RSBM has a fairly low concentration of carbon other than RS.


(a)
IBUN-02694
IBUN-02712
IBUN-00175
IBUN-00241
IBUN-00328
SDW
IBUN-02711
IBUN-02723
IBUN-00248
IBUN-00263
IBUN-00466
mg PO4 L-1
Days
(b)
IBUN-02704
IBUN-00255
IBUN-00270
IBUN-00466
IBUN-02724
IBUN-00362
IBUN-02755
SDW
mg PO4 L-1
Days
(c)
% Dry weight reduction
IBUN-00241
IBUN-00248
IBUN-00263
IBUN-00175
IBUN-02712
IBUN-00328
IBUN-02723
IBUN-00466
IBUN-02694
IBUN-02711
Treatment
(d)
% Dry weight reduction
IBUN-02724
IBUN-00255
IBUN-00270
IBUN-02755
IBUN-02704
IBUN-00466
IBUN-00362
Treatment
(e)
y = 0,0775x - 12,456
R² = 0,3525
P-value = 0,0005
Dry weight reduction
P Soluble
(f)
y = 0,0311x - 2,3625
R² = 0,2656
P-value = 0,0059
Dry weight reduction
P Soluble
(g)
CFU mL-1
IBUN-00328
IBUN-00466
IBUN-00263
IBUN-02694
IBUN-00248
IBUN-00175
IBUN-02723
IBUN-02712
IBUN-00241
IBUN-02711
Treatment
(h)
CFU mL-1
IBUN-02755
IBUN-00270
IBUN-00255
IBUN-02724
IBUN-02704
IBUN-00362
IBUN-00466
Treatment

◂**Fig. 1** Results obtained during 20 days of incubating *Bacillus* in liquid culture with RS as the only source of P. Released P (**a** and **b**), dry weight reduction of RS (**c** and **d**), linear correlation coefficient between P solubilization from RS and percentage of dry weight reduction in RS (**e** and **f**), and biomass of Bacillus in the 20 day of the incubation (**g** and **h**). SDW: negative control without bacterial inoculation; *P. fl.* IBUN-00466: positive control. The data correspond to the mean of three experimental units per treatment. The experiment was repeated one time with similar results. Different letters indicate statistical differences with the multiple comparison test of Duncan ($\alpha = 0.05$)

Molecular Characterization

The amplification results showed the presence of the *phy* gene in the strains IBUN-02724, -02755, -02704, -00263 and -00270 (Accession Numbers in NCBI database KX900420, KX900421, KX900422, KX900418, KX900419 respectively). All of them, with the exception of IBUN-02704, showed best results in the release of P and dry weight reduction with the submerged liquid culture. It is important to mention also that strains like IBUN-00241, -00248 and -00255, despite having a good release of P and dry weight reduction from RS, did not show phytase activity nor *phy* gene sequences (Table 2).

A comparative analysis of sequences encoding the *phy* gene was conducted using the clustering method of neighbor joining. The analysis showed three clusters (with a bootstrap value of 1000), after comparing the sequences of our strains with similar sequences belonging to *Bacillus* strains obtained from the database of microbes at NCBI (Fig. 2). The first group was formed by strains of the *B. velezensis* where IBUN-00263, -02704, -02755 and -00270 strains were clustered. The second group has strains of *B. subtilis and B. amyloliquefaciens*, in which the IBUN-02724 strain was associated. Finally, the third clade involved *phy* sequences of strains of the species *Bacillus licheniformis* group (Fig. 2), having a percentage of maximum identity of 99% with *phy* gene sequences that belong to this species (data not shown).

Plant Growth Promotion by Bacillus Strains Under Greenhouse Conditions Using Soil and Rice Straw as Substrate

The IBUN-02755 strain showed a significant increase in terms of length and dry weight of shoots when compared with un-inoculated plants only grown with RS. Similarly, the IBUN-02704 strain increased the dry weight of root seedlings by 195%, compared to an un-inoculated treatment (Fig. 3). When treatments are compared with the control treatment without RS, the IBUN-02755 and -02704 showed statistically significant differences in shoot length with an increase of 24% and 19%. In terms of plant biomass three strains significantly increased the dry weight of stem and root of the seedlings compared to treatment without RS ($P \leq 0.05$ Duncan test) (Fig. 3).

Compared to the RS treatment, there is a higher soluble P content in the soils with the RS treatments inoculated with *Bacillus* strains, highlighting the RS + IBUN-02704 treatment, which showed the highest concentrations of P in the soil (13.5 $mg.kg^{-1}$ of the soil) (Table 3). This result suggests a mineralization capacity of the *Bacillus* strains from the RS, similar to the previous results obtained in the submerged culture with RS as the only source of P. On the other hand, addition of *Bacillus* strains in RS amended soil increased the microbial biomass of P in comparison to RS. More importantly, inoculated plants showed an increase in assimilated P up to 160% (RS + IBUN-02724), in comparison to the treatment with RS after 40 days of growth (Table 3).

Discussion

We hypothesize that the plant growth promotion activity of 15 selected strains should be associated with phytoestimulation through the production of plant growth regulator substances or the solubilization/mineralization of phosphates. Therefore, 15 mentioned isolates were evaluated in terms of their production of IAA-like compounds and their potential as phosphate solubilizers/mineralizers. Auxin phytohormones such as indole acetic acid are strong plant growth regulators able to mediate in formation of plant biomass with strong effects on shoot and root structure and vascular differentiation. Auxins of microbial origin have also been recognized as having an effect on plant growth promotion [35, 36]. This suggests that this is one of the pivotal plant growths promoting compounds produced by many PGPR strains. IAA-like compounds have been associated as major traits for plant growth promotion in rice plants [37], and several PGPR of rice plants producers of IAA-like compounds have been identified [24]. However, it is not clear yet which level of IAA-like compound productions by a particular rhizosphere strain is the most adequate for the promotion of growth in rice plants, given the fact that high levels of IAA may also reduce plant growth [38]. In this context, our study suggests that PGPR producing low levels of IAA-like compounds is adequate for plant growth promotion in rice, however further studies confirming such condition as a prerequisite for the selection of PGPR in rice is needed.

NBRIP supplemented with tricalcium phosphate is one of the techniques most used in the isolation and selection of PSB. Such bacteria secrete different types of organic acids that act mainly as chelating agents over forms of inorganic insoluble phosphate releasing P that is then available for plants [17, 39, 40]. Several studies have reported isolates with the ability to promote growth in different crops trying to associate such ability with solubilization of tricalcium

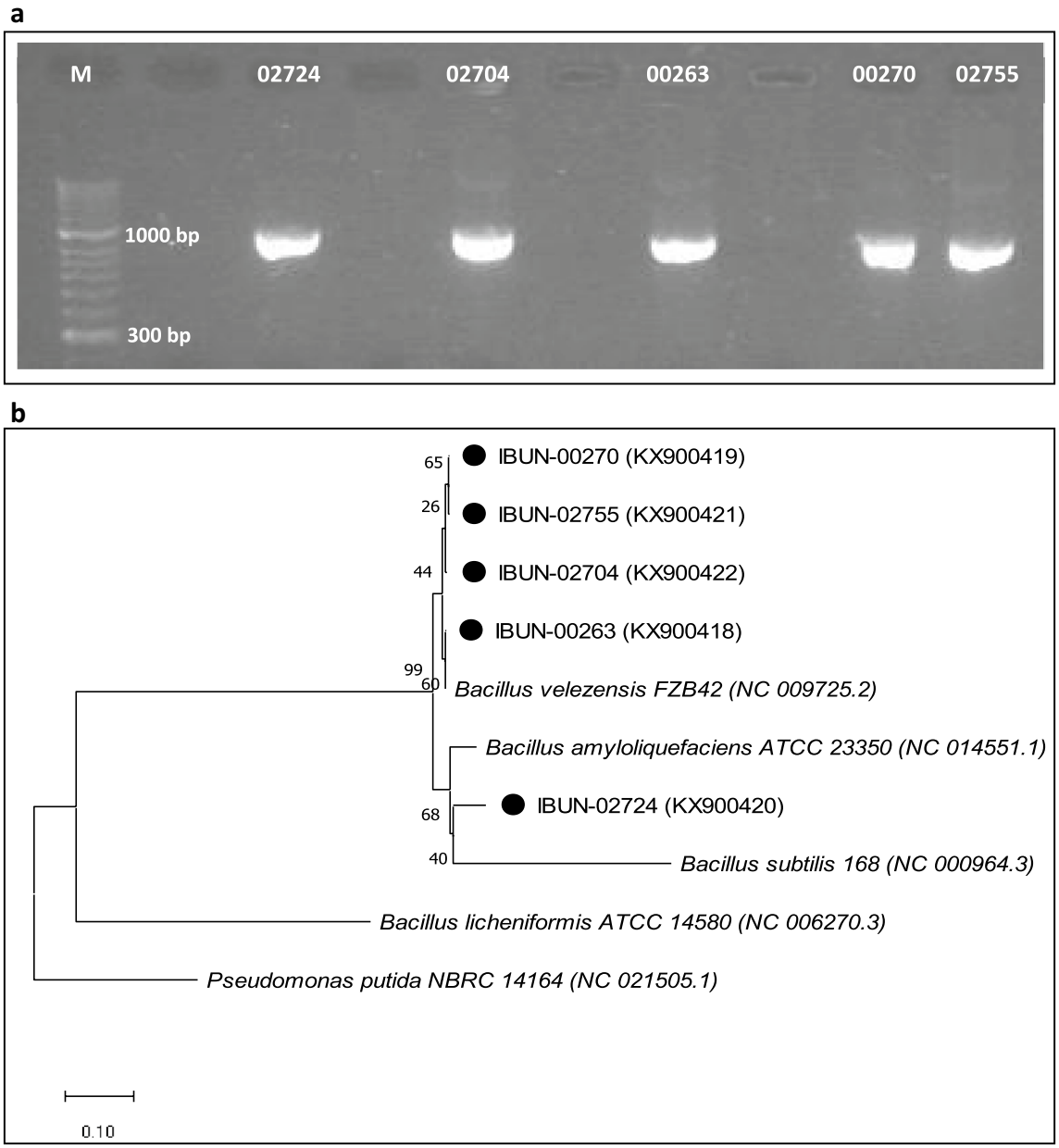


Fig. 2 PCR-amplified product (**a**) and Phylogenetic tree (**b**) derived from Neighbor-Joining analysis based on partial gene coding sequences for the enzyme phytase (*phy*) from *Bacillus* sp. M: DNA Ladder. Sequence alignment was performed using BioEdit Sequence Alignment Editor. The NCBI accession numbers of GenBank sequences are shown in parentheses. The numbers at each node represents the bootstrap number obtained after 1000 replications with the program MEGA 10.2.0. ● Strains from this study. *Pseudomonas fluorescens* (LT907842.1) *phy* sequence was used as outgroup

phosphate [41–43]. However, Bashan et al. [44] have indicated the need to form a more holistic characterization of PSB before claiming this as the mode of action of a particular PGPR. Attending this call, the solubilization capacity of our strains using AlPO4 was also evaluated, especially due to the fact that agricultural production in Neotropical areas mostly occurs on acid soils where such kinds of P salts are formed [45].

Rice straw incorporation has been considered in rice crops not only as a carbon amendment to nurture soil organic matter, but also as a management strategy to increase phosphate availability and P plant uptake [11, 12]. In this context and considering the results mentioned above, in terms of the mineralization of phytic acid in vitro, the capacity of *Bacillus* strains to release P from an organic substrate such as RS was evaluated. Four of the isolates (IBUN-00263, -02724, -02755 and -00270), that were able to release P from RS

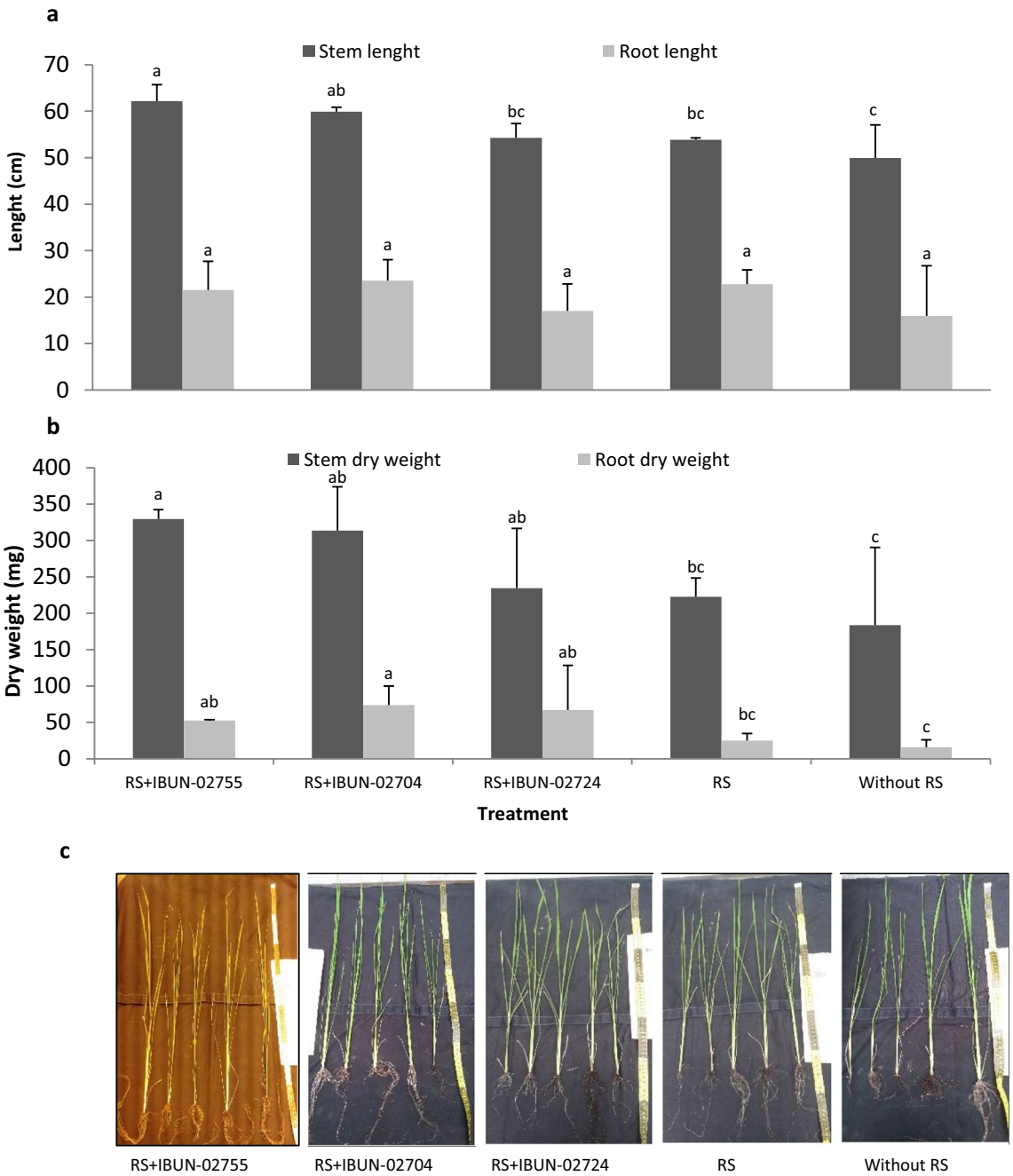


Fig. 3 Seedling length (**a**) and dry weight (**b**) after 40 days of growth in soil-rice straw using three *Bacillus* strains. Pictures showing the effect of treatments on rice plants. (**c**). *RS* treatment without application of PGPR. Without RS: treatment without rice straw amendment, without PGPR and with direct application of urea on the soil (conventional fertilization treatment). The data correspond to the average of five experimental units per treatment. Different letters indicate significant differences between treatments with multiple comparison test of Duncan ($\alpha = 0.05$)

also showed activity on a solid medium with phytic acid (Table 2), demonstrating consistency in the degradation of organic P sources. Phosphate solubilizing and mineralizing bacteria have been reported to produce several kinds of phosphoric hydrolases including phosphomonoesterases and phytases [5, 18, 33, 46]. Such enzymatic activity has been associated with an increased release of P from organic substrates such as phytic acid added with rice brand [47] or compost-based RS, vermicompost, and compost farm waste [48]. The significant correlation of up to 35% found in our study between the release of P and the dry weight reduction of RS is interesting as the content of P in the RS is

Table 3 Available P in soil, soil microbial biomass of P and, P in plant tissue, after 40 days of growth of inoculated rice plants of F733 cv under greenhouse conditions

Treatment	Soil P mg $PO_4.Kg^{-1}$ soil	Microbial biomass P mg $P.pot^{-1*}$	P in plant tissue
RS	10.1 ± 0.4^{b}	1.1 ± 0.8^{b}	1.1 ± 0.1^{b}
RS + IBUN-02755	11.2 ± 0.3^{ab}	2.8 ± 0.6^{a}	1.9 ± 0.2^{ab}
RS + IBUN-02704	13.5 ± 2.4^{a}	2.2 ± 0.9^{ab}	2.3 ± 0.1^{a}
RS + IBUN-02724	11.3 ± 1.2^{ab}	1.7 ± 0.2^{ab}	2.9 ± 0.3^{a}

RS treatment without application of PGPR. The data correspond to the mean of three experimental units per treatment. Different letters indicate significant differences between treatments with multiple comparison test of Duncan ($\alpha = 0.05$)

*mg of P in stem dry weight

just around 0.13% [30]. This indicates that other enzymes besides phosphatases should explain such dry weight reduction. These reports support the hypothesis that the *Bacillus* strains selected in this study have the potential to contribute to the degradation of RS and make essential nutrients such as P available to plants, contributing to its growth.

It is important to mention also, that strains like IBUN-00241, -00248 and -00255, which despite having good releases of P and dry weight reductions from RS, showed neither phytase activity nor *phy* gene sequences (Table 2), suggesting that other kind of phosphatases such as phosphomonoesterases produced by *Bacillus* strains, like those reported by Jorquera et al. [46], may contribute to the release of P from RS. However, as mentioned below it should not be the only activity involved. Studies show that in terms of chemical composition RS predominantly contains cellulose (32–47%), hemicellulose (19–27%) and lignin (5–24%) [30]. Therefore, large percentages of dry weight reduction that was obtained should be mediated by ligninolytic and cellulolytic activities [49] or by the action of other enzymes as proteases, amylases, xylanases and, lipases, some of which have been identified also in some of the strains mentioned in this study (results not shown).

As previously mentioned, the neighbor-joining algorithm applied to the *phy* sequences, clustered the *phy* positive strains within the *B. velezensis* and *B. subtilis*/*B. amyloliquefaciens* group (Fig. 2). However, a phylogenetic analysis using sequences of 16S rDNA, showed that strains of IBUN-02704 and –00270 belong to the group of *B. megaterium*/*B. aryabhattai*, -00263 to *B. subterraneus* group, and -02724 and -02755 to the *B. velezensis*/*B. amyloliquefaciens* group (Fig. S2). This result, in addition to the higher power of the *16S rDNA* gene as a phylogenetic analytic tool can be related to the lack of *phy* gene sequences available for most *Bacillus* species. In fact, to the best of our knowledge, this is the first report in the literature of a *phy* gene partial sequence for a *B. subterraneus* strain. Alternatively, the non-amplification of the *BPP* gene from six of the phytate-hydrolysing isolates (Table 2) described in this study may be also an indication of the presence of an unknown *BPP* genetic diversity or the presence of other classes of phytase in *Bacillus* strains, as suggested by Sanguin et al. [7], when they found similar results after using three degenerate BPP-specific primers.

Tropical soils usually contain total P at concentrations that are orders of magnitude greater than those of plant-available P. Large proportions of this P correspond to organic forms or are fixed at the surface of soil minerals that are two P reservoirs unavailable to plants [45]. PSB may affect the P supply to plants in different ways by immobilizing P in a microbial biomass or by releasing P from its natural reservoirs, through P mineralization and/or by solubilization of mineral fixed phosphate [45]. To know if evaluated *Bacillus* strains may have any of these effects on rice plant growth, the labile P was determined in the soil as well as the microbial biomass of P. The results can be related to the higher capacity of strains for releasing P out of RS, suggesting that this P was directly available and taken up by the rice plants. In a similar assay Duarah et al. [50], found higher phosphatase activity in rice planted soils inoculated with PSB. They even found a reduction of soluble P in the soil, and a higher P assimilation by the rice plants after 30 days of growth. This allowed them to suggest that such effects should be mediated by the inoculation with at least some PSB. Ramesh et al. [33] finds increases in the microbial biomass of P in the soil after application of microbial bioinoculants. This was expected by the effect of the P assimilation of the microorganisms applied to the soil. This may not be a negative effect for plant growth, as this can be a source of P of short renewal's time and therefore, it could provide a kind of slow release of inorganic P that can be used by plants later during the crop cycle.

Selected bacterial strains, IBUN-02704, -02724 and -02755, were able to release P from RS in comparison with treatments without bacteria. Besides, when bacterial strains were inoculated in rice seeds planted in soil amended with RS as the only source of P, two strains (IBUN-02755 and -02704) showed significantly higher plant length and biomass. However, rice plantlets did not show a higher P uptake, suggesting that their capacity for mineralization of P is not directly related to their plant growth promoting

capacity, at least not at the initial vegetative stages evaluated in this study. It is worth mentioning that inoculation of those strains increased the microbial biomass of P in the soil, such assimilated P could become available for plants later during the crop cycle when the microorganisms die and are subject to decomposition [5]. On the other hand, the strain IBUN-02724 fulfills the criteria as P solubilizer and mineralizer bacteria, since it not only is able to release organic P (from RS and phytic acid), and from insoluble mineral P sources (Ca_3 $(PO_4)_2$ and $AlPO_4$), but this strain also contribute to higher assimilation of P into the plants. The results obtained in this study suggest that the selected strains have great potential as promoters of plant growth, contributing beside to the degradation of crop residues such as RS. These strains could be implemented for the formulation of the future generation of inoculants in rice cultivation. However, additional studies are needed to confirm the benefits in field trials.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00284-021-02354-7.

Acknowledgements The authors would like to acknowledge the Program of Science, Technology and Agricultural Innovation of COLCIENCIAS (RC No. 0145-2013), and the Research Direction of the Universidad Nacional de Colombia, Bogotá for the economic support of this study.

Author contributions LFGR carried out experimental work. LFGR and DUV co-wrote the manuscript. LFGR and DUV conceived and designed the study and contributed to interpretation of results, read and approved the final draft.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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