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Sustainable Management with Mycorrhizae and Phosphate Solubilizing Bacteria for Enhanced Phosphorus Uptake in Calcareous Soils

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Abstract: Low availability of phosphorus (P) in calcareous soils is a major problem for sustainable improvement in cereals crops yield. A higher amount of calcium in soils precipitates the P, thus making it immobile in soil. Inoculation of arbuscular mycorrhizal fungi (AMF) and phosphate solubilizing bacteria (PSB) could be helpful in the sustainable management of immobile P in soil. However, their combined use in releasing P from rock phosphate (RP) in alkaline calcareous soils have been little investigated. In this regard, two successive field experiments were conducted to assess the interactive inoculation potential of AMF and PSB strain *Bacillus* sp. PIS7 with RP on the yield and P uptake of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) crops in alkaline calcareous soil. The first field experiment was conducted in a complete randomized block design with 10 treatments and three replications by inoculating maize seeds with AMF and *Bacillus* sp. PIS7 inoculum alone and in combination with RP. Their performance was compared with single super phosphate (SSP) inorganic fertilizer. Afterward, the residual effects of inoculated AMF and *Bacillus* sp. PIS7 were investigated on wheat as a subsequent crop. Maize and wheat yield parameters, P uptake, AMF root colonization, and PSB population was measured. The results of both trials indicated the beneficial effects of AMF and *Bacillus* sp. PIS7 with RP in increasing the plants grain yield and P uptake until the second season after inoculation, as compared to controls. Likewise, maize and wheat roots colonization, PSB population density, and post-harvest soil properties were also improved by the combined inoculation of AMF and *Bacillus* sp. PIS7 with RP. It is concluded that PSB solubilizes the unavailable forms of P in combination with RP fertilizers in soil, and AMF ultimately transfers it to plants for growth promotion. Moreover, the combined inoculation of AMF and PSB with ground RP

had more potential to improve maize-wheat yields and P uptake comparable to those obtained by using expensive phosphatic fertilizers in P deficient calcareous pH soils.

Keywords: maize; mycorrhizae; plant nutrients; rhizobacteria; rock phosphate; wheat; *Zea mays* L.

1. Introduction

Plant growth and mineral nutrition depend largely on the phosphorus (P) content of the soil. Phosphorus is the second essential macronutrient required for plant development and productivity [1,2] and is involved in almost all the major metabolic and most important plant biochemical processes [3]. In many agricultural soils, P is abundantly distributed in both inorganic and organic forms, but its concentration and availability to plants is limited due to fixation, precipitation, and formation of soils complexes with other soil nutrients [4]. In order to compensate for this deficiency and sustain crop productivity, high doses of chemical P fertilizers are nowadays widely applied in agricultural soils [5].

The repeated use of chemical fertilizers at supra-optimum rates in modern agriculture is expensive [6] and has the potential to adversely affect the environment [7], soil microbial population [8] and soil health both in grain and bioenergy production systems [9–11]. However, there is also some risk of xenobiotic contaminated in manure used to fertilize agricultural soils [12–14]. A cheaper and more environmentally friendly alternative to commercial P fertilizers is the use of naturally occurring rock phosphate (RP) [15]. The north-western region of Pakistan contains millions of tons of recoverable RP deposits that could be used as an economical source of P fertilizers to increase the country's overall grain production. Despite this, the direct use of RP as a source of P fertilizers is more suitable for alkaline soils in the presence of certain amendments that can increase overall microbial solubilization of RP [16]. In the face of a growing global concern regarding climate change and the impact that this may have in current agricultural systems, research aiming to understand the agronomic implications following the utilization of these amendments is receiving greater attention. Soil microorganisms play a key role in the bioavailability of insoluble nutrients and source of nutrients as RP through different soil functional processes and are the key drivers in the biogeochemical cycling of nutrients, particularly N and P [17].

The rhizosphere contains a very broad and diverse group of beneficial soil microorganisms. Among these, arbuscular mycorrhizal fungi (AMF) and phosphate solubilizing bacteria (PSB) are essential in P availability and plant growth-promoting activities [18,19]. According to recent calculations, the total population of PSB in the soil can range between 1% and 50%, while that of AMF fluctuates between 0.1% and 0.5% [1]. AMF release protons and extends their hyphae for the uptake of both soluble and insoluble soil P by the plants [20], while PSB releases organic acids and phosphatases for mineralization of organic P in soil [21].

Dual inoculations of beneficial soil microorganisms, including rhizobacteria and AMF have been observed to enhance nutrients' uptake and production of different crops to a greater extent than single inoculation even under stress environment [22–29]. Research shows that the interaction of AMF with certain plant growth-promoting rhizobacteria enhances the activity of AMF in the rhizosphere, resulting in increases in P, Zn, and Cu uptake by maize plants [17,30]. In the specific case of P, this interaction allows the mycorrhizal mycelium to take the phosphate previously released by PSB, with a subsequent improvement in the overall P acquisition by the host plant [31]. As a result of this symbiotic interaction, the application of RP with the dual inoculation of P solubilizing microorganisms and AMF has the potential to provide a faster, increased, and more economical supply of P that would result in better and higher-yielding crops in the near future [32].

Additionally, the interaction between AMF and PSB not only solubilizes P from RP and increases crop performance and plant biological yield, but may also increase AMF spore number, mycorrhization, and PSB population density [16]. The bacteria with P solubilizing ability has also been found to

change the growth of mycorrhizal fungi in roots (outside and inside) and enhanced root length and phosphatase activity in the presence of soil native microorganisms [33].

The synergistic role between AMF and PSB is not well understood, and the application of these microbial groups is often masked and confounded by variations in both biotic and abiotic environmental factors for which the interaction of these microorganisms have often not been tested. Furthermore, in Pakistan, limited research has been conducted to understand the interactive inoculation potential of PSB and AMF on P solubility, and plant uptake both in laboratory and greenhouse conditions, and no attempts have been made to investigate these responses in maize and wheat productivity and P uptake under field conditions. In view of the importance of these microorganisms, and the use of powdered indigenous RP as a possible substitute for costly commercial P fertilizers over the long term, the present study was conducted to evaluate the field inoculation potential of AMF and PSB with RP on maize and wheat productivity and nutrient uptake in alkaline calcareous soil.

2. Materials and Methods

2.1. Arbuscular Mycorrhizal Fungi (AMF) Inoculum

The AMF commercial inoculum (Symbivit, Zivojin Rilakovic, Guntramsdorf, Austria) was obtained from the Division of Plant Protection, University of Natural Resources and Life Sciences Vienna, Austria. There were approximately 80,000 AMF spores L^{-1} in this inoculum with six different species of AMF, including *Glomus microagregatum*, *Funneliformis geosporum*, *Claroideoglomus etunicatum*, *Funneliformis mosseae*, *Rhizophagus intraradices*, and *Glomus claroideum* [34].

2.2. Preparation of Mycorrhizal Substrate for Field Application

Mycorrhizal substrate applied in the field experiment was prepared as a mixture of sand and soil (1:3 ratio) in eight big plastic pots (10 kg each). Previously, the substrate was analyzed for different physiochemical properties by the following standard procedures. Soil pH (7.88) was determined by adding 50 mL water to 10 g of soil sample. Following shaking and filtrate recovery, pH was determined with a pH meter by the method of McClean [35]. Substrate organic matter content (0.6%) was determined by the titration method of Nelson and Sommers [36]. Similarly, lime content (15.2%) was determined by the acid neutralization method of Richard [37]. The ammonium bicarbonate-diethylenetriaminepentaacetic acid (AB-DTPA) available P (2.1 mg·kg^{-1}) was determined by the procedure of Soltanpour and Schwab [38] by mixing 15 g soil with 30 mL of AB-DTPA solution in a beaker. After shaking and filtration, the sample was mixed with boric acid indicator for the determination of P on a spectrophotometer. Prior to adding it into the pots, the substrate was autoclaved at 121 °C for 30 min to eliminate natural microorganisms. Then, each pot was planted with three maize seeds and inoculated with 100 mL of commercial crude AMF inoculum. The plants were properly irrigated and maintained throughout the growing season with a mean temperature of 28 °C in March and 35 °C in May. The plants were harvested after 80 days, and the whole root system with soil was removed from each pot. The air-dried soil and roots were having an average of 90 spores/20 g soil with 80% AMF colonization, similar to the set up previously obtained by McGonigle et al. [39]. All roots were cut carefully and mixed with the soil and stored at 4 °C at the Soil Biology laboratory of the Department of Soil and Environmental Sciences, The University of Agriculture, Peshawar, Pakistan, before field application.

2.3. Phosphate-Solubilizing Bacteria (PSB) Inoculum

Inoculation effects of the most efficient PSB strain (*Bacillus* sp. PIS7) selected previously in pot experiment [16] was further investigated in combination with AMF inoculum at field conditions on different maize growth parameters. Initially, this bacteria was isolated from the soil attached to sorghum roots in irrigated areas of Peshawar, Pakistan (Latitude 34°0′ N and 71°35′ E) [16]. The inoculum was prepared by growing a single colony of *Bacillus* sp. PIS7 in 100 mL of Pikovskaya's nutrients broth in 250 mL flasks that were incubated in a growth chamber for five days on an orbital shaker

(120 rpm) at 28 °C. To measure the microbial concentration, 1 mL of the broth culture was spread on the plates containing Pikovskaya's agar media, according to the method described by Pikovskaya's [40]. Finally, the strength of the culture was adjusted to approximately 108 colony-forming unit (cfu)/mL [41]. The broth cultures (100 mL) were mixed with 1 kg autoclaved soil (carrier material) and stored at 4 °C before field application.

2.4. Field Set Up for Maize Growth (Experiment 1)

This field experiment was conducted at the New Developmental Malakandher Research Farm of The University of Agriculture, Peshawar, Pakistan. The mean air temperatures during the crop growth cycle (i.e., between 5 June to 10 September 2016) ranged between 34 and 37 °C. The experimental site was plowed twice with a cultivator to a depth of 30 cm, followed by one passage of a rotavator, and then harrowed for soil leveling and proper seedbed preparation. Each experimental unit (EU) was 3 m wide by 4 m in length (total of 12 m^2 per EU) with six rows planted at a 65-cm distance in each EU. The plant to plant distance within a row was maintained at 25 cm with a uniform population of 12 maize (cv. "Azam") plants per row at each EU at the three-four leaves (V3-V4) emerging stage. The soil used in this experiment was silty clay, alkaline calcareous in reactions with low N (0.08%), AB-DTPA available P (2.8 $mg{\cdot}kg^{-1}$), organic matter content (0.9%), pH (7.83), lime (15.3%), and electrical conductivity EC*e* (0.18 $dS{\cdot}m^{-1}$). In Pakistani soils, the availability of P to plants is low due to its alkaline calcareous nature and only 10 to 20% applied P is utilized, while the remaining 80 to 90% P is fixed due to complex formation with Al, Fe, or Ca in soil. Starting doses of N as urea (three splits) and K_2O as muriate of potash at the rate of 120 and 60 $kg{\cdot}ha^{-1}$, respectively, were applied to all treatments except for the control, while single super phosphate (SSP) or powdered RP (20% P_2O_5) were applied at the sowing time at the rate of 90 kg P_2O_5 ha^{-1}. The maize seeds were surface-sterilized with 3.5% NaOCl (household bleach) for 10 min and rinsed three times with distilled water. Afterward, the sterilized maize seeds were soaked for 10 min in 30% concentrated sugar solution to be used as an adhesive agent. The required sticky maize seeds were then well mixed with phosphate solubilizing bacteria (PSB) inoculated carrier material for proper seeds coating. Approximately 6 kg of AMF crude inoculum per EU [42] was used at each required treatment before seed sowing. The coated maize seeds were applied alone and in combination with AMF inoculum in the field. The following treatment combinations were used in the field: (1) Control (no fertilizers), (2) only N and K fertilizers, (3) single super phosphate (SSP), (4) rock phosphate (RP), (5) *Bacillus* sp., (6) arbuscular mycorrhizal fungi (AMF), (7) AMF + *Bacillus* sp., (8) RP + *Bacillus* sp., (9) RP + AMF, and (10) RP + AMF + *Bacillus* sp. The experiment was conducted as a complete randomized block design with 10 treatments and three replicates per treatment. The field was irrigated after one week of seeds germination, and then subsequently irrigated according to the crop requirements. After germination, all agronomic practices were strictly followed for optimum plant growth throughout the growing season until crop harvesting.

2.5. Residual Effects of Inoculated AMF and PSB with RP on Wheat Crop (Experiment 2)

To confirm the residual effects of inoculated AMF and PSB with RP on the yield and P uptake of plants, a subsequent experiment was conducted by sowing wheat seeds in the same layout of the maize crop during the fall of 2016–2017. The field was irrigated and tilled with hand hoe without disturbing the experimental design and treatment combination. The wheat variety "Siren" was sown at a seed rate of 120 $kg{\cdot}ha^{-1}$. There were 13 rows per EU, each spaced 20 cm from the other. The recommended doses of N and K_2O were applied to all the treatments at the sowing time. Post-harvest soil and plant parameters were recorded as mentioned for the maize crop above.

2.6. Analysis of Post-Harvest Plant Parameters

The maize crop was harvested after three months from of planting. The three central rows were uprooted with the whole root system, and the adhering soil particles were removed by gentle tapping and then thoroughly washed with tap water. To estimate the maize grain yield, the shoots were

air-dried for seven days. Afterward, the grains were removed from the shoots and converted into kg·ha^{-1} by the following formula as described by Norman et al. [43].

$$\text{Grain yield (kg·ha}^{-1}\text{)} = \text{Sample yield per plot (kg)} \times 10{,}000/\text{number sub-samples} \times \text{segment length (m)} \times \text{row spacing (m)} \quad (1)$$

Following drying, the plant's samples were brought to the laboratory for grinding to pass a 1-mm screen, as suggested by Weidhuner et al. [33]. The P contents of the maize samples were determined by the molybdate blue method of Jones [44]. The P uptake by maize plants was determined by the formula, as given by Sharma et al. [45].

$$\text{Nutrient uptake (kg·ha}^{-1}\text{)} = \text{Plant nutrients concentration (\%)} \times \text{Total dry biomass (kg·ha}^{-1}\text{)}/100 \quad (2)$$

In maize roots, the mycorrhizal colonization was estimated by the roots clearing and staining method of Phillips and Hayman [46]. The maize roots of all three central rows from every treatment were cut (1 g) into pieces at 2.5 cm length from the base of the stem and preserved in 50% ethanol. The roots were then cleared with 10% KOH solution and heated in a water bath at 65 °C for 30 to 35 min. Afterward, the roots were washed and stained with 0.1% trypan blue in water bath at 65 °C for 10 min. Then, roots were washed three times with tap water and the mycorrhizal colonization was estimated under the microscope by the line intersect method suggested by McGonigle et al. [39].

2.7. Analysis of Post-Harvest Soil Parameters

Following maize harvest, six soil samples were randomly collected from each EU to determine the nutrient concentration, soil pH, EC*e*, and organic matter content. Soil samples were collected at a depth of 15 cm, air-dried, and ground to pass a 2-mm sieve [47] to remove pebbles, gravels, roots, and other larger materials. The physiochemical properties of all soil samples were determined by the standard procedures abovementioned at the substrate preparation stage. Physiochemical properties vary with the land use [48–50]. The AMF spores were isolated from post-harvest rhizosphere soil of maize plants uprooted from three central rows of each treatment by wet-sieving and decanting techniques, as described by Gerdeman and Nicolson [51].

Similarly, the PSB population density was also carried out in the same rhizosphere soil by using colony-forming unit (CFU) count method. The viable PSB was calculated in post-harvest soil samples by the procedure and formula, as described by James [52].

$$\text{Colony forming unit (CFU)/mL} = \text{Number of colonies} \times \text{dilution factor/Volume of inoculums} \quad (3)$$

2.8. Statistical Analysis

Statistical analysis was performed by using the Fisher (F) test for randomized complete block design (RCBD) through statistical package SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) [53]. For any significant difference in the means, the data were further subjected to the least significant difference (LSD) test used at a $p \leq 0.01$ level of significance.

3. Results

3.1. Maize Grain Yield and Post-Harvest Soil and Plant P Concentration (Experiment 1)

The grain yield of maize recorded in the treatment supplied with SSP (3460 kg·ha^{-1}) and RP + AMF + *Bacillus* sp. (3387 kg·ha^{-1}) was significantly ($p \leq 0.01$) higher than control and other treatments inoculated alone with AMF and the *Bacillus* sp. or their sole inoculation with RP (Tables 1 and 2). The grain yield of SSP was almost equivalent to the treatments having combined inoculation of AMF and *Bacillus* sp. with and without RP. Likewise, there was no significant difference in the grains yield of the treatments inoculated with AMF or *Bacillus* sp. alone and together with RP. However,

in comparison with control, the percentage increase in the maize grain yield was maximum in all the fertilized and inoculated treatments. Overall, Table 1 indicates that the role of the applied AMF and *Bacillus* sp. with RP was significant in increasing maize growth parameters under field conditions. Similarly, the statistical analysis of the data shows that the maximum soil P content of 6.73 mg·kg^{-1} was recorded in the treatment co-inoculated with the AMF and the *Bacillus* sp. with RP, but it was not significantly different than SSP and the co-inoculated treatments without RP (Tables 1 and 2). However, this trend was significantly different from the rest of the treatments. The data also indicate that a significant increase in P uptake by maize plants occurred in the treatment of SSP, and the combined inoculated treatment of AMF and *Bacillus* sp. with RP as compared to control and single inoculated treatments with and without RP. Compared to the controls, the maximum P uptake of 19 kg·ha^{-1} was recorded in the treatment fertilized with SSP. Thus, the increase in post-harvest soil and maize plants' P contents by the application of powdered RP with the dual inoculation of AMF and *Bacillus* sp. was comparable to that of SSP (Tables 1 and 2).

Table 1. Maize grain yield and post-harvest soil and plant P concentration as influenced by the inoculation of AMF and PSB strain (*Bacillus* sp. PIS7) with RP.

Treatments	Grain Yield (kg·ha^{-1})	Plant P Uptake (kg·ha^{-1})	Soil P Concentration (mg·kg^{-1})
Control (No fertilizers)	1668 ± 480 f *	3 ± 0.7 g *	1.40± 0.72 f *
N & K fertilizers	2136 ± 115 ef	5 ± 2.4 fg	1.63 ± 0.87 f
Single super phosphate (SSP)	3460 ± 562 a	19 ± 0.9 a	6.45 ± 0.82 a
Rock phosphate (RP)	2984 ± 382 abcd	8 ± 2.1 ef	2.8 ± 1 e
Bacillus sp.	2823 ± 197 bcd	11 ± 3.4 cd	4.8 ± 1.31 cd
Arbuscular mycorrhizal fungi (AMF)	2545 ± 622 de	10 ± 2.6 de	3.93 ± 1.1 d
AMF + *Bacillus* sp.	3198 ± 536 abc	15 ± 2.3 b	6.2 ± 0.87 ab
RP + *Bacillus* sp.	2752 ± 545 cde	14 ± 2.9 bc	5.67 ± 1.9 bc
RP + AMF	2717 ± 136 cde	12 ± 2.8 cd	4.93 ± 0.5 c
RP + AMF + *Bacillus* sp.	3387 ± 547 ab	18 ± 1.6 a	6.73 ± 0.92 a
LSD ($p \leq 0.01$)	621	2.7	0.99

* Means with different letter(s) in the same columns are significantly different at ($p \leq 0.01$).

Table 2. Mean square values for the soil and plant parameters as influenced by the applied AMF and PSB with RP in maize experiments.

SOV	DF	Grain Yield (kg·ha^{-1})	Plant P Uptake (kg·ha^{-1})	Soil P (mg·kg^{-1})	AMF Spores (20 g Soil)	Roots Colonization (%)	PSB Population (×10^8 cfu/mL)	Soil pH (1:5)	Soil EC (dS·m^{-1})	Organic Matter (%)
Rep	2	1,228,628	0.0047	8.03	104.63	58.43	566.03	0.03	04	0.11
Treatments	9	437,860.3 **	0.0059	11.68 **	159.63 **	532.07 **	1558.96 **	0.07 **	0.06 **	0.97 **
Error	18	47,421.87	0.0004	0.33	8.63	16.17	39.88	0.01	0.003	0.06
Total	29									

** Represents the significance at $p \leq 0.01$; DF = degree of freedom; Rep = Replication

3.2. Soil Mycorrhiza Status and Phosphate Solubilizing Bacteria Population

The combined inoculation of AMF and *Bacillus* sp. PIS7 with RP had a significant impact on maize roots colonization and PSB population counts (Tables 2 and 3). In the absence of any RP fertilization and inoculation, the AMF was colonized only 9% of maize roots. Following inoculation and fertilization, an increasing trend in roots colonization and PSB population was recorded in all treatments when compared with the control. The root colonization percentage and PSB population were significantly higher in the treatment of RP + AMF + *Bacillus* sp. (42%, 81 × 108 cfu/mL) over the control, respectively, which is significantly different from all the treatments having SSP or RP fertilization alone or in combination with the sole inoculation of AMF or *Bacillus* sp. except AMF + *Bacillus* sp. treatment for root colonization. Similarly, the number of AM fungal spores were increased by the combined application of P solubilizing microorganisms. The maximum number of AMF spores of 36 per 20 g soil

were recorded by the inoculation of AMF and *Bacillus* sp., followed by the dual inoculation of AMF and *Bacillus* sp. with RP (33 per 20 g soil) as compared to control and single inoculated treatments. Furthermore, the development of AMF spores showed a positive relation with the percentage root colonization, while the relationship between the PSB population and the percentage root colonization by AMF was also positive in maize rhizospheres due to the inoculation of AMF and PSB with RP.

Table 3. Soil spore's density and roots colonization of AMF and PSB population in maize as affected by the inoculation of AMF and PSB with RP.

Treatments	No of Spores (per 20 g Soil)	Root Colonization (%)	PSB Population ($\times 10^8$ cfu/mL)
Control (No fertilizers)	15 ± 3 f *	6 ± 3 f *	15 ± 7 e *
N & K fertilizers	21 ± 3 de	9 ± 4 ef	21 ± 6 e
Single super phosphate (SSP)	16 ± 2 ef	12 ± 4 def	22 ± 5 de
Rock phosphate (RP)	17 ± 2 ef	15 ± de	33 ± 10 d
Bacillus sp.	18 ± 3 ef	12 ± 3 def	54 ± 10 c
Arbuscular mycorrhizal fungi (AMF)	29 ± 4 bc	28 ± 4 c	25 ± 7 de
AMF + *Bacillus* sp.	36 ± 4 a	38 ± 4 ab	67 ± 15 b
RP + *Bacillus* sp.	25 ± 9 cd	18 ± 5 d	55 ± 10 c
RP + AMF	25 ± 4 cd	35 ± 8 b	23 ± 3 de
RP + AMF + *Bacillus* sp.	33 ± 5 ab	42 ± 3 a	81 ± 14 a
LSD ($p \leq 0.01$)	5.0	7.0	11.0

* Means with different letter(s) in the same columns are significantly different at ($p \leq 0.01$).

3.3. Post-harvest Soil Properties

The results of post-harvest soil organic matter (SOM) content, pH], and EC*e*, as influenced by the dual inoculation of AMF and *Bacillus* sp. with RP can be seen in Tables 2 and 4. The highest soil organic matter (SOM) value recorded in this experiment was observed in this RP + AMF + *Bacillus* treatment (i.e., 1.4%), which was not different than that achieved with the SSP and co-inoculation of AMF and PSB without RP (1.3% in both cases), as compared to control and all the treatments having AMF or PSB inoculation alone with and without RP. A similar trend was also observed for soil EC*e*, with maximum values occurring in the RP + AMF + *Bacillus* (0.67 dS·m^{-1}), which was not different than EC*e* for AMF + *Bacillus* (0.66 dS·m^{-1}) and RP + *Bacillus* (0.56 dS·m^{-1}) treatments. Significant changes in soil pH were observed due to the activities of the applied beneficial microorganisms in the plant rhizosphere. The minimum pH value of 7.3 was recorded for the RP + AMF + *Bacillus* treatment when compared to the controls and the rest of the treatments containing a single inoculation with and without RP. Thus, the physiochemical properties of soil could be improved by the combined inoculation of AMF and PSB.

Table 4. Maize post-harvest soil organic matter content, pH, and EC*e* as influenced by AMF and PSB inoculation with RP.

Treatments	Organic Matter (%)	pH (1:5)	EC*e* (dS·m^{-1})
Control (No fertilizers)	0.7 ± 0.02 f *	7.8 ± 0.04 a *	0.26 ± 0.07 f *
N and K fertilizers	0.8 ± 0.07 e	7.6 ± 0.03 ab	0.28 ± 0.12 f
Single super phosphate (SSP)	1.3 ± 0.06 ab	7.5 ± 0.04 bc	0.45 ± 0.03 cd
Rock phosphate (RP)	0.9 ± 0.13 de	7.5 ± 0.04 bc	0.34 ± 0.13 ef
Bacillus sp.	1.1 ± 0.2 c	7.4 ± 0.03 bcd	0.46 ± 0.1 cd
Arbuscular mycorrhizal fungi (AMF)	1.0 ± 0.13 cd	7.4 ± 0.02 cd	0.39 ± 0.09 de
AMF + *Bacillus* sp.	1.3 ± 0.06 ab	7.4 ± 0.02 cd	0.66 ± 0.08 a
RP + *Bacillus* sp.	1.1 ± 0.2 c	7.5 ± 0.07 bc	0.56 ± 0.06 ab
RP + AMF	1.2 ± 0.07 bc	7.5 ± 0.02 bc	0.5 ± 0.04 bc
RP + AMF + *Bacillus* sp.	1.4 ± 0.07 a	7.3 ± 0.27 d	0.67 ± 0.04 a
LSD ($p \leq 0.01$)	0.12	0.2	0.1

* Means with different letter(s) in the same columns are significantly different at ($p \leq 0.01$).

3.4. Residual Effect of AMF and PSB with RP on Post Harvest Soil and Wheat Crop

The maximum grain yield of wheat (3101 kg·ha^{-1}) was found in the treatment of AMF and PSB inoculated with RP (Tables 5 and 6). Compared to the control, AMF and PSB improved the P levels of both RP-fertilized and non-fertilized soils. Higher AB-DTPA available soil P was recorded by the combined application of AMF and PSB with RP. Moreover, the co-inoculation of these microorganisms with RP caused a significant decrease in soil pH as compared to the un-inoculated treatments. However, we did not observe significant changes in soil pH between both the direct and residual trials. Similarly, P uptake by wheat plants showed a trend similar to growth parameters; therefore, the maximum P uptake of 24 kg·ha^{-1} was observed in the co-inoculated treatment RP + AMF + *Bacillus* as compared to control and single inoculated treatments with and without RP (Tables 6 and 7). In this study, although the AMF root infection percentage in wheat roots and PSB population in some treatments were not significantly different as those recorded in the maize experiment, significant positive increases were observed both in AMF root colonization and PSB population with the application of the treatment having combined inoculation of AMF and PSB with RP as compared to the remaining fertilized or inoculated treatments. A maximum root infection intensity and PSB population of 70% and 64% was observed in the treatment having AMF and PSB with RP as compared to the control, respectively. Moreover, after the harvesting of wheat crop, we noted an increase in the AMF soil spores density and PSB population in the field soil (Tables 6 and 7). The increase in wheat growth and P uptake, as well as the decrease in soil pH in this experiment, shows that the beneficial effects of AMF and PSB inoculum with RP were persistent till the second year of its application in the field.

Table 5. The residual effect of AMF and PSB with RP on soil P concentration in post-harvest wheat crop.

Treatments	Grain Yield (kg·ha^{-1})	Plant P Uptake (kg·ha^{-1})	Soil P Concentration (mg·kg^{-1})
Control (No fertilizers)	1720 ± 12 h *	2.04 ± 0.71 h *	1.11 ± 0.21 g *
N & K fertilizers	1981 ± 13 g	4.64 ± 0.96 gh	1.25 ± 0.22 g
Single super phosphate (SSP)	2292 ± 84 ef	10.02 ± 1.3 ef	3.75 ± 0.39 ef
Rock phosphate (RP)	2555 ± 67 d	13.58 ± 1.5 cd	4.29 ± 0.49 e
Bacillus sp.	2420 ± 38 de	10.48 ± 0.97 de	5.55 ± 1.71 d
Arbuscular mycorrhizal fungi (AMF)	2150 ± 17 fg	7.13 ± 0.76 fg	3.1 ± 0.31 f
AMF + *Bacillus* sp.	3061 ± 14 b	20.98 ± 3.75 b	5.99 ± 0.22 cd
RP + *Bacillus* sp.	3316 ± 27 a	18.79 ± 3.5 b	7.4 ± 0.22 ab
RP + AMF	2770 ± 83 c	15.36 ± 2 c	6.7 ± 0.37 bc
RP + AMF + *Bacillus* sp.	3101 ± 18 b	24.49 ± 5.4 a	8.05 ± 0.17 a
LSD ($p \leq 0.01$)	179	3.2	1.11

* Means with different letter(s) in the same columns are significantly different at ($p \leq 0.01$).

Table 6. Mean square values for the soil and plant parameters as influenced by the applied AMF and PSB with RP in wheat experiments.

SOV	DF	Grain Yield (kg·ha^{-1})	Plant P Uptake (kg·ha^{-1})	Soil P (mg·kg^{-1})	AMF Spores (20 g soil)	Roots Colonization (%)	PSB Population (×10^{8} cfu/mL)	Soil pH (1:5)	Soil EC (dS·m^{-1})	Organic Matter (%)
Rep	2	26,294.53	35.67	0.03	20.233	64.93	38.53	0.008	0.04	0.785
Treat	9	818,194 **	158.24 **	17.78 **	432.38 **	644.66 **	895.88 **	0.044 **	0.09 **	0.592 **
Error	18	10,791.87	3.5	0.42	18.455	16.93	29.49	0.0007	0.01	0.046
Total	29									

** Represents the significance at $p \leq 0.01$.

Table 7. Soil spore's density and PSB population, and AMF wheat roots colonization as affected by the inoculation of AMF and PSB with RP.

Treatments	No of Spores (per 20 g Soil)	Root Colonization (%)	PSB Population ($\times 10^8$ cfu/mL)
Control (No fertilizers)	13 ± 3 f *	11 ± 2 f *	21 ± 2 g *
N & K fertilizers	18 ± 4 ef	17 ± 3 ef	28 ± 3 f
Single super phosphate (SSP)	24 ± 2 de	23 ± 3 de	35 ± 3 e
Rock phosphate (RP)	21 ± 1 e	27 ± 4 cd	32 ± 2 ef
Bacillus sp.	13 ± 3 f	29 ± 8 cd	46 ± 1 d
Arbuscular mycorrhizal fungi (AMF)	30 ± 8 cd	33 ± 7 c	32 ± 2 ef
AMF + *Bacillus* sp.	50 ± 5 a	49 ± 3 b	57 ± 5 c
RP + *Bacillus* sp.	25 ± 3 de	31 ± 5 c	68 ± 5 b
RP + AMF	35 ± 4 bc	42 ± 5 b	32 ± 2 ef
RP + AMF + *Bacillus* sp.	41 ± 4 b	60 ± 4 a	78 ± 3 a
LSD ($p \leq 0.01$)	7.2	7	4.4

* Means with different letter(s) in the same columns are significantly different at ($p \leq 0.01$).

4. Discussion

Due to the depleting reservoirs of available phosphorus (P) and high costs of phosphate fertilizers, RP serves as an alternative and sustainable source of P to modern agriculture [16]. In the present study, we determined the direct and residual beneficial effects of AMF inoculum alone and together with native *Bacillus* sp. PIS7 on maize and wheat growth, yield and P uptake, both in RP fertilized and unfertilized treatments in P-deficient field soil. Compared to the control (1668 kg·ha^{-1}), single inoculation and our previous pot experiment on maize plants (54 g·pot^{-1}), the combined inoculation of RP with AMF and *Bacillus* sp. significantly increased the maize grain yield (3387 kg·ha^{-1}) and P accumulation (19 kg·ha^{-1}), as well as the growth of wheat plants under field conditions. This increase in grain yield of maize and wheat could be due to the increased solubility of P from RP and soil organic P reserves by PSB, which ultimately increased the amount of P that is readily available for direct plant and mediated-AMF P uptake by plants [18]. Our results also indicate that in the presence of RP, not only the PSB strain (*Bacillus* sp. PIS7) but also the AMF inoculum played a significant role in plant growth and productivity. The AMF has the ability to increase plant roots infectivity and root hairs in the soil for water and nutrients uptake by plants [54]. Mukherjee and Rai [55] found that the combined application of *Glomus fasciculatum* and *Bacillus megatarium* var *phosphaticum* with P fertilizers increased growth, yield, and nutrients' uptake by wheat and chickpea compared to the application of P alone. Singh et al. [56] found that the inoculation of P-solubilizing fungus increased nutrient uptake, grain yield, P balance, and availability by wheat and maize in the presence of RP. Similarly, according to Chu et al. [57], AMF enhanced plant P uptake when soil-available P was present at intermediate levels, and that lower or higher than optimum P levels inhibited the contribution of mycorrhizal fungi.

In many treatments, the results of SSP in our previous pot experiment, as well as field experiments on maize and wheat crop, were comparable to inoculated treatments together with RP. When this occurred, the better performance of SSP could be explained by its higher solubility and bioavailability to plants when compared to RP in alkaline calcareous soils [58]. However, when RP was inoculated with PSB, its solubility increased because the inoculated PSB in combination with native microorganisms released different acids in the soil and made the soil environment more favorable for the release of P from low soluble RP in the presence of AMF. Similarly, besides co-inoculation of RP with AMF and PSB, we observed a significant increase not only in the P uptake of AMF + PSB treatment for maize (15 kg·ha^{-1}) and wheat (21 kg·ha^{-1}) plants but also in all the remaining treatments amended without RP in both the experiments. In our field conditions, alkaline calcareous soils could fix more than 80% of the applied P. In this context, the use of AMF and PSB inoculants following fertilizer P application most likely explained the increased release, and subsequent greater bioavailability, of the soil fixed P in these soils. Similar results were also reported by Abarchi et al. [59], who observed high P contents

in legumes treated with and without RP. Our results also imply that the role of applied *Bacillus* sp. alone and together with RP was effective in P solubilization and; therefore, after inoculation with AMF, its transfer was increased to both maize and wheat plants. These results are in agreement with findings from Cao et al. [60] who found that during these interactions, bacteria released organic acids, gibberellins, abscisic acid citric acid, oxalic acid, and phosphatases which consequently converted hardly soluble RP into soluble forms of P. Similarly, the increase in P uptake by maize and wheat plants by the combined inoculation of AMF and *Bacillus* sp. with RP might be due to the fact that during their interaction, the fungi extended their hyphae beyond plant rhizosphere and increased nutrients' uptake such as P, N by the plants [17]. These results are consistent with reports from Singh et al. [61] who found that both *Pseudomonas monteilii* and *Glomus fasciculatum* had the ability to increase NPK uptake and above the biomass of *Coleus forskohlii* plants. Martins da Costa et al. [5] also conducted a greenhouse experiment and proved that the growth and nutrient uptake by rice plants could be enhanced by the combined inoculation of bacterial strains with RP.

A non-significant increase in the number of AMF spores per 20 g soil over controls (15 ± 3) was observed in the treatment of the sole inoculation of *Bacillus* (18 ± 3), while a significant increase in this parameter was found following the application of RP + *Bacillus* (25 ± 9) in maize crop. It is because both of our direct and residual experiments were conducted under natural field soil conditions, and the number of indigenous AMF spores in the field soil was 15 ± 3. Therefore, the increase in soil spore's density (25 ± 9) in the rhizosphere soil in the treatment inoculated with *Bacillus* stimulated the development and the activities of native AMF spores and was comparable to N and K-treated plots. Compared to the control, the increase in the population of AMF spores and PSB population in non-inoculated treatments was due to the application of nutrients element (NK) and RP [62]. Furthermore, the increase in AMF spore density with the inoculation of *Bacillus* sp. behaved as mycorrhizal helper bacteria [63]. Similarly, the abundant spore's proliferation and colonization of mycorrhiza in roots could be due to the presence of RP and the synergistic effect with rhizobacteria in plant rhizospheres. Additionally, according to Barea et al. [64], different soil microorganisms produce some chemicals that could improve the amount of root exudates, resulting in higher rates of mycorrhizal colonization. Babana et al. [65] also reported that phosphate-solubilizing microorganisms inoculated with AMF and RP increased the yield and colonization by wheat crops under field conditions. The increase in PSB population density was also recorded in all treatments of post-harvest maize and wheat rhizosphere soils having dual inoculation of *Bacillus* sp. and AMF with RP. The reason for this increase in the PSB population might be attributed to the ability of AM fungi to stimulate hyphae-associated bacteria and improve P uptake in the soil [18]. On the other hand, compared to AMF + PSB without RP, the less number of AMF spores in the treatment of AMF + PSB + RP might be attributed to the presence of high levels of P in the soil, which directly inhibited hyphal growth and spore germination [66]. Another reason might be the different abilities of some native microorganisms to weather RP and solubilize mineral phosphate. The bacterial inoculation with AMF in rhizospheres also assisted large numbers of spore germination and, thus led to high colonization [67]. Similarly, the post-harvest soil samples were taken from the vicinity of the plants; therefore, the combined inoculation of AMF and *Bacillus* sp. with RP was effective in decreasing soil pH and improving organic matter contents as compared to controls and single inoculated treatments. These changes might be credited to the ability of PSB to secrete organic acids for P solubilization and decrease the pH of the surrounding bulk and rhizosphere soil [68–73] and, thus create a favorable environment for the growth of maize and wheat plants in alkaline calcareous soil.

5. Conclusions

In phosphorus-deficient soils, it is possible to attain greater maize and wheat yields by increasing P bioavailability, and subsequent plant uptake, by the direct application of locally available powder RP fertilizer in combination with arbuscular mycorrhizal fungi and phosphate solubilizing bacteria inoculants. Moreover, to ensure the profitable availability of these inoculants to farmers, a more

thorough understanding and formulation with locally available fertilizers are needed for optimal utilization. Further field trials are suggested to investigate the synergistic effects of arbuscular mycorrhizal fungi and phosphate-solubilizing bacteria inoculants with different native soil microbial communities on crop performance under different agro-ecological conditions.

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