Pak. J. Bot., 48(2): 739-747, 2016.

INOCULATION OF ARBUSCULAR MYCORRHIZAL FUNGI AND PHOSPHATE SOLUBILIZING BACTERIA IN THE PRESENCE OF ROCK PHOSPHATE IMPROVES PHOSPHORUS UPTAKE AND GROWTH OF MAIZE

FAZLI WAHID[1*], MUHAMMAD SHARIF[1], SIEGRID STEINKELLNER[2], M. AZIM KHAN[1], K.B. MARWAT[3] AND S.A. KHAN[1]

[1]*The University of Agriculture Peshawar, 25130, Pakistan.*
[2] *Division of Plant Protection, University of Natural Resources and Life Sciences Vienna, Konrad Lorenz Strasse 24, 3430 Tulln, Austria.*
[3] *SBB University Sheringal, Upper Dir, KP, Pakistan*
**Corresponding author's email: fazliwahid@aup.edu.pk*

Abstract

The beneficial microbes like arbuscular mycorrhizal fungi (AMF) and phosphate solubilizing bacteria (PSB) are known to play an important role in phosphorous (P) supply to plants in a sustainable manner in P deficient soils. In this scenario, a pot experiment was conducted under greenhouse condition to assess the synergistic effect of AMF and PSB strains (*Coccus* DIM7 *Streptococcus* PIM6 and *Bacillus* sp. PIS7) on P solubility from RP and their successive uptake by maize (*Zea-mays* L. Azam**)** crop at alkaline soil. The experiment was completely randomized design with three replications having calcareous silty clay loam soil, low in organic matter, nitrogen and phosphorus contents. RP was used as a crude phosphate alone and/or in combination with the native AMF and PSB inoculum. The results indicated that the rhizosphere interactions between AMF and PSB significantly promote RP mineralization in soil and improved all growth parameters including shoot (56%), root yield (52%), height (41%), N (80%) and P (91%) uptake by the maize plants as compared to control and single inoculation. A remarkable increase in soil spore density, PSB population and percent root colonization in maize plants were also recorded by the combined inoculation of AMF and PSB with RP. From this study, it is concluded that the combined application of AMF and PSB with RP has the potential to improve maize growth and nutrients uptake. Moreover, AMF and PSB inoculants are recommended as useful biofertilizers for enhancing P solubility and bioavailability in P deficient agricultural soils.

Key words: AMF, PSB strains, RP, Microbial populations, Nutrients uptake and maize crop.

Introduction

Phosphorus (P) is a vital nutrient that plays a key role in maintaining soil fertility for optimum plant growth and productivity. The mineral nutrition of plants mainly depends on soil P content that can be assimilated as a soluble phosphate (Ehteshami *et al*., 2011). Like other nutrients, organic matter in the soil also plays an important role in the growth of plants (Khan *et al*., 2012). P is involved in all major metabolic processes in plants such as biosynthesis of macromolecules, energy transfer, photosynthesis, signal transduction and respiration (Khan *et al*., 2010). Regardless of its importance, P is the most deficient macro-nutrient in agricultural soils and its natural soil reserves are depleting at a higher rate. According to an estimate there will be no soil P reserves by the end of 2050 for sustainable crop production especially at the tropical and subtropical regions of the world **(**Balemi & Negisho, 2012**).** P is present both in organic and inorganic forms in soils but its availability is limited to plants and mostly restricted due to complex formations with other nutrients (Sharma *et al*., 2013). However, to compensate this poor availability of P, the frequent supplementation of P fertilizers is essential in P deficient soils (Costa *et al*., 2015) to maintain an adequate level of P for crop sustainability (Naseer, 2014) as well as to ensure food security for the world growing population. On the other hand, the regular and huge application of P fertilizers enable the introduction of additional P which requires a lot of energy for processing, distribution, transportation and thus increases the cost of production as well as environmental risks (Pizzeghello *et al*., 2011).

In recent years the use of rock phosphate (RP) as a substitute to commercial P fertilizers has received a significant interest because it is natural, inexpensive and provides abundant P, necessary for plant growth and sustainable plant production in P-deficient soils (Zapata & Zaharah, 2002). It is the major source of P in nature and is being used as a raw-material for manufacturing commercial P fertilizers (90%) and elemental P (10%). In the north-western part of Pakistan, the Hazara phosphorite deposits are the major sources of raw materials for P fertilizers production. These deposits are predictable at 6.9 million tons of which 4.58 million tons are considered as recoverable (Anon., 1989). In alkaline calcareous soils, the direct application of RP as a source of phosphate fertilizers is agronomically more suitable for crop growth and development in the presence of certain amendments (Xiong *et al*. 1994: Sharif *et al*., 2015).

In agricultural sustainability, the use of beneficial soil microbes for P solubility and crop productivity is getting more attention. Currently inoculation of these microorganisms have gained popularity (Parkash *et al*., 2011) to be used as biofertilizers instead of high input chemical fertilizers in crop production system (Kennedy *et al*., 2004). In this regard, the inoculation of AM fungi in combination with some rhizobacteria to boost P availability, plant growth and productivity is relatively recent developments in natural and agricultural ecosystem. These microorganisms possessing the potential to solubilize inorganic phosphate and to supply nutrients in a readily available form, such as arbuscular mycorrhizal fungi (AMF) and phosphate solubilizing

bacteria (PSB) have been found in the rhizosphere of most of the plants (Yadav *et al*., 2014). These microorganisms release organic acids and reduce pH of the medium by increasing P availability in P deficient soils (Gulati *et al*., 2010). Moreover, now a day's various strategies are in considerations to use efficient microbes to enhance the bioavailability of P from different types of phosphates of low solubility. However, the direct application of RP with the combined inoculation of P solubilizing microorganisms is effective for faster supply of P for crop growth and productivity. Maize is one of the major crops worldwide. Its productivity potential is high as compared to other cereal crops and therefore it is called "King of cereals" (Umesha *et al*., 2014). It is a staple food in many parts of the world, but also used as livestock feed and as a raw material for industrial purposes. However, maize has very high nutrient requirement and the given levels of N and P are not enough to maintain its yield levels. The soils in Pakistan are calcareous in nature and due to the high pH much of P is not available for plant uptake and growth promotion. To achieve high productivity based on a cost effective and native source of fertilizers, the combined application of RP, AMF and PSB might be alternative approach. In Pakistan no attempts have been made so far to investigate the effect of PSB and AMF inoculation on maize crop. Therefore, the aim of the present study was to investigate RP solubility as influenced by AMF and PSB strains and its affect on growth and P uptake of maize crop in alkaline calcareous soil.

Materials and Methods

Phosphate solubilizing bacteria (PSB): Rhizosphere soil samples were collected from maize and sorghum irrigated and unirrigated areas of Chitral, Peshawar & Dera Ismail Khan (Pakistan). The PSB were isolated from each rhizosphere soil sample on LB agar media (Miniatis *et al*., 1982) using the serial dilution counting method (Johnson & Curl, 1972) and the prominent colonies were picked and purified on Pikovskaya's agar. *Coccus* sp., *Streptococcus* sp. and *Bacillus* sp. were identified based on their microscopic, phenotypic and morphological characters and classified for their capability to solubilize P from hardly soluble rock phosphate according to Pikovskaya (1948). Therefore each of the pure PSB strain was placed on Pikovskaya's agar plates (5.0 g RP, 10.0 glucose, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4.7\ H_2O$, 0.2 g KCl, 0.5 g yeast extract, 15.0 g agar and 1000 ml distilled water 1000 ml) (Gupta *et al*., 1994). The capability of PSB strains to solubilize insoluble P compound was qualitatively determined from the clear light zone surrounding colonies according to Edi-Premono *et al*. (1996). The quantitative analysis has been done by spectrophotometer according to Murphy & Riley (1962). The three most efficient P solubilizing bacterial strains (*Coccus* DIM7, *Streptococcus* PIM6 and *Bacillus* sp. PIS7) were selected and stored at 4^oC at the Institute of Soil Biology and Biochemistry, LLRI, NARC, Islamabad for further research.

For the inoculum preparation the selected PSB strains were inoculated separately in 250 ml flasks containing 100 ml of Pikovskaya's broth. The flasks were kept in a growth chamber at 28°C on an orbital shaker at 120 rpm. After seven days 1 ml of the broth culture was spread on Pikovskaya's agar plates to determine the microbial concentration. Finally, the bacterial cultures were adjusted to a concentration of approximately 10^8 cfu /ml (Murray *et al*., 2003). The bacterial cultures (100 ml) were mixed with 1 kg of an autoclaved carrier material (organic soil) and packed in biofertilizer packets.

Arbuscular mycorrhizal fungi: AMF spores were isolated from 20 g alkaline calcareous silty clay loam rhizosphere soil by the wet-sieving and decanting technique as described by Gerdeman & Nicolson (1963). The binocular microscope with magnification of 40X was used for spore observation in 20 g soil. Approximately 100 spores were isolated and counted for each pot and stored as suspension in Petri plates at 4°C for a maximum of two days before pots application.

Plant growth conditions: The experiment was conducted under greenhouse conditions at the University of Agriculture Peshawar, Pakistan at a mean temperature of 24-28°C. Pots were filled with 10 kg unsterilized field soil. The soil was silty clay in texture, alkaline calcareous in reactions with low N (0.07%), P (2.5 mg kg^{-1}), organic matter content (0.6%), pH (7.85), lime (16.5%) and ECe (0.17%). N, P and K were applied at the rate of 120-90-60 kg ha^{-1} in the form of urea, single superphosphate (SSP) or RP (20% P) and potassium sulfate (SOP) fertilizers at sowing time. The N (three splits) and K fertilizers were constantly applied to all the pots as basal dose. The maize seeds were surface disinfected with 3.5% NaOCl (house hold bleach) and then rinsed with distilled water 3 times after 10 min. The disinfected maize seeds were then soaked in 30% concentrated sugar solution as an adhesive agent for 15 minutes. Afterwards the sticky maize seeds were mixed with the above mentioned carrier material for proper seeds coating. AM fungal spores were applied as suspension at the rate of 100 AMF spores per pot, each containing five inoculated seeds. The three PSB isolated strains (*Coccus* DIM7, *Streptococcus* PIM6 and *Bacillus* sp. PIS7) were used alone and/or in combination with AMF. The five maize seeds (*Zea mays* L. cv. Azam) sown at the beginning were thinned to two plants per pot after one week of germination. The experiment includes the following treatments: 1) Control (N&K fertilizers only), 2) SSP, 3) RP, 4) *Streptococcus*, 5) *Coccus*, 6) *Bacillus*, 7) AMF, 8) RP+ *Streptococcus*, 9) RP+*Bacillus*, 10) RP+Coccus, 11) RP+AMF, 12) RP+AMF+*Streptococcus*, 13) RP+AMF+*Bacillus*, 14) RP+AMF+*Coccus*). The experiment was conducted as complete randomized design. There were three replicates in each treatment and each replicate comprise of one pot with two maize plants. To maintain the field capacity, water was applied to each pot uniformly after germination at a regular interval till maturity. All agronomic practices were strictly followed throughout the growing season for optimum plant growth. The plants grew for 70 days up to maturity stage.

Determination of plant parameters: All maize plants were harvested after 70 days and the growth parameters, such as plant height and dry weight were recorded. The shoots were separated from the roots at 0.5 cm above the surface of soil. The roots were washed out with tap water to remove the soil particles. The dry weight of roots and shoots were measured after drying in an oven for 48 hours at 60°C. After drying the samples were weighted, grinded and further analyzed for phosphorus concentration and accumulation by maize plants by the method as described by Walsh & Beaten (1977). The nutrients uptake by plants was calculated by the formula as described by Sharma *et al*. (2012).

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

The AMF colonization of the roots was determined by staining the mycorrhizal chitin with lactic–trypan blue according to the method of Philips & Hyman (1970). Approximately 1 g of roots (2 cm in length), starting 2 cm from the base of shoots were separated and preserved in 50% ethanol. Afterwards the roots were cut into small pieces and cleared in 10% KOH solution. The roots were then cooked in water bath at 65°C for 30 min. After washing the roots were stained with 0.1% trypan blue and placed in water bath at 65°C for 10 min. The stained roots were removed from water bath and rinsed with tap water 3 times. The percentage of AMF colonization rate was determined under the microscope by the method as described by McGonigle *et al*. (1990).

Post harvest soil analyses: Post harvest soil samples (1 kg) were collected from each treatment at the time of harvest. These soil samples were air dried ground and sieved through a 2 mm sieve to remove gravels and pebbles and closed in plastic bags for analysis in laboratory. Soil samples were analyzed for their various physico-chemical properties by the following standard procedures. Soil texture, soil pH, SOM, EC and lime were determined according to Koehler *et al*. (1984), McClean (1982), Nelson & Sommers (1982), Black (1965), Richard (1954) respectively. AB-DTPA extractable P and K was determined as mentioned in Soltanpour & Schwab (1977), total N by the Kjeldhal method according to Bremner (1982). AM fungal spores were isolated from 20 g post-harvest rhizosphere soil of each treatment by wet-sieving and decanting techniques as described by Gerdeman & Nicolson (1963). The isolated spores from the soil sample were identified according to their morphological characteristics including shape, size, colour, distinct wall layer, attached hyphae and surface orientation of spores as described by Schenck & Parez (1990).

Enumeration of post harvest viable PSB were carried out by using colony forming unit (CFU) count method. The population density of PSB in the rhizosphere soil samples were calculated by the formula and procedure as given by James (1978).

$$\text{Colony forming unit (CFU) g}^{-1}\text{ soil} = \frac{\text{Number of colonies} \times \text{dilution factor}}{\text{Volume of inoculum}}$$

Statistical analysis: The data were subjected analysis of variance (ANOVA) and correlation analysis using Statistic, 2000 package. Statistical significance was determined at the 5% level. Means were compared by the least significant difference (LSD).

Results

Plant growth parameters: The growth parameters of maize plants are summarized in Table 1. The results reveal that the inoculation of AMF and PSB strains with RP significantly ($p \leq 0.05$) influenced the growth parameters of maize as compared to the treatments having single or no inoculation. The maximum maize shoot biomass of 58.00 g pot^{-1} ($p<0.001$) with 56% increase over control was found in the treatments of AMF and *Streptococcus* sp. with RP. It was followed by the treatment having SSP fertilizer (56.33 g pot^{-1}) without inoculation of AMF and PSB with RP and the treatment containing AMF and *Bacillus* with RP (54. 70 g pot^{-1}). The increase in shoot biomass of maize crop also enhanced roots elongation due to microbial activity and RP solubility. The AMF inoculation with PSB and RP increased root biomass of maize significantly ($p \leq 0.05$) over single inoculation and control. The maximum (17.33 g pot^{-1}) root dry biomass with 52 % increase over control was observed by the having SSP fertilizer. The higher shoot and root biomass resulted in better plant height of maize. The maximum height of maize plants (118.67 cm) was noted in the combined inoculation of AMF and *Coccus* sp. with RP (Table 1).

Post harvest soil and plant nutrients contents: Post harvest soil and plant nutrients (N and P) contents were affected by the dual inoculation of AMF and PSB strains in the presence of RP in alkaline calcareous soil (Table 2). Nitrogen applied in the form of urea at recommended rate of 120 kg N ha^{-1} stimulated the growth of maize plants. The maximum (0.56%) soil N concentration was recorded in the treatment of RP+AMF, followed by RP+AMF+*Bacillus* (0.54%). In maize plants the higher N concentration (2.70%) was observed in inoculated treatments of AMF both with *Bacillus* and *Streptoccocus* sp. with RP, respectively. The AMF and PSB application enhanced N concentration in soil as well as in maize plants. The N range in plants co-inoculated with AMF and PSB was higher than the single inoculated plants with RP. Therefore, the minimum values of N observed in both soil and plant control treatments were 0.10% and 1.01%, respectively. The P contents showed a trend almost similar to N in both soil and plant. It was observed that the total available P contents were significantly increased by the dual inoculation of AMF and PSB strains with RP (Table 2). Similarly, the higher AB-DTPA extractable soil P content of 13.33 mg kg^{-1} and maximum P content of 0.19% were recorded in the treatment having inoculation of AMF and *Bacillus* with RP, respectively. The natural field soil used in this pot experiment with no P fertilizer added showed the minimum P values both in soil and plant (1.70 mg kg^{-1} and 0.05%). The combined inoculation of PSB strains and AMF improved nutrients solubility and their bioavailability.

Table 1. Shoot and root dry matter yields and plants height of maize as influenced by the inoculation of AMF and PSB with RP*.

Treatments	Dry shoot biomass (g pot^{-1})	Dry root biomass (g pot^{-1})	Plants height (cm)
Control	25.70 ± 4.16^{g}	8.33 ± 0.57^{g}	69.70 ± 7.23^{h}
SSP	56.33 ± 4.73^{a}	17.33±0.56^{a}	106.00±8.65^{ab}
RP	37.00 ± 5.30^{f}	11.70 ± 1.53^{f}	74.00 ± 6.10^{gh}
Streptococcus	39.00 ± 4.58^{ef}	13.00 ± 1.00^{def}	78.00 ± 6.56^{fgh}
Bacillus	42.00 ± 4.00^{def}	13.70 ± 0.57^{cdef}	84.00 ± 9.53^{efg}
Coccus	41.33 ± 4.04^{def}	13.33 ± 1.53^{cdef}	81.00 ± 6.56^{fgh}
AMF	37.33 ± 5.51^{f}	12.00 ± 1.00^{ef}	76.00 ± 6.56^{gh}
RP + *Streptococcus*	45.70±3.51^{cde}	15.33 ± 1.53^{abcd}	100.33 ± 10.02^{bc}
RP + *Bacillus*	53.00 ± 1.73^{abc}	15.70 ± 1.15^{abc}	98.70 ± 6.51^{bcd}
RP + *Coccus*	52.33 ± 6.11^{abc}	16.33±1.15^{ab}	95.70 ± 14.01^{bcde}
RP + AMF	48.00 ± 6.56^{bcd}	14.33 ± 1.15^{bcde}	90.70 ± 9.02^{cdef}
RP + AMF + *Streptococcus*	58.00 ± 3.00^{a}	16.70 ± 1.15^{ab}	108.67 ± 6.51^{ab}
RP + AMF + *Bacillus*	54.70 ± 3.51^{ab}	15.70 ± 0.58^{abc}	107.00 ± 8.72^{ab}
RP + AMF + *Coccus*	53.70±2.88^{ab}	16.70 ± 1.15^{ab}	118.67 ± 8.08^{a}

*Values are means ± SD (n=03). Values followed by different letters within a column indicate significant differences according to LSD ($p<0.05$)

Table 2. Post harvest soil and plant N and P concentrations and plants uptake as affected by AMF and PSB strains inoculated with RP*.

Treatments	Soil N (%)	Plant N (%)	Plant P (%)	Soil P (mgkg^{-1})	N uptake (g pot^{-1})	P uptake (g pot^{-1})
Control	0.10±0.02^{g}	1.01±0.02^{f}	0.05±0.03^{c}	1.70±0.36^{e}	0.26±0.05^{g}	0.01±0.01^{d}
SSP	0.15±0.03^{fg}	1.60±0.10^{de}	0.13±0.04^{abc}	9.70±1.05^{bcd}	0.71±0.05^{def}	0.06±0.02^{abcd}
RP	0.13±0.02^{fg}	1.40±0.20^{e}	0.06±0.05^{c}	2.43±0.50^{e}	0.52± 0.10^{f}	0.02± 0.03^{bcd}
Streptococcus	0.18±0.05^{ef}	1.70±0.25^{de}	0.09±0.04^{bc}	8.00±1.50^{cd}	0.66±0.17^{f}	0.03±0.02^{bcd}
Bacillus	0.25±0.05^{e}	1.73±0.15^{de}	0.09±0.05^{bc}	7.70±0.95^{cd}	0.73±0.07^{def}	0.04±0.02^{bcd}
Coccus	0.19±0.05^{ef}	1.80±0.15^{d}	0.08±0.06^{c}	7.40±1.95^{d}	0.72±0.08^{def}	0.03±0.03^{bcd}
AMF	0.41±0.04^{cd}	1.90±0.12^{bcd}	0.06±0.05^{c}	7.00±2.00^{d}	0.70±0.10^{ef}	0.02±0.02^{cd}
RP+*Streptoco.*	0.35±0.07^{d}	1.70±0.00^{de}	0.13±0.06^{abc}	9.70±2.00^{bcd}	0.92±0.05^{cde}	0.07±0.03^{ab}
RP+*Bacillus*	0.46±0.05^{bc}	1.80±0.00^{cd}	0.13±0.05^{abc}	10.70±2.00^{abc}	0.96±0.03^{cd}	0.07±0.03^{ab}
RP+*Coccus*	0.49±0.06^{abc}	1.90±0.02^{bcd}	0.12±0.07^{abc}	9.70±2.00^{bcd}	0.98±0.11^{bc}	0.06±0.04^{abc}
RP+AMF	0.56±0.06^{a}	2.14±0.22^{abc}	0.11±0.04^{abc}	8.70±1.50^{bcd}	1.03±0.15^{bc}	0.05±0.02^{bcd}
RP+AMF+*Strepto*	0.54±0.06^{a}	2.70±0.36a	0.18±0.05^{a}	13.33±2.51^{a}	1.33±0.30^{a}	0.11±0.04^{a}
RP+AMF+*Bacillus*	0.54±0.04^{a}	2.70±0.45^{a}	0.19±0.08^{a}	10.70±2.95^{abc}	1.32±0.32^{a}	0.11±0.05^{a}
RP+AMF+*Coccus*	0.51±0.03^{ab}	2.18±0.19^{ab}	0.18±0.06^{ab}	11.70±2.90^{ab}	1.21±0.02^{ab}	0.10±0.05^{a}

*Values are means ± SD (n=03). Values followed by different letters within a column indicate significant differences according to LSD ($p<0.05$)

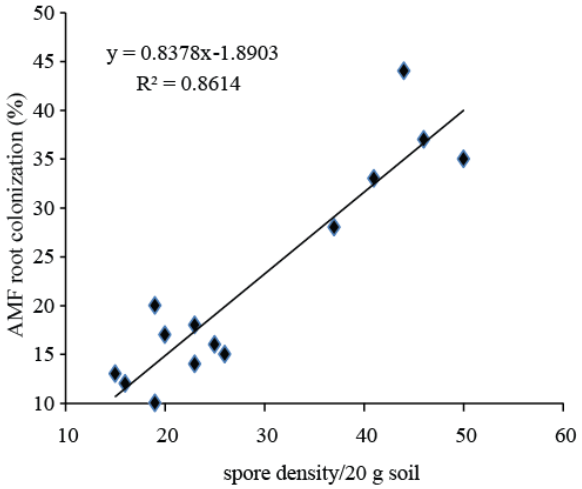

Fig. 1. Correlation between AMF spores density and maize root infection.

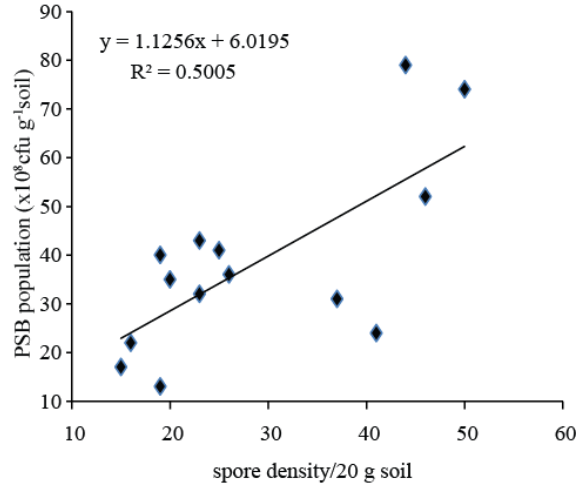

Fig. 2. Correlation between AMF spores and PSB population in maiz.

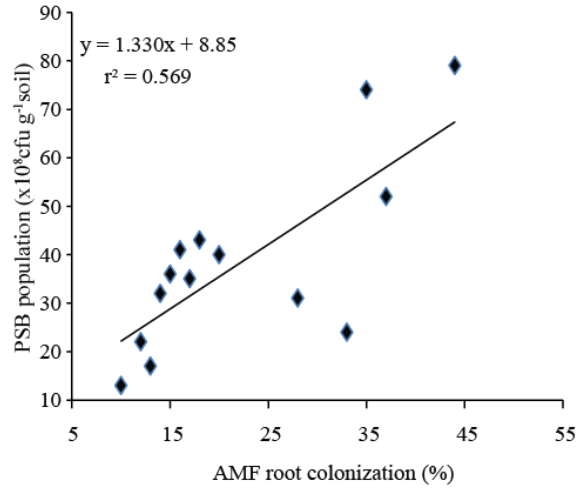

Fig. 3. Correlation between AMF roots infection and PSB population in maize.

The PSB and AMF inoculation with RP significantly stimulated the growth and P uptake by maize plants (Table 2). The highest P uptake was recorded in the treatment of AMF inoculated with *Bacillus* and *Streptococcus* sp. (0.11 g pot^{-1}) in the presence of RP as compared to control. It was followed by *Coccus* sp. (0.10 g pot^{-1}) with RP and SSP (0.10 g pot^{-1}). The minimum (0.01 g pot^{-1}) P uptake was found in control treatment having no P fertilizers. Improved P solubility and plants uptake by AMF and PSB inoculation enhanced N uptake by maize plants and thus its maximum value of 1.33 g pot^{-1} was noted by the inoculation of AMF with *Streptococcus* and 1.32 g pot^{-1} with *Bacillus* sp. as compared to control (Table 2).

Soil mycorrhiza status and phosphate solubilizing bacteria population: Data regarding the microscopic examination of soil mycorrhiza status and phosphate solubilizing bacteria population are presented in Table 3. All interactions of AMF and PSB strains combined with RP were highly significant. The different numbers of AMF spores were observed in all treatments treated with and without RP. Maximum numbers of AMF (50 and 46 spores per 20 g soil) were observed by the combined application of AMF with *Coccus* and *Streptococcus* sp. along with RP, respectively. The application of indigenous AMF inoculated with PSB strains enhanced AMF roots colonization in maize significantly (as compared to PSB inoculated alone or in combination with RP. The presence of mycorrhizal spores affected the percentage of root colonization of maize plants and ranged between 10-44%. Highest percentage of AMF colonization (44 and 37%) was found in plants inoculated with AMF, *Bacillus* and *Streptococcus* in the presence of RP. The inoculation of AMF and PSB with RP significantly increased the PSB population in maize rhizosphere soil after crop harvest (Table 3). Higher PSB population of 79×10^8 CFU g^{-1} soil was noted in AMF treated with *Bacillus*. The minimum number (13×10^8 CFU g^{-1} Soil) of PSB was recorded in control treatment which was at par with the treatments having fertilizers and PSB or AMF inoculation alone. In addition, it was found that AMF spores development was positively (R^2=0.86) correlated with percent root colonization (Fig. 1) and PSB population (R^2=0.51) in maize rhizosphere (Fig. 2). While the relationship between AMF root colonization and PSB population (R^2 = 0.56) was also positive due to the combined application of AMF and PSB with RP (Fig. 3).

Post harvest soil properties: The interactive effects of AMF and PSB strains on post harvest soil properties is depicted in Table 4. The soil organic matter (SOM) contents recorded after maize harvest were influenced by the dual inoculation of AMF and PSB with RP. High value of 1.34% SOM content was observed in RP+AMF+*Coccus* treatments, followed by inoculation of AMF and *Streptococcus* with RP (1.32). The microbial symbiosis and soil organic matter content affects the pH of soil. Lowest pH value of 7.31 was observed by the inoculation of AMF with *Streptococcus* sp. followed by *Coccus* sp. The minimum value of pH in control treatments was 7.82. A maximum increase in ECe of 0.36 dSm^{-1} was recorded in the treatments having combination of AMF and *Streptococcus* sp. with RP. The minimum ECe of 0.19 dsm^{-1} was observed in control which is at par with the treatments having single or no inoculation (Table 4).

Table 3. Soil spores 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 g^{-1} soil)
Control	19 ± 3.8^{def}	10 ± 3.6^{f}	13 ± 1.7^{i}
SSP	16 ± 4.0^{ef}	12 ± 3.5^{ef}	22 ± 3.6^{gh}
RP	15 ± 4.5^{f}	13 ± 3.6^{def}	17 ± 3.0^{hi}
Streptococcus	20 ± 5.3^{def}	17 ± 4.0^{def}	35 ± 4.5^{cde}
Bacillus	23 ± 2.9^{de}	14 ± 5.5^{def}	32 ± 4.5^{def}
Coccus	19 ± 5.5^{def}	20 ± 4.0^{d}	40 ± 4.5^{cd}
AMF	41 ± 4.6^{bc}	33 ± 4.5^{bc}	24 ± 4.0^{fgh}
RP+*Streptococcus*	26 ± 5.0^{d}	15 ± 4.5^{def}	36 ± 3.5^{cde}
RP+Bacillus	23 ± 5.0^{def}	18 ± 2.5^{de}	43 ± 3.5^{c}
RP+Coccus	25 ± 4.5^{d}	16 ± 4.04^{def}	41 ± 2.3^{c}
RP+AMF	37 ± 2.6^{c}	28 ± 3.6^{c}	31 ± 4.0^{efg}
RP+AMF+*Streptoco*	46 ± 6.5^{ab}	37 ± 4.5^{ab}	52 ± 7.6^{b}
RP+ AMF+*Bacillus*	44 ± 5.7^{abc}	44 ± 5.5^{a}	79 ± 5.6^{a}
RP+AMF+*Coccus*	50 ± 4.0^{a}	35 ± 4.5^{bc}	74 ± 12.2^{a}

Values are means ± SD (n=03). Values followed by different letters within a column indicate significant differences according to LSD ($p<0.05$)

Table 4. Post harvest soil organic matter, pH and ECe as influenced by AMF and PSB inoculation with RP*.

Treatments	Organic matter (%)	pH	ECe (dSm^{-1})
Control	0.8 ± 0.41^{c}	7.82 ± 0.07^{a}	0.19 ± 0.03^{d}
SSP	1.23 ± 0.45^{abc}	7.73 ± 0.03^{abc}	0.19 ± 0.03^{d}
RP	1.13 ± 0.30^{bc}	7.76 ± 0.01^{ab}	0.19 ± 0.02^{d}
Streptococcus	1.13 ± 0.30^{bc}	7.66 ± 0.10^{abcd}	0.21 ± 0.07^{cd}
Bacillus	1.15 ± 0.35^{abc}	7.63 ± 0.04^{abcde}	0.22 ± 0.05^{cd}
Coccus	1.18 ± 0.45^{abc}	7.57 ± 0.06^{bcdef}	0.23 ± 0.05^{bcd}
AM Fungi	1.20 ± 0.30^{abc}	7.55 ± 0.00^{cdef}	0.29 ± 0.04^{abcd}
RP + *Streptococcus*	1.22 ± 0.45^{abc}	7.47 ± 0.06^{defg}	0.26 ± 0.05^{abcd}
RP + *Bacillus*	1.26 ± 0.46^{abc}	7.42 ± 0.14^{fg}	0.27 ± 0.04^{abcd}
RP + *Coccus*	1.28 ± 0.35^{abc}	7.42 ± 0.13^{efg}	0.33 ± 0.04^{abc}
RP + AMF	1.28 ± 0.15^{abc}	7.42 ± 0.15^{efg}	0.32 ± 0.04^{abc}
RP + AMF + *Streptococcus*	1.32 ± 0.56^{ab}	7.31 ± 0.13^{g}	0.36 ± 0.21^{a}
RP + AMF + *Bacillus*	1.31 ± 0.11^{ab}	7.41 ± 0.15^{fg}	0.34 ± 0.03^{ab}
RP + AMF + *Coccus*	1.34 ± 0.60^{a}	7.42 ± 0.30^{efg}	0.35 ± 0.02^{a}

*Values are means ±SD (n=03). Values followed by different letters within a column indicate significant differences according to LSD ($p<0.05$)

Discussion

Rock phosphate is a potential substitute to replenish the depleting reservoirs of P, particularly in tropical and subtropical soils. The direct application of low cost RP with the combined inoculation of P solubilizing microorganisms is an important approach to decrease the use of chemical fertilizers and improve P supply for sustainable crop production. In our study, we inoculate three strains of PSB (*Coccus* DIM7, *Streptococcus* PIM6 and *Bacillus* sp. PIS7) with AMF and RP and investigated its effect on different plant growth parameters. The response of each inoculated species on maize shoot, root dry biomass and height were found significantly higher than the un-inoculated treatments. The synergistic effect of AMF and PSB influenced RP solubility and stimulated maize roots to absorb nutrients from soil and thus enhanced the overall plant growth as compared to the treatments having SSP and RP alone. In this experiment, the higher growth of maize observed was due to plant growth promoting activities of AMF and PSB in the rhizosphere. The microbial activities stimulated nutrients uptake and plant growth may be due to hormones such as auxin or gibberellic acid production as stated by Minaxi *et al*., 2013: Kang *et al*., 2012) The increase in maize plant is also attributed to absorbance of more P from the soil and its accumulation towards shoots, resulting in increased shoot and roots dry weight and plant height. This pot trial clearly indicates the synergistic effects of inoculation of AMF and PSB on overall growth of maize plants as supported by Saxena *et al*. (2015) who evaluated that the growth of chickpea can be enhanced by the co-inoculation of *Bacillus* sp. RM2 and *Aspergillus niger* S36 with AMF in the presence of tricalcium phosphate.

In this experiment, we have shown that the single inoculation of AMF and/or PSB with RP did not significantly enhance the nutrients (N and P) concentrations of both the soil and maize plants as compared to the un-inoculated treatments. The possible reason therefore is that P fertilizers quickly converted into insoluble compounds in calcareous soils and thus its solubility and bioavailability was restricted due to complex formation with other nutrients (He *et al*., 2002). Moreover, the inoculation of AMF alone did not significantly influence the concentration of plant P and total N (Tanwar *et al*., 2013), although it is well known that mycorrhiza is effective in increasing nutrients uptake in low P soil (Parewa *et al*., 2010). All bacterial strains used in our study were efficient in enhancing P uptake and solubility from RP in interaction with AMF due to their different mechanisms. The synergistic effects of AMF and PSB on nutrients solubility and uptake have also been reported by Zhang *et al*., 2014 and Saxena *et al*., 2015. During interaction and nutrients solubility the PSB produce enzymes and secrete organic acids and biological materials such as auxin gibberlic acid, vitamins and hormones that increase the dissolution of phosphate (He *et al*., 2002). The PSB increases the soil P pool available for AM fungal extraradical hyphae to pass on to the plant (Smith & Read, 1997). The AM fungi develop extramatrical mycelium which help in reducing the distance between AM fungi and P for plant uptake (Howler *et al*., 1987).

The highest soil spores density and root colonization of maize were observed by the co-inoculation of AMF and PSB with RP. The applied PSB strains stimulated the development and activities of AMF spores and their root infection intensity and thus the correlation between AM fungi and PSB strains in the maize rhizosphere was positive. In some of the combined treatments of AMF and PSB with RP, we found an irregular increase between AMF spores formation and their maize roots colonization (Tanwar *et al*., 2013). It might be due the presence of some rhizosphere organisms which affect the pre-symbiotic stages of AMF development (Giovannetti, 2000). The percentage of mycorrhization produced by AMF was also further modified by the inoculation of our three indigenous bacterial strains. In general, we found more colonization in maize roots by combining AMF and PSB strains with RP. This abundant proliferation and colonization of mycorrhiza in roots could be due to the presence of RP, microbial inoculants (changes morphology and physiology) and synergistic effect with rhizobacteria (Singh & Singh, 1993) in maize rhizosphere. In our findings, we observe high colonization by AMF in the presence of RP fertilizers and PSB but there are also evidences that the application of P fertilizers reduced the colonization rate of plant roots. Bacterial population was also enhanced in the treatments having AMF and PSB interaction with RP over control. It shows that the indigenous bacterial strains can survive, proliferate and colonize in the maize plants rhizosphere (Minaxi *et al*., 2013). The AMF or PSB alone without fertilizers didn't improve the total microbial population (AMF+PSB) in rhizosphere of maize. Reyes *et al*. (2002) observed a reduction of PSB population in the rhizosphere of maize in the absence of P fertilizers after inoculation with *Penicillium rugulosum*. Thus it is clear from this pot trial that the co-inoculation of AMF and PSB strains increased the growth and number of AMF spores, root colonization (Babana *et al*., 2012) of maize plants and PSB population in the presence of RP. In our study, the observed differences in percent soil organic matter (SOM), pH and ECe (dsm^{-1}) also resulted from various microbial activities (AMF and PSB) in maize rhizosphere. The AMF inoculated with PSB strains enhanced SOM and ECe of the soil in the treatments together with RP. Initially, the pH of our experimental soil was 7.85 but slowly dropped down after inoculation with AMF and PSB strains. The PSB solubilized RP and released P into the soil by organic acid production and thus lowered pH of the surrounding bulk and rhizosphere soil (Zhang *et al*., 2011). These results clearly showed that the combined application of AMF and PSB strains can produce a favorable environment for RP mineralization and P solubilization in maize rhizosphere at alkaline calcareous soils.

Conclusion

It is concluded that the combined inoculation of AM fungi and phosphate solubilizing bacteria strains (*Coccus* DIM7, *Streptococcus* PIM6 and *Bacillus* PIS7) with RP is a promising approach to manage P sustainability and maize productivity in P deficient soils. Moreover, inoculation of plants by AMF and PSB with RP is beneficial to minimize dependence on phosphatic fertilizers by locally available RP sources. Hence, AMF and PSB can be used as a useful strategy to prepare biofertilizers for enhancing RP mineralization and solving the P problems in alkaline soils. Further research work is suggested to investigate the synergistic effects of combined use of AMF with PSB strains as inoculants with RP for various crops under different agro ecological conditions.

Acknowledgement

The Authors would like to acknowledge Higher Education Commission (HEC) of Pakistan for financial support of this study.

References

Anonymous. 1989. Crop response to fertilizer. NFDC Publication 4/89. Islamabad, Pakistan.

Anonymous. 1989. Crop response to fertilizer. NFDC Publication 4/89. Islamabad, Pakistan.

Babana, A.H., A. Hani, H.D Amadou, M. Kadia and T. Diakaridia. 2012. Effect of *Pseudomonas* sp. on wheat roots colonization by mycorhizal fungi and phosphate-solubilizing microorganisms, wheat growth and P-uptake. *Intercontinental Journal of Microbiology*, 1(1): 01-07.

Balemi, T. and K. Negisho. 2012. Management of soil phosphorus and plant adaptation mechanisms to phosphorus stress for sustainable crop production: a review. *J. of Soil Sci. and Plant Nut*., 12(3): 547-562.

Baquall, M.F. and M.F. Das. 2006. Influence of biofertilizers on macronutrient uptake by the mulberry plant and its impact on silkworm bioassay. *Caspian J. Environmental Sci*., 4(2): 98-109.

Barea, J.M., C. Azco´n-Aguilar and R. Azco´n. 1997. Interactions between mycorrhizal fungi and rhizosphere microorganisms within the context of sustainable soil-plant systems, p. 65-77. In: *Multitrophic interactions in terrestrial systems*. (Ed.): Gange, A.C. and V. K. Brown. blackwell Science, Cambridge, England.

Black, C.A. 1965. Methods of Soil Analysis. Part-ii. Soc. Agron. Inc. Publ. Madison, Wiconsin. USA.

Bremner, J.M. and C.S. Mulvaney. 1982. Nitrogen-total. In: *Methods of soil analysis*. (Ed.): Page, A.L., R.H. Miller and D.R. Keenay. Part 22nd ed. *Agron*., 9: 595-621.

Costa, E.M.d., L. Wellington de, M. Silvia, Oliveira-Longatti, M. Fatima and de Souza. 2015. Phosphate-solubilising bacteria enhance Oryza sativa growth and nutrient accumulation in an oxisol fertilized with rock phosphate. *Ecol. Eng*., 83: 380-385.

Edi-Premono., M.A. Moawad and P.L.G. Vleck. 1996. Effect of phosphate solubilizing Pseudmonas putida on the growth of maize and its survival in the rhizosphere. *Indonasian J. Crop Sci*., 11: 13-23.

Ehteshami, S.M.R. 2011. Phosphorus acquisition by two wheat cultivars supplied with rock phosphate and inoculated with Glomus intraradices in an alkaline soil. *Tech. J. Engin. & App. Sci*., 1(1): 20-25.

Gerdeman, J.W. and T.H. Nicolson.1963. Spores of mycorrhiza, Endogone species extracted from soil by wet sieving and decenting. *Mycology Society*, 46(2): 235-244.

Giovannetti, M. 2000. Spore germination and pre-symbiotic mycelial growth. In: *Arbuscular mycorrhizas: physiology and function*. (Eds.): Kapulnik, Y. and D.D. Douds Jr. Kluwer Academic, Dordrecht, The Netherlands, pp. 3-18.

Gulati, A., P. Vyas, P. Rahi and R.C. Kasana. 2010. Plant growth promoting and rhizosphere competent *Acinetobacter rhizosphere* strain BIHB 723 from the cold desert of Himalayas. Current Microbiology, New York, v. 58. 371-377.

He, Z.L., W. Bian and J. Zhu, 2002. Screening and identification of microorganisms capable of utilizing phosphate adsorbed by goethite. *Communications in Soil Science and Plant Analysis*, 33: 647-663.

Hodge, A., C. D. Campbell and A. H. Fitter. 2001. An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Nature*, 413: 297-299.

Howler, R.H., E. Sieverding and S. Saif. 1987. Practical aspects of mycorrhizal technology in some tropical crops and pastures. *Plant and Soil*, 10: 249-283.

James, G.C. 1978. Native Sherman Rockland Community College, State University of New York. The Benjamin/Cummins Publishing Co., Inc., pp: 75-80.

Jandel Scientific. 1991. Jandel Scientific Table Curve User's manual v. 3.0 software. AISN Software, Corte Madera, California.

Johnson, L.F. and E.A. Curl. 1972. Methods for research on the ecology of soil borne plant pathogens. 426 So. Sixth St., Minneapolis, MN 55415: Burgess Publishing Company.

Kang, S.M., A.L. Khan, M. Hamayun, Z.K. Shinwari, Y.H. Kim, G.J. Joo and I.J. Lee. 2012. *Acinetobacter calcoaceticus* ameliorated plant growth and influenced gibberellins and functional biochemicals. *Pak. J. Bot*., 44(1): 365-372.

Kennedy, I.R., A. T. M. A. Choudhury and M. L Kecskés. 2004. Non–symbiotic bacterial diazotrophs in crop–farming systems: can their potential for plant growth promotion is better exploited?. *Soil Biol. Biochem*., 36: 1229-1244.

Khan M.S., A. Zaidi, M. Ahemad, M. Oves and P.A. Wani. 2010. Plant growth promotion by phosphate solubilizing fungi – current perspective. Arch. *Agron. Soil Sci*., 56:73-98.

Khan, M.A., K. B. Marwat, A. Amin, A. Nawaz and H. Khan. 2012. Soil solarization: an organic weed management approach in cauliflower. *Comm. Soil Sci. Plant Anal*., 43(13): 1847-1860.

Koehler, F.E., C.D. Moudre and B.L. McNeal. 1984. Laboratory manual for soil fertility. Washington State University Pluman, USA.

McClean, E.O. 1982. Soil pH and lime requirement. P. 209-223. In: *Methods of Soil Analysis*, (Ed.): Page, A. L., R.H. Miller and D. R. Keeny, Part 2 2nd ed. *Am. Soc., Agron*., 9: 199-208.

McGonigle, T.P., M.H. Miller, D.G. Evans, G.L. Farchild and J.A. Swan. 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytol*., 115: 495-501.

Minaxi, J. Saxena, S. Chandra and L. Nain. 2013. Synergistic effect of phosphate solubilizing rhizobacteria and arbuscular mycorrhiza on growth and yield of wheat plants. *J. Soil Sci. Plant Nut*., 13(2): 511-525.

Miniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, U.S.A. pp. 545.

Murphy and J.P. Riley. 1962. A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimia Acta*, 27: 31-36.

Murray, P., E. Baron, J. Jorgensen, M.A. Pfaller and R.H. Yorken. 2003. Susceptibility testing methods yeast and filamentous fungi, manual of clinical microbiology 8th ed. Vol. 2 American Society Microbiology press Washington DC.

Naseer, M. and M. Dost. 2014. Direct and residual effect of Hazara rock phosphate (HRP) on wheat and succeeding maize in alkaline calcareous soils. *Pak. J. Bot*., 46(5): 1755-1761.

Nelson, D.W and L.E. Sommer. 1982. Total carbon, organic carbon and organic matter. pp. 539-577. In: *Methods of Soil Analysis*, (Ed.): Page, A.L., R.H. Miller and D.R. Keeny, Part 2 2nd ed. Am. Soc. Agron. Madison. WI. Organization, New Delhi, India.

Parewa, H.P., A. Rakshit, A. M. Rao, N. C. Sarkar and P. Raha. 2010. Evaluation of maize cultivars for phosphorus use efficiency in an Inceptisol. *Inter. J. of Agri. Envir. & Biotechnolog*, 3(2): 195-198.

Parkash, V., S. Sharma and A. Aggarwal. 2011. Symbiotic and synergistic efficacy of endomycorrhizae with *Dendrocalamus strictus* L. *Plant Soil Environ*., 57(10): 447-452.

Phillips, J.M. and D.S. Hayman. 1970. Improved producers for clearing roots and staining parasitic and VAM fungi for rapid assessment of infection. Trans, B.R. *Mycology Society,* 84(1): 168-170.

Pikovskaya, R.I. 1948. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species, *Mikrobiologiya,* 17: 362-370.

Pizzeghello, D., A. Berti, S. Nardi and F. Morari. 2011. Phosphorus forms and P-sorption properties in three alkaline soils after long-term mineral and manure applications in north-eastern Italy. *Agri. Eco. Environ*., 141: 58-66.

Rajan, S.S.S., J.H. Watkinson and A.G. Sinclair. 1996. Phosphate rocks for direct application to soils. *Adv. Agron*., 57: 77-159.

Reyes, I., L. Bernier and H. Antoun. 2002. Rock phosphate solubilization and colonization of maize rhizosphere by wild and genetically modified strains of *Penicillium rugulosum*. *Microbial Ecology*, 44: 39-48.

Richards, L.A., ed. 1954. Diagnose and Improvement of Saline and Alkaline Soils. U.S.D.A. Hand Book No. 60. Washington D.C.

Richardson, A.E., J.M. Barea, A.M. Mcneill and C. Prigent-Combaret. 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil*, The Hague., v. 321, p. 305-339.

Saxena, J., S. Amita, R. Indu, C. Shalini and G. Veena. 2015. Consortium of phosphate-solubilizing bacteria and fungi for promotion of growth and yield of chickpea (*Cicer arietinum*) *J. of Crop Impro.*, 29: 353-369.

Schenck, N.C. and Y. Perez. 1990. Manual for the identification of VA mycorrhizal fungi, 3rd edn. Synergistic, Gainesville, Fla Schüssler A, Gehrig H, Schwarzott D, Walker C (2001) Analysis.

Sharif, M., M. Khan, M.A. Khan, F. Wahid, K.B. Marwat, A.M. Khattak and M. Naseer 2015. Effect of rock phosphate and farmyard manure applied with effective microorganisms on the yield and nutrients uptake of wheat and sunflower. *Pak. J. Bot*., 47(SI): 219-226.

Sharma, N.K., Raman Jeet Singh and Kuldeep Kumar. 2012 "Dry Matter Accumulation and Nutrient Uptake by Wheat (*Triticum aestivum* L.) under Poplar (*Populus deltoides*) Based Agroforestry System. *Agronomy,* 1-7.

Sharma, S.B., R.Z. Sayyed, M.H. Trivedi and T.A. Gobi. 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. http://www.springerplus.com/content/2/1/587.

Singh, H.P. and T.A. Singh. 1993. The interaction of rockphosphate *Bradyrhizobium* vesicular-arbuscular mycorrhizae and phosphate- solubilizing microbe on soybean grown in a sub Himalayan mollisol. *Mycorrhiza*, 4: 37-43.

Smith, S.E. and D.J. Read. 1997. *Mycorrhizal Symbiosis*. San Diego, CA, USA: Academic Press.

Soltanpour, P.N. and A.P. Schwab. 1977. A new soil test for simultaneous extraction of macro and micronutrients in alkaline soils Comm. Soil Sci. *Plant Anal*., 8: 195-207.

Tanwar, A., A. Aggarwal N. Kadian and A. Gupta. 2013. Arbuscular mycorrhizal inoculation and super phosphate application influence plant growth and yield of *Capsicum annuum. J. Soil Sci. Plant Nut*., 3(1): 55-66.

Umesha, S., M. Divya, K.S. Prasanna, R.N. Lakshmipathi and K.P. Sreeramulu. 2014. Comparative effect of organics and biofertilizers on growth and yield of maize (*Zea mays* L). *Curr. Agri. Res*., 2(1). 55-62.

Walpola, C.B. and M. Yoon. 2013. Phosphate solubilizing bacteria: Assessment of their effect on growth promotion and phosphorous uptake of mung bean (*Vigna radiata* [L.] R. Wilczek) *Chil. J. of Agric. Res*., 73(3): 275-281.

Walsh, L.M. and J.D. Beaton. 1977. Soil testing and plant anal. *Soil Sci. Am. Inc*., Madison. WI.

Wilson, G.W.T., C.W. Rice, M.C. Rillig, A. Springer and D.C. Hartnett. 2009. Soil aggregation and carbon sequestration are tightly correlated with the abundance of arbuscular mycorrhizal fungi: Results from long-term field experiments. *Ecology Letters*, 12: 452-461.

Xiong, L.M., R.K. Lu and B. Truong. 1994. An evaluation of the agronomic potential of partially acidulated rock phosphates in calcareous soil. *Fert. Res*., 38: 205-212.

Yadav, A. and A. Ashok. 2014. Effect of dual inoculation of AM fungi and pseudomonas with phosphorus fertilizer rates on growth performance, nutrient uptake and yield of soybean. *Researcher*, 6(11): 5-13.

Zapata, F. and A.R. Zaharah. 2002. Phosphorus availability from phosphate rock and sewage sludge as influenced by the addition of water-soluble phosphate fertilizer. *Nut. Cyc. in Agroeco*., 63: 43-48.

Zhang, H., W. Xianghua, L. Gang and Q. Pei. 2011. Interactions between arbuscular mycorrhizal fungi and phosphate-solubilizing fungus (*Mortierella* sp.) and their effects on *Kostelelzkya virginica* growth and enzyme activities of rhizosphere and bulk soils at different salinities. *Biol. Fertil. Soils*, 47: 543-554.

Zhang, L.A., B.J. Fan, A.C.X. Ding, D.X. He, A.F. Zhang and G. Feng. 2014. Hyphosphere interactions between an arbuscular mycorrhizal fungus and a phosphate solubilizing bacterium promote phytate mineralization in soil. *Soil Biol. & Biochem*., 74 177-183.

(Receive for publication 22 March 2015)