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ORIGINAL ARTICLE

Phytase, Phosphatase Activity and P-Nutrition of Soybean as Influenced by Inoculation of *Bacillus*

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Abstract The efficiency of different *Bacillus* isolates on rhizosphere soil enzyme activities and P-nutrition of soybean was carried out under microcosm conditions. Significant increase in enzyme activities viz., fluorescein diacetate activity, phosphatase and phytase activity and consequent effects on P-nutrition were observed with the inoculation of *Bacillus* isolates over uninoculated control. Among the isolates, BD-3-1B, KHBD-6, BDKH-3, *Bacillus amyloliquefacians*, and *Bacillus cereus* were found to be promising. The phytic acid-P as a percentage of total P content in soybean seeds decreased with the inoculation of *Bacillus* isolates as compared to un-inoculated control. A decrease in phytic-P in soybean seeds not only results in better digestibility and increased feed efficiency. Pearson correlation studies revealed a significant positive association between acid, alkaline phosphatases, phytase activity on available P content in soil and P content in seeds with the inoculation of *Bacillus* isolates, indicating role of these enzymes in P mobilization and acquisition by soybean.

Keywords *Bacillus* · Fluorescein diacetate activity · Phosphatases · Phytase · Phytic-P · Soybean

Introduction

Phosphorus (P) is an essential plant nutrient, a deficiency of which limits crop production. Coupled with low native soil P availability is the problem of low utilization efficiency of applied P due to sorption and precipitation reactions. Consequently, large inputs of mineral P fertilizers to soil are required to meet the P needs of crops. Since the farmers are largely cash limited and P fertilizers being costly, restricts their capacity to procure fertilizers, and hence it is pertinent to develop production systems that are P efficient. Synchrony of P supply with crop requirements is the way to attain this objective. In soils, the cycling of P from organic pools, rather than equilibration of the solution with inorganic P, is of a practical significance. Synthesis and mineralization of organic P by soil microorganisms is integral to this process [1, 2].

Plants have developed a range of mechanisms to enhance their acquisition of P from soil [3]. Plants and microorganisms together increase P availability by solubilising inorganic P and mineralizing organic P. These include beneficial rooting characteristics, mycorrhizae, fungi, bacteria, root exudates and production of phosphatase enzymes. Enzyme activities have been proposed as a tool to monitor changes in soil nutrient cycling resulting from the interaction between inoculants and indigenous microbial population [4]. They found perturbation in soil enzyme activities following the inoculation of *Pseudomonas fluorescens* in the rhizosphere of wheat.

Production of phosphatases which importantly catalyse the hydrolysis of P from organic forms of P [5] is a potential way for plants to enhance P availability as a large proportion of soil P (up to 80%) occurs in organic forms. Phosphatase activity in soil originates from many sources including plant roots [6], Fungi [7] and bacteria [8]. Microorganisms may produce acid and alkaline phosphatase, but plants can only secrete acid phosphatase [9]. Enzyme that hydrolyzes derivatives of inositol penta- and hexakisphosphate (phytate) are of particular interest because phytate constitutes up to 50% of total organic P in soil [10]. The abundance of phytic acid in soils seems to be

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associated with their low solubility, their firm association with the solid phase, and their high stability [11]. The importance of these compounds as a source of P for plants depends on their hydrolysis. Studies have shown that phytate-P is of limited availability to plants [12]. Poor availability of phytate in soils is a major limitation to its utilization by plant roots. Some of the plant growth-promoting microorganism (PGPM) strains of *Bacillus amyloliquefaciens, Bacillus laevolacticus, Bacillus subtilis,* and *Aspergillus niger, Penicillium rubrum* are known to be active phytases producer [2, 13, 14]. As for as seed quality parlance, phytic acid is the storage form of P in seeds and accounts for 65–80% [15] and is considered as anti-nutritional factor to animals and human beings. It is, therefore, our endeavor to reduce the phytic acid-P content in seeds.

The aim of the present study is to assess impact on soil phosphatase and phytase activity and their role in P-nutrition of soybean as influenced by inoculation of *Bacillus* isolates recovered from soybean rhizosphere.

Materials and Methods

Isolation and Characterization of *Bacillus* Isolates

To isolate of *Bacillus* isolates, 1.0 g moist soil sample was suspended in 9 ml of sterile distilled water in test tubes and these tubes were placed in hot water bath at 60°C for 60 min to kill all vegetative cells of microbes leaving only spore forming bacilli in suspension [16]. After cooling the tubes for a short period, the suspensions of different soil sample are diluted up to 10^{-3} dilution and thereafter 100 µl of this dilution was spread on nutrient agar (NA) medium and incubated at 30°C for 24–48 h. Morphologically diverse bacterial isolates were purified from each plate and these isolates were maintained as glycerol stock at −20°C in deep freezer. All the isolates were subjected to morphological and biochemical characterization following standard methods [17–21]. Parameter investigated were Gram's reaction, colony morphology, pigmentation, spore formation, catalase, oxidase, nitrate reduction, arginine hydrolase, IAA production, starch hydrolysis, citrate utilization, VP test, casein hydrolysis etc.

Microcosm Experiment

Sixteen soil samples from soybean rhizosphere were collected from Burhanpur, Badwani, Khandwa and Khargone districts located in *Nimar* region of Madhya Pradesh, during *kharif* 2007. Among the 134 isolates recovered from these soil samples, only ten zinc-phosphate solubilizing *Bacillus* isolates were taken into account for this study. These soils are deficient in zinc and P and acts as a deterrent to sustained productivity of soybean. Therefore, efforts were made to isolate bacilli which can solubilize unavailable forms of these nutrient elements to available form efficiently. Two standard cultures *B. amyloliquefaciens* sks_bnj-1 AY 932823 and *Bacillus cereus* ATCC 13061 were also tested along with our isolates. The experiment was conducted during *kharif* 2008 with soil belonging to Sarol series (Iso-hyperthermic, montmorollinitic, typic Haplusterts) and collected from Directorate of Soybean Research, Indore. The pertinent characteristics of the soil were: pH 8.2, OC 4.6 g/kg, clay content 56.2%, and available P 5.82 ppm. The soil had an initial fluorescein diacetate, acid phosphatase, alkaline phosphatase, and phytase activity of 51.2, 18.5, 43.2 and 123.8 pKat/g soils, respectively. The air dried soil was passed through a 2 mm sieve and filled in polythene bags (5 kg/bag) and the moisture content was brought to field capacity. No fertilizer was applied to the experimental bags. The soybean seeds were subjected to washing one time with sterile distilled water followed by surface sterilization with three-step surface sterilization procedure: a 30-s wash in 90% ethanol, followed by a 3-min wash in 3.0% NaOCl, and a final rinse in sterile distilled water. Four seeds were pre inoculated with *Bradyrhizobium* followed by the inoculation with appropriate *Bacillus* isolates/strains and were sown in each bag and after germination two plants were maintained in each bag. The treatments were replicated six times and arranged in a complete randomized block design. Three replications were utilized for R5 stage (beginning seed fill) and remaining three replicates were harvested at maturity. At R5 stage of crop growth, shoots from two plants were cut 5 mm above the soil surface, roots uprooted and thereafter, rhizosphere soil samples were collected by standard method [22] for soil enzymatic analysis. The soil closely associated to the roots was considered as rhizosphere soil. At maturity, plants were harvested for recording seed yield and for estimation of seed P and phytic acid content.

Soil Enzyme Assay

Fluorescein diacetate activity (FDA) in soil was determined using standard method [13]. Briefly, soil samples (0.1 g) were placed in plastic tubes to which 10 ml of potassium phosphate buffer (pH 7.6, 60 mM) was added. The reaction was initiated with addition of 1 ml of fluorescein diacetate (1 mg/ml in acetone) and incubated at 37°C for 4 h. Addition of 10 ml of acetone terminated the reaction, and after centrifugation at 3200 rpm for 10 min the supernatant was read at 490 nm. Phosphatases (acid and alkaline) were measured [23] by using acetate buffer (pH 5.4) and sodium tetraborate–NaOH buffer (pH 9.4),

respectively. The enzyme substrate (4-nitrophenyl phosphate) mixture was incubated at 35°C for 1 h. Measurement on phytase activity was carried out [24]. Briefly, the amount of inorganic P released by hydrolysis of sodium phytate (1 mM) in 0.2 M sodium acetate (pH 4.5) after incubation for 1 h at 37°C. The reaction was terminated with the addition of 0.5 ml 10% trichloroacetic acid. After centrifugation at 10,000*g* for 10 min, the supernatant was analysed for inorganic P content. The enzyme activities of all the enzymes were expressed in pico Katals (pKat), (i.e., pico moles of substrate hydrolyzed per second) on dry weight soil basis.

Soil and Plant Analysis

Olsen-P in soil was estimated by the standard method [25]. At harvest, seed samples were dried at 60°C, weighed and ground to powder and total P content in seed was estimated by standard Vanadomolybdate method [26]. Phytic acid-P was estimated by the modified colorimetric procedure (Wade reagent) [27]. Samples of 0.50 g of soy flour were thoroughly mixed with 2.4% HCl and shaken for 16 h at 220 rpm and centrifuge at 10000 rpm for 10 min. The crude extracts were then transferred to another centrifuge tube containing 1 g of NaCl. The contents were shaken for 20 min and allowed to settle at 4°C for 60 min. Later, the contents were centrifuged at 10000 rpm for 10 min and 1 ml of clear supernatant was diluted to 25 ml with distilled water. Three millilitre of the diluted sample was combined with 1 ml of Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$ + 0.3% sulfosalyclic acid) and contents read at 500 nm.

Statistical Analysis

Analysis of variance was carried out and the least significant differences (LSD) were used to separate the treatment means using DMRT test (COSTAT statistical software, Cohart, Berkeley, California). Pearson correlation between variables was analyzed using SPSS version 10.0 software.

Results and Discussion

A total of 134 putative *Bacillus* isolates were recovered from 16 soil samples. Most of the isolates recovered on NA had smooth surface, while a few were wrinkled. The colonies observed were white, orange and yellow in color while texture was opaque, shiny, smooth or wrinkled, mucoid or dry on NA medium. All isolates and a standard were found gram positive, endospore-forming, positive for catalase, oxidase and nitrate reduction tests while isolates BD-3-1B and BDN-5 which were gram positive, endospore-forming but positive for catalase only (Table 1). According to the results obtained showed that the isolates belonged to the genus *Bacillus* [28, 29].

Table 1 Morphological and biochemical characterization of zinc-solubilizing *Bacillus* isolates

Characteristics	Isolates/Standards									
	KHBD-2-1A	BDKH-3	BD-3-1B	BDSD-2-2C	BDN-5	KHTH-4-1	KHBAR-1	KHBD-6	KDMR-1-1	*B. cereus*
Appearance	D	S	D	S	S	S	S	Sm	S	Sm
Form	C	C	C	C	C	C	C	Ir	C	C
Margin	Sr	Fil	L	Un	E	Un	Ir	Ir	Fil	L
Elevation	F	F	F	F	R	Um	F	F	F	F
Pigmentation	W	W	Y	W	O	W	W	Y	W	W
Gram reaction	+	+	+	+	+	+	+	+	+	+
Spore staining	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	−	+	−	+	+	+	+	+
Nitrate reduction	+	+	+	+	−	+	+	+	+	+
Arginine dihydrolase	+	+	−	+	−	−	+	+	+	−
Indole-3-acetic acid	−	−	+	−	−	−	−	−	+	−
Starch hydrolysis	+	+	NA	+	+	NA	NA	−	+	−
Casein hydrolysis	+	+	+	+	+	+	+	+	+	+
VP test	+	+	+	+	+	+	+	−	+	+
Citrate utilization	−	−	−	−	−	−	−	+	−	−
Methyl red	+	+	+	+	+	+	+	−	+	+

D Dull, *C* Circular, *S* Shiny, *Sr* Serrate, *F* Flat, *W* White, *R* Raised, *E* Entire, *Fil* Filamentous, *Y* Yellow, *O* Orange, *L* Lobate, *Un* Undulated, *Um* Umbonate, *Ir* Irregular, *Sm* Smooth, *NA* Not assessed

Table 2 Activities of fluorescein diacetate, phosphatases and phytase in the rhizosphere soils of soybean as influenced by inoculation of *Bacillus*

Isolates/Standard	Fluorescein diacetate activity	Acid phosphatase activity	Alkaline phosphatase activity	Phytase activity
KHBAR-1	63.74^{ef}	28.54^{de}	52.57^{def}	181.04^{f}
BD-3-1B	102.50^{a}	31.98^{abc}	61.86^{b}	273.47^{c}
BDN-5	67.06^{def}	26.84^{ef}	50.28^{f}	228.31^{d}
KHTH-4-1	94.18^{ab}	31.74^{abc}	53.87^{b}	230.68^{d}
KHBD-6	93.40^{bc}	30.79^{bcd}	59.57^{bc}	284.40^{c}
KHBD-7	85.55^{c}	25.26^{fg}	55.10^{d}	202.77^{e}
KDMR-1-1	74.37^{d}	30.04^{cd}	53.60^{de}	166.43^{g}
KHBD-2-2A	75.47^{d}	27.08^{ef}	58.26^{c}	280.45^{c}
BDKH-3	98.27^{ab}	30.04^{cd}	67.75^{a}	334.43^{a}
BDSD-2-2C	98.83^{ab}	33.28^{a}	68.30^{a}	241.35^{d}
B.amyloliquefaciens	70.16^{de}	32.28^{ab}	69.45^{a}	305.07^{b}
B. cereus	69.94^{de}	31.46^{abc}	53.12^{def}	193.29^{ef}
Uninoculated control	60.65^{f}	23.60^{g}	50.63^{ef}	139.44^{h}
LSD ($P = 0.05$)	7.99	2.26	2.92	14.12

Different letters represent significant differences ($P = 0.05$) (DMRT). *Bacillus* isolates are from different locations with contrasting agricultural management practices. Enzyme activity is expressed as pKat/g soil

Soil enzyme assays have been used to investigate biochemical processes such as soil organic matter formation and degradation, nutrient cycling and interaction between inoculants and indigenous microbial population in soils and is a possible integrative measure of soil quality to reflect biological status of soils [4, 30]. Fluorescein diacetate activity was considered in the present study as it was reported to be a better biological indicator of soils than dehydrogenase activity [31].

Inoculation of *Bacillus* isolates significantly increased FDA over uninoculated control (Table 2). However, significant variation in FDA was observed among *Bacillus* isolates. The data revealed that isolates BD-3-1B, followed by KHTH-4-1, KHBD-6, BDKH-3 and BDSD-2-2C had higher FDA as compared to the other isolates. It has been reported that FDA to be a measure of total microbial activity in soil is mainly contributed by the activity of fungi and bacteria [32].

In general, increases in acid- and alkaline- phosphatase activities were recorded with the inoculation of *Bacillus* isolates over control (Table 2). The *Bacillus* isolates BD-3-1B, KHTH-4-1, KHBD-6, BDKH-3, BDSD-2-2C, and *B. amyloliquefaciens*, increased both acid- and alkaline-phosphatases activity as compared to the rest of the *Bacillus* isolates. This may be attributed to increased growth of plant roots [33], which in turn stimulating the proliferation of soil microorganisms in the rhizosphere. Alkaline phosphatase activity is solely of microbial origin, while acid phosphate activity is contributed both by plant roots and microorganisms. The activity of alkaline phosphatase was comparatively higher than acid phosphatase irrespective of inoculation. The increase in FDA supports this hypothesis as it is an indication of microbial build-up.

A large variation in phytase activity was recorded upon inoculation of different *Bacillus* isolates. Among the *Bacillus* isolates, phytase activity was found to be higher with the inoculation of BD-3-1B, KDBD-6 BDKH-3 and *B. amyloliquefaciens*. The potential role of soil microorganisms and in particular *B. amyloliquefaciens* FZB45 and *B. subtilis* for increasing the availability of P from phytase activity and presumably by affecting the availability of phytate itself has been reported [2, 34, 35]. Furthermore, the ineffectiveness of endogenous wheat root phytases was further highlighted by the observation that inoculation of seedlings with *Pseudomonas* sp. CCAR59 significantly enhanced the availability of inositol hexa phosphate (IHP), such that the growth and P content of these plants was equivalent to that observed in Pi-fed controls. This study further supports the findings that phytase was active in the hydrolysis of phytin-P and increase in plant available P status in the rhizosphere for plant nutrition [13].

The available P content was also observed to be increased with the inoculation of *Bacillus* isolates. The isolates BD-3-1B, KHBD-6, BDKH-3, *B. amyloliquefaciens*, and *B. cereus* increased available P content in soils vis-à-vis other *Bacillus* isolates (Table 3). The increased phosphatase and phytase activity with inoculation of *Bacillus* isolates might have been responsible for increased P mobilization and acquisition by plants. This is in

Table 3 P-nutrition and yield of soybean as influenced by inoculation of *Bacillus*

Isolates/standard	Olsen- P (mg/kg soil)	Total P content (g/Kg seed)	Phytic-P (g/Kg seed)	Phytic-P as a % of total P	Yield (g/plant)
KHBAR-1	6.41^{f}	1.99^{a}	1.74^{e}	87.4	1.56^{bc}
BD-3-1B	8.91^{ab}	2.77^{cd}	1.62^{ab}	59.9	1.16^{bc}
BDN-5	8.50^{bcd}	2.57^{ab}	1.73^{bc}	67.3	1.93^{bc}
KHTH-4-1	7.15^{de}	2.42^{cd}	1.61^{d}	66.5	1.61^{bc}
KHBD-6	9.09^{bcd}	2.61^{bcd}	1.62^{a}	62.1	1.93^{bc}
KHBD-7	8.07^{e}	2.30^{ef}	1.48^{c}	64.3	1.26^{c}
KDMR-1-1	6.09^{de}	2.44^{cde}	1.59^{ef}	65.2	1.46^{bc}
KHBD-2-2A	8.61^{cde}	2.52^{cde}	1.57^{abc}	62.3	2.16^{bc}
BDKH-3	8.90^{abc}	2.75^{cd}	1.62^{ab}	58.9	1.83^{bc}
BDSD-2-2C	6.57^{bcd}	2.64^{def}	1.53^{e}	57.9	1.75^{bc}
B.amyloliquefaciens	8.13^{a}	2.90^{cde}	1.59^{c}	54.8	2.28^{ab}
B. cereus	8.07^{cde}	2.52^{abc}	1.65^{c}	65.5	3.11^{a}
Uninoculated control	5.64^{f}	1.91^{f}	1.45^{f}	75.9	1.76^{bc}
LSD ($P = 0.05$)	0.22	0.10	0.52		0.85

Different letters represent significant differences ($P = 0.05$) (DMRT). *Bacillus* isolates are from different locations with contrasting agricultural management practices

Table 4 Pearson correlation between phosphatase, phytase activity and P-nutrition of soybean as influenced by inoculation of *Bacillus*

Isolates/Standard	Fluorescien diacetate activity	Acid phosphatase	Alkaline phosphatase	Phytase activity	Olsen-P content	Total P content in seed
Fluorescien diacetate						
Acid phosphatase	0.544					
Alkaline phosphatase	0.571*	0.604*				
Phytase activity	0.574*	0.466	0.785**			
Olsen-P	0.418	0.201	0.355	0.782**		
Total P content	0.539	0.683*	0.736**	0.831**	0.690**	
Phytic-P	−0.137	0.302	−0.160	0.165	0.288	0.172

* significant at $P = 0.05$

** significant at $P = 0.01$

consonance with the study revealing that inoculation of A*spergillus* strains significantly improved P uptake by plants and extractable P status in soil [33]. The phytic acid-P as a percentage of total P content in soybean seeds decreased with the inoculation of *Bacillus* isolates as compared to un-inoculated control (Table 3). More than 75% of the total P in soybean exists in phytic acid or its complex anion form, phytate salt. Moreover, phytate P content in animal feed and those meant for human consumption cannot be readily digestible as they do not produce sufficient phytase to breakdown phytate. To meet the nutritional requirements, a decrease in phytic-P in soybean seeds not only results in better digestibility and increased feed efficiency which can be achieved with inoculation of *Bacillus* isolates as they decreased phytic acid-P. This calls for further investigation. The increased seed yield achieved upon inoculation of different *Bacillus* isolates as compared to control are mainly due to higher enzyme activities in the rhizosphere which resulted in increased P uptake. Pearson correlation studies (Table 4) revealed a significant positive association between acid, alkaline phosphatases, phytase activity on available P content and P content in seeds with the inoculation of *Bacillus* isolates, indicating role of these enzymes in P mobilization and acquisition by soybean.

The activity of acid and alkaline phosphatase, phytase determine the P mobilization and acquisition by soybean crop with the inoculation of *Bacillus* isolates. The present results demonstrates that inoculation of *Bacillus* isolates BD-3-1B, KHBD-6, BDKH-3, *B. amyloliquefacians*, and *B. cereus* increased phytase, phosphatase activity and P-nutrition of soybean. Furthermore, field evaluation is necessary to confirm soil enzymes activities in soil and P-uptake by soybean to assess practical utility of these bacilli since they have capacity to form spore which can easily survive for longer period even in adverse conditions.

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