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ORIGINAL PAPER

Effect of Seed Bio-priming with *Trichoderma viride* Strain BHU-2953 for Enhancing Soil Phosphorus Solubilization and Uptake in Soybean (*Glycine max*)

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Abstract

The main objective of our study was to evaluate the effectiveness of *Trichoderma viride* BHU-2953 as a single inoculant during seed-priming to enhance phosphorus (P) uptake in soybean. A pot experiment was conducted, taking six treatments, in a completely randomized block design to assess the P uptake, root length, apparent phosphorus recovery (APR), rhizospheric phosphatase activity, pH, soil-P status, dehydrogenase activity, and fungal colony-forming unit (CFU g^{-1}) in three different soybean growth stages through seed-priming with *T. viride* along with graded fertilizer P-doses. Significantly ($P < 0.05$) higher soil phosphatase activity, dehydrogenase activity, CFU of *T. viride*, and P-content in soybean vegetative parts were observed in bio-primed treatments as compared to control and RDF (full recommended dose of P-fertilizer without seed-priming), while seed-P (%), APR (%), and root lengths of RDF were found significantly ($P < 0.05$) lower than bio-primed soybeans with 90% RDF but were at par with 80% RDF. Higher positive correlations between CFU and acid phosphatase ($R^2 = 0.89$, 0.9, and 0.89; $P < 0.05$) and between CFU and alkaline phosphatase activities ($R^2 = 0.98$, 0.96, and 0.97; $P < 0.05$) at 30, 50, and 75 DAS indicate that *T. viride* mediated higher soil phosphatase activities. Higher P-recoveries of bio-primed soybeans, received 90% and 80% RDF, were achieved mainly due to soil applied-P solubilization through enhanced acid phosphatase activities along with better soil exploration by plant roots. Thus, seed-priming with *T. viride* BHU-2953 can reduce up to 20% of the recommended P-dose in soybeans.

Keywords Apparent phosphorus recovery · *Trichoderma viride* · Bio-priming · Phosphatase · Dehydrogenase

1 Introduction

Phosphorus (P) is the second major element, essential for plant growth and development after nitrogen (N). The concentration of phosphorus in plant dry matter varies from 0.05 to 0.5% (Malhotra et al. 2018). Being a constituent of nucleic acids, proteins, and different triose phosphates, phosphorus plays a major role in plants. Major functions of phosphorus include energy transfer, cell division, photosynthesis, and activation of different plant enzymes that resulted in better seed germination, membrane integrity, optimum plant metabolism, formation of plant reproductive parts, and nutrient balance in plants (Julia et al. 2016; Razaq et al. 2017; Singh and Singh 2016; Yamaji et al. 2017). Soil is a vast source of phosphorus for terrestrial plants, but the availability is primarily governed by the release of soluble inorganic phosphate ions ($H_2PO_4^{-}$, HPO_4^{2-} and PO_3^{3-}) in soil-solution, reaction with dominant soil-cations (Fe^{2+}, Fe^{3+}, Al^{3+}, Ca^{2+}, and Mg^{2+}), type of soils, and soil-pH (Penn and Camberato 2019). In agricultural fields, the majority of the phosphorus (natural source, externally applied, or both) in top soils gets converted to plant unavailable forms and interferes with crop uptake. Low use efficiency of phosphorus (15–20%) in field crops has resulted in higher consumption of fertilizer-P (Malhotra et al. 2018). Consumption of phosphatic fertilizers has been increased from 4.2 (2000–2001) to 6.9 Mt (2018–2019) in Indian soils, making field practices more cost-intensive (FAI 2019).

The recent trend towards sustainability follows the use of beneficial microbes to increase the use efficiency of nutrients in field crops without degrading soil health (Meena et al. 2017). Bio-priming is a very promising technology in this

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aspect. It incorporates beneficial microbes to obtain various desirable plant attributes including higher nutrient use efficiencies, high-density microbial proliferation in soils, enhanced crop performances, abiotic and biotic stress resistance, and disposal of crop residues (Devika et al. 2019; Rakshit et al. 2015). Among different inoculants, *Trichoderma* is the most culturable fungus, behaving like an opportunistic and avirulent plant symbiont (Harman et al. 2004). Under the genus, *T. harzianum*, *T. viride*, *T. virens*, *T. atroviride*, *T. hamatum*, *T. asperellum*, and *T. reesei* have been extensively used in the field of agriculture. They were primarily found to boost up the defense in target plants and antagonize soil-borne pathogens. Zhang et al. (2018) had seen that soil application of *Trichoderma* bio-fertilizer reduces the soil-borne pathogenic bacteria and increased the biological yield of *Leymus chinensis*. Similarly, *T. asperellum* was found to be as effective as synthetic fungicides when applied against cucumber wilt (*Fusarium oxysporum*) at the seedling stage and rapid growth stage (He et al. 2018). The fungi were found to be harmlessly associated with plant roots. Beneficial root colonization of *Trichoderma* spp., *Pseudomonas fluorescens*, and arbuscular mycorrhizal (AM) fungi was observed by Yadav and Aggarwal (2015). In the past few decades, several researchers have focused on the application of *Trichoderma viride* species because of its disease suppression abilities, better crop germination, and vigor. Shahid et al. (2011) observed that seed treatment with *T. viride* enhanced chickpea germination and vigor. Enhancement of root length and seed germination in mustard was found by Lalitha and Arunalakshmi (2012). Afzal et al. (2013) found that *T. viride* was able to decrease the disease incidence in okra (causal organisms: *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium* spp., and *Meloidogyne* spp.) when applied with *Pseudomonas aeruginosa*. Recently, plant growth-promoting activities, soil nutrient loss management, and crop nutrient use efficiency have also been reported through the application of this species. Wang et al. (2018) incubated alkaline soils of China with *Trichoderma viride* bio-fertilizer and found that there were decreased levels of ammonia (NH_3) volatilization losses from the treated soils. Mahato et al. (2018) found that the application of *T. viride* can increase the plant height, root length, leaf length, number of leaves, and grain yield of wheat. Tanwar et al. (2013) inoculated broccoli with *T. viride*, *P. fluorescens*, and AM fungi to increase its P-acquisition. The usefulness of *T. viride* was confined to different consortia in most of these cases rather than single inoculation. Inoculation of chickpea with *T. viride* and *Azotobacter chroococcum* biofilm was done by Velmourougane et al. (2017). The existence of a beneficial association between the microbes and chickpea roots was confirmed by them. Yadav et al. (2018) had also reported a similar kind of association among *T. viride*, AM fungi, and baby corn roots that reduced the doses of mineral fertilizer, while higher acid and alkaline phosphatase activities were recorded from sunflower-growing soils when applied with *T. viride*, AM fungi, and *P. fluorescens* (Yadav et al. 2015). Therefore, the potential of *T. viride* isolate needs to be evaluated to see its impact upon priming. Soybean is very sensitive to P-deficient soil conditions (Zhang et al. 2017). Being a leguminous crop, it can fix atmospheric N_2, build up soil-N economy, and reduce succeeding crop-N requirements under intensive cropping-systems (Paul et al. 2019). Works have been carried out by different researchers to enhance the phosphorus use efficiency in soybean. Janegitz et al. (2016) found higher phosphorus use efficiency in soybean when cultivated with ruzi grass as a cover crop and reactive phosphates. Varietal differences in soybean in response to low P-status were evaluated by Vengavasi and Pandey (2018). Similar genotypic differences between P-efficient soybean cultivars were also evaluated by Zhou et al. (2016). However, these methods are very much conventional and require a substantial period to achieve higher P-use efficiency. The recovery of phosphorus is also very less in these methods.

Keeping the above aspects in mind, we hypothesized that seed bio-priming of soybean with single inoculation of *Trichoderma viride* can enhance the P-acquisition in plant and reduce the application of fertilizer-P. This experiment might generate some additional information for future studies and can be a new dimension to maximize crop production with a minimum application of P-fertilizers.

2 Materials and Methods

2.1 Experimental Soil

The pot experiment was conducted in the Department of Soil Science and Agricultural Chemistry, Institute of Agricultural Sciences (IAS), Banaras Hindu University (BHU), Varanasi (25° 15′ N, 82° 59′ E), during the late monsoon season, 2018. Soils in this region have developed from Quaternary alluvium of river Ganga and fallen under Typic Ustifluvents (Soil Survey Staff 2014). To represent major arable soil of Varanasi, soil samples (0–15 cm) were collected from Agricultural Research Farm, IAS, BHU. Previously, a rice-wheat cropping system was practiced in these soils for 2 years. These soils were then analyzed for the determination of different physical and chemical parameters, presented in Table 1.

2.2 Experimental and Treatment Details

The fungal inoculum, *Trichoderma viride* BHU-2953, was collected from the Department of Mycology and Plant Pathology, IAS, BHU. It was then cultured on potato dextrose agar (PDA) medium (pH 5.6) containing

Table 1 Soil physical and chemical parameters before starting the experiment

Initial soil parameters	Value
Texture	Sandy loam
Bulk density (Mg m^{-3})	1.48
pH	7.6
EC (ds m^{-1})	0.41
Oxidizable organic carbon (%)	0.44
Cation exchange capacity (Cmol (p^+) kg^{-1})	32.27
Soil available-P (mg kg^{-1})	15.7

potato broth (obtained from boiling 200 g of potato), 20 g of dextrose (Merck, India), and 15 g of agar-agar powder (Merck, India) in 1 L sterilized distilled water. The cultured *T. viride* in PDA slants were incubated at 27 ± 1 °C for 5 days. The fungal biomass was obtained by scraping with a sterile scraper followed by collecting in 25 mL sterile distilled water. Through serial dilutions, the fungal colony-forming unit was estimated 2×10^5 CFU mL^{-1} in Trichoderma Selective Medium (TSM). The TSM was prepared by adding 20.5 g rose-bengal-chloramphenicol agar (Merck Life Science Pvt. Ltd., India), 3 g dextrose, 1 g ammonium nitrate, 0.9 g di-potassium hydrogen phosphate, 0.3 g fenaminosulf (Merck, India), 0.2 g magnesium sulfate heptahydrate, and 0.15 g potassium chloride in 1 L sterile distilled water as mentioned by Elad et al. (1981).

In this study, soybean (*Glycine max* [L.] Merr.) cv. JS95-60 was selected because of its early maturity, lower shattering nature, and higher productivity under Indian conditions (Dixit et al. 2009). Seeds, weighing 500 g, were sterilized for 1 min in sodium hypochlorite solution, washed thereafter with distilled water, and air-dried. Dried seeds were coated with 10 g talcum powder carrying *Trichoderma viride* BHU-2953 spore suspension (2×10^5 CFU mL^{-1}). Previously, this novel strain of *T. viride* (strain no. BHU-2953) was found to suppress *Fusarium oxysporum* (wilting of tomato) and *Pythium aphanidermatum* (damping-off of chili) pathogens effectively (Singh et al. 2014). For uniform coating, powdery seeds were blended with clear hands along with distilled water and shade dried at room temperature for 1 day before the date of sowing. No strains of *Rhizobium* spp. or *Bradyrhizobium* spp. were used in this experiment.

Soil samples (0–15 cm depth) were filled in 10 kg pots after weighing. Except for control and full recommended doses of fertilizers (RDF), graded doses of nitrogen (N), phosphorus (P), and potassium (K) were applied in all pots through urea, di-ammonium phosphate (DAP), and potassium sulfate (K_2SO_4). Higher doses of urea were applied to supplement the N nutrition as there was no inoculation of *Bradyrhizobium* culture. Moistening of pots was done with a rose can on alternate days along with manual weeding. Day and night temperatures were maintained at 35 °C and 23 °C, respectively, during the growing season with relative humidity at ~74%. Soil and plant samples were taken during 30 (peak vegetative stage), 50 (pod development stage), and 75 (harvesting stage) days after sowing (DAS), but records of root length were taken after harvesting (75 DAS) for laboratory analysis.

The pot experiment was carried out in a completely randomized block design (CRD). Total six treatments were allocated in eighteen pots keeping three replications for each treatment. The treatments, used, were as follows: T_1 (control; without fertilizers and *T. viride*), T_2 (RDF; 29 mg N kg^{-1}, 17.9 mg P kg^{-1}, and 17.9 mg K kg^{-1} without *T. viride*), T_3 (90% of RDF; 26.1 mg N kg^{-1}, 16.1 mg P kg^{-1}, and 16.1 mg K kg^{-1} + *T. viride*), T_4 (80% of RDF; 23.2 mg N kg^{-1}, 14.3 mg P kg^{-1}, and 14.3 mg K kg^{-1} + *T. viride*), T_5 (75% of RDF; 21.8 mg N kg^{-1}, 13.4 mg P kg^{-1}, and 13.4 mg K kg^{-1} + *T. viride*), and T_6 (70% of RDF; 20.3 mg N kg^{-1}, 12.5 mg P kg^{-1}, and 12.5 mg K kg^{-1} + *T. viride*).

2.3 Plant Parameters

Effective root lengths were measured with Tennant (1975) method at the end of the experiment (75 DAS). Oven-dried root samples (1 cm length cut pieces) were spread over a 1 cm × 1 cm grid with 20% ethyl alcohol (C_2H_5OH) (Merck Millipore, India). The vertical and horizontal root interceptions on the grid were then calculated using the following formula:

$$\text{Effective root length (cm)} = \frac{(11 \times R \times 0.7857)}{14}$$

where R was the number of vertical and horizontal root intercepts with grid lines and value 0.7857 was grid constant for 1 cm × 1 cm grid area.

Concentrations of phosphorus in the powdered plant (combining roots, shoots, and leaves) as well as in seed samples were determined separately by nitric (HNO_3) and per-chloric acid ($HClO_4$) digestion at a 4:1 ratio. The colorimetric phosphorus estimation (420 nm) was accomplished in spectrophotometer (μ Controller-Based Visible Spectrophotometer Type-104, Systronics India Ltd., India), using yellow color (vanado-molybdate) method as described by Jackson (1973). The vanado-molybdate solution was prepared by dissolving ammonium molybdate tetrahydrate (Central Drug House (P) Ltd., India) and ammonium metavanadate (Sisco Research Laboratories Pvt. Ltd., India) solutions in concentrated acid media (HNO_3). The final volume was made up of double-distilled water.

2.4 Soil Parameters

Soil-pH (1:2.5, soil/distilled water) was measured with a digital pH meter (Digital pH Meter with Electrode (LCD) type-802, Systronics India Ltd., India) as described by Jackson (1973).

Oxidizable soil organic carbon (C) was measured following the method of Walkley and Black (1934). Soil samples (0.5 g) were oxidized with 10 mL 1 N potassium di-chromate (Merck Ltd., India) in the presence of concentrated H_2SO_4 for 30 min. The reaction was stopped by adding 200 mL distilled water and titrated against 0.5 N ferrous ammonium sulfate hexahydrate (FAS) (Emplura®, Merck Specialities Pvt. Ltd., India) while taking diphenylamine (Molychem, India) and 85% H_3PO_4 as an indicator to detect the sharp color change from violet to bright green at the end point. Similar titration was carried out with a blank. Organic C (%) was calculated by the amount of FAS consumed to titrate unreacted potassium di-chromate, multiplied by 1.33 (76% recovery).

Soil available-P was extracted with Bray-1 reagent (0.03 N NH_4F in 0.025 N HCl) as mentioned in the method by Bray and Kurtz (1945). Colorimetric determination of extracted soil-P was carried out (660 nm) with 5% ammonium molybdate tetrahydrate and 40% stannous chloride in dilute HCl as outlined by Dickman and Bray (1940).

2.5 Fungal Colonization

Counts of *T. viride* colonies had been taken using TSM. Rhizospheric soils (within 2 cm of roots) were serially diluted up to 10^{-3} times with sterile distilled water before placing on TSM media, while the root colonization study was carried out by the method of Zhang et al. (2015). Sterilized root bids were smashed in the suspension of 0.05% agar, diluted 10^{-4} times, smeared on Petri dishes containing TSM, and incubated (5 days) at 27 °C. The counts were expressed in CFU g^{-1} of soil or root.

2.6 Soil Biochemical Properties

Acid and alkaline phosphatase activities of rhizospheric soils (within 2 cm of root zone) were measured according to Tabatabai and Bremner (1969) method. We used modified universal buffer (MUB), prepared by dissolving H_3BO_3, maleic acid (Merck Specialities Pvt. Ltd., India), citric acid (Merck Sigma-Aldrich, India), and tris-hydroxy-methyl amino-methane (Himedia® Laboratories Pvt. Ltd., India) in NaOH followed by making the 1 L volume with double distilled water. Buffer pH was maintained at 6.5 (for acid phosphatase) and 11 (for alkaline phosphatase). Again *p*-nitrophenyl phosphate hexahydrate (Sisco Research Laboratories Pvt. Ltd., India) solution was prepared with MUB at both pHs. Then two sets of soils (1 g) along with their blanks were incubated with toluene (Merck Emsure® ACS, ISO, Reag. PhEur, India), MUB, and *p*-nitrophenyl phosphate solutions (pH 6.5 and 11). The suspensions were filtered through Whatman No. 1 filter paper after the addition of 0.5 M calcium chloride di-hydrate and 0.5 M NaOH. The color intensities were measured at 440 nm wavelength. Activities of soil phosphatases (μg *p*-nitrophenol g^{-1} h^{-1}) were determined from the standard curve, prepared with standard *p*-nitrophenol solutions (Sisco Research Laboratories Pvt. Ltd., India).

Dehydrogenase activities of soils were measured by incubating 1 g of air-dried soil in 3% 2,3,5-triphenyltetrazolium chloride (Merck Millipore, India) and 1% glucose solution (Merck Sigma-Aldrich, India) at 28 °C for 1 day. After incubation, methanol (Merck Sigma-Aldrich, India) was added and allowed to stand for 6 h. Determination of dehydrogenase activities was carried out by taking absorbance of pink colored 1,3,5-triphenyltetrazolium formazan (TPF) (Himedia® Laboratories Pvt. Ltd., India) solutions at 485 nm as done by Klein et al. (1971).

2.7 Use Efficiency of Phosphorus

Apparent seed-P recovery (APR) percentage was calculated after harvesting of soybeans (except control) by the following equation (Fageria and Baligar 2005):

$$\text{APR}\ (\%) = \frac{\text{Seed–P uptake (kg) from (fertilized pot–control pot)} \times 100}{\text{Phosphorus (kg) applied to the fertilized pot}}$$

2.8 Statistical Analysis

Data analysis was carried out using SPSS (version 16.0). One-way ANOVA was prepared along with *F* test to compare treatment means and critical difference values to justify test significance. Pearson's correlation test ($P < 0.05$) was done to assess the relationships between variables at different stages of crop growth. Mean differences between treatments were shown using Duncan's multiple range test (DMRT) at 95% confidence level.

3 Results

3.1 Root Length

Data of root lengths are depicted in Table 2. Soybeans, treated with *T. viride*, showed significantly higher ($P < 0.05$) root lengths compared to untreated ones. However, the root length of untreated T_2 was statistically at par with T_5. Maximum root length was obtained from T_3 (141.33 cm), and it was nearly 1.36 times longer than untreated T_2. At harvest, root lengths

Table 2 Root length, seed phosphorus content, apparent phosphorus recovery at harvesting stage (75 DAS), phosphorus content in vegetative plant parts, and root colonization at peak vegetative (30 DAS), pod development (50 DAS), and harvesting stages (75 DAS) ($n = 3$; mean ± SE)

Treatments	Root length (cm)	P-content (%) in vegetative plant parts			Apparent phosphorus recovery (%)	Seed-P content (%)	Root colonization by *T. viride* (CFU g^{-1})		
		30 DAS	50 DAS	75 DAS			30 DAS	50 DAS	75 DAS
T_1	$84.67^d±6.468$	$0.107^c±0.007$	$0.143^d±0.003$	$0.162^e±0.001$	–	$0.29^c±0.012$	$3.7^{bc}±0.33×10^4$	$11.7^b±2.03×10^4$	$14^c±1.73×10^4$
T_2	$103.67^c±4.296$	$0.123^b±0.009$	$0.162^c±0.002$	$0.192^d±0.001$	$7.596^b±0.225$	$0.343^{ab}±0.012$	$2.3^c±0.33×10^4$	$8.7^b±1.2×10^4$	$9.7^c±2.33×10^4$
T_3	$141.33^a±10.82$	$0.143^a±0.003$	$0.190^a±0.001$	$0.224^a±0.002$	$11.767^a±0.094$	$0.373^a±0.009$	$7.7^{ab}±1.33×10^4$	$19.3^a±1.2×10^4$	$24^{ab}±1.73×10^4$
T_4	$121.67^b±2.39$	$0.137^{ab}±0.001$	$0.180^b±0.001$	$0.214^b±0.002$	$8.071^b±0.15$	$0.350^{ab}±0.015$	$7.3^{ab}±2.33×10^4$	$17.7^a±1.76×10^4$	$23.3^b±1.86×10^4$
T_5	$99.33^c±5.949$	$0.136^{ab}±0.001$	$0.184^b±0.001$	$0.211^b±0.001$	$5.64^c±0.174$	$0.333^b±0.015$	$8.7^a±1.2×10^4$	$20.7^a±1.2×10^4$	$28.3^{ab}±2.03×10^4$
T_6	$110.67^{bc}±5.42$	$0.139^a±0.001$	$0.180^b±0.001$	$0.198^c±0.001$	$4.774^d±0.139$	$0.323^{bc}±0.013$	$9^a±1.52×10^4$	$20.3^a±1.86×10^4$	$29.7^a±2.19×10^4$
CD (0.05)	19.8	0.015	0.006	0.004	0.511	0.039	$4.214×10^4$	$4.872×10^4$	$6.13×10^4$

T_1, control; T_2, RDF (29 mg N kg^{-1}, 17.9 mg P kg^{-1}, and 17.9 mg K kg^{-1}) without *T. viride*; T_3: 90% RDF + *T. viride*; T_4: 80% RDF + *T. viride*; T_5: 75% RDF + *T. viride*; T_6: 70% RDF + *T. viride*. *CD*, critical difference; *CFU*, colony-forming unit; *DAS*, days after sowing; *RDF*, recommended doses of fertilizers. Different lower case letters on mean values are significantly different at 95% confidence limit within columns

followed a decreasing order of $T_3 > T_4 > T_6 > T_2 > T_5 > T_1$ during harvesting (Table 2).

3.2 Plant Phosphorus

Highest P-concentrations (%) in vegetative plant parts of soybean were found in T_3 (0.143%, 0.190%, and 0.224% at 30, 50, and 75 DAS), while T_1 showed the least concentrations (0.107%, 0.143%, and 0.162% at 30, 50, and 75 DAS) (Table 2). Similar results were recorded in the case of seed-P (%) at the harvesting stage where significantly higher ($P < 0.05$) seed-P was found in T_3 (0.373%) followed by T_4, T_2, T_5, T_6, and T_1 (Table 2). Among untreated soybeans, seed-P (%) of T_2 had shown to be at par with bio-primed treatments, but P-concentrations in vegetative parts of untreated soybeans were significantly ($P < 0.05$) lower as compared to bio-primed treatments.

3.3 Soil Phosphatase Activity

Phosphatase activities were recorded at 30, 50, and 75 DAS of soybean growth. Data depicted in Fig. 1a show that rhizospheric soils of bio-primed treatments had shown relatively higher acid phosphatase activities than soils of untreated soybeans. There were no significant differences ($P < 0.05$) in acid phosphatase activities among T_3 (129.9 μg *p*-nitrophenol g^{-1} h^{-1}), T_4 (129.67 μg *p*-nitrophenol g^{-1} h^{-1}), T_5 (128.31 μg *p*-nitrophenol g^{-1} h^{-1}), and T_6 (128.24 μg *p*-nitrophenol g^{-1} h^{-1}) at 50 DAS. A similar result was found at the harvesting stage. However, at 30 DAS, significantly lower ($P < 0.05$) soil acid phosphatase activity was found in T_6 as compared to T_4 and T_3, but T_6 was statistically at par with T_5. Compared to T_6, T_5 did not vary significantly ($P < 0.05$) from T_3 and T_4 at 30 DAS (Fig. 1a). Untreated T_2 was statistically at par with T_5 and T_6 at 30 and 75 DAS but found lower ($P < 0.05$) at 50 DAS. Control was recorded with the lowest ($P < 0.05$) acid phosphatase activities, except at 30 DAS when it was found significantly at par with T_5. Similarly, rhizospheric soils of bio-primed soybeans showed higher alkaline phosphatase activities than treatments without priming, irrespective of fertilizer doses (Fig. 1b). At 30 DAS, T_3 (31 μg *p*-nitrophenol g^{-1} h^{-1}), T_4 (27.57 μg *p*-nitrophenol g^{-1} h^{-1}), and T_5 (29.33 μg *p*-nitrophenol g^{-1} h^{-1}) were recorded with high soil alkaline phosphatase activities with no significant differences within them, but T_3 was found significantly higher ($P < 0.05$) than T_6 (24.03 μg *p*-nitrophenol g^{-1} h^{-1}). Alkaline phosphatase activities of T_3, T_4, and T_5 were found about twofold higher than T_2 during this stage (Fig. 1b). At 50 DAS and 75 DAS, very low soil alkaline phosphatase activities were found in T_1 and T_2, while higher activities were recorded from bio-

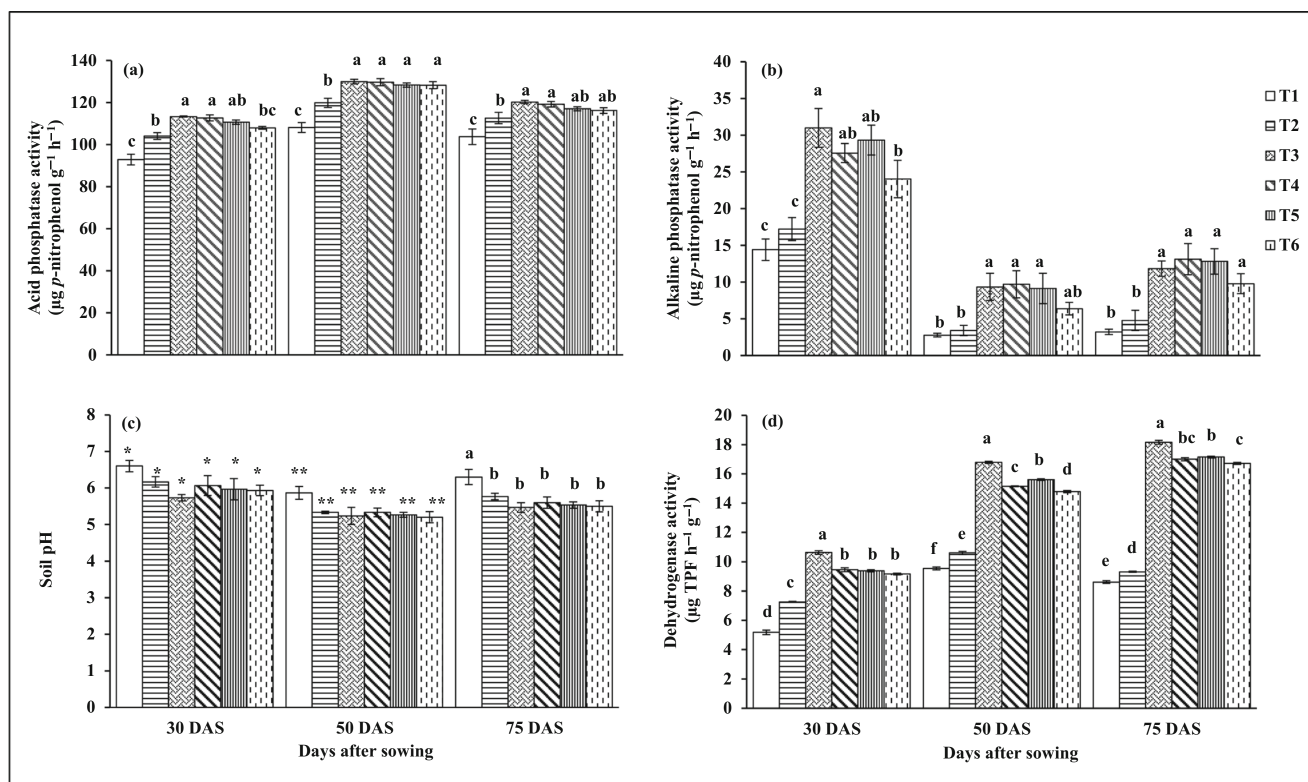

Fig. 1 Effect of soybean bio-priming along with graded levels of NPK on (**a**) soil acid phosphatase activity (µg *p*-nitro-phenol g^{-1} h^{-1}), (**b**) soil alkaline phosphatase activity (µg *p*-nitrophenol g^{-1} h^{-1}), (**c**) soil pH, and (**d**) soil dehydrogenase activity (µg TPF h^{-1} g^{-1} soil) in peak vegetative stage (30 DAS), pod development stage (50 DAS), and harvesting stage (75 DAS) of soybean ($n = 3$; mean ± SE). T_1, control; T_2, RDF (29 mg N kg^{-1}, 17.9 mg P kg^{-1}, and 17.9 mg K kg^{-1}) without *T. viride*; T_3, 90% RDF + *T. viride*; T_4, 80% RDF + *T. viride*; T_5, 75% RDF + *T. viride*; T_6: 70% RDF + *T. viride*. DAS, days after sowing; RDF, recommended doses of fertilizers; TPF, triphenyl formazan. Different letters indicate significant differences between treatment means according to Duncan's multiple range tests at 95% confidence level within each day after sowing. Single asterisks denote non-significant values at $P < 0.05$ at 30 DAS. Double asterisks denote non-significant values at $P < 0.05$ at 50 DAS

primed treatments with no significant differences among them (Fig. 1b). At 50 DAS, alkaline phosphatase activities in soils of T_3, T_4, and T_5 were more than threefold higher than T_1 and more than two and half-fold higher than T_2. Around fourfold soil alkaline phosphatase activities were seen in T_4 and T_5 as compared to T_1 during the harvesting stage, while T_6 showed threefold and T_3 more than threefold activity than control (Fig. 1b). Almost twofold higher alkaline phosphatase activities were recorded in soils of T_3, T_4, and T_5 as compared to T_2 at this stage. However, in the case of T_6, this activity was nearly twofold higher than T_2 (Fig. 1b). For all cases, the lowest soil phosphatase activities were recorded in T_1 followed by T_2 (Fig. 1a, b).

3.4 Soil Acidity

At 75 DAS, the highest soil-pH value was recorded in T_1 (6.3), while T_2 was statistically at par with T_3, T_4, T_5, and T_6 (Fig. 1c). These values were lower compared to control. No significant differences in soil-pH were observed at 30 DAS and 50 DAS. The soil-pH was found to decrease at the pod development stage but again increased a little at the time of harvesting.

3.5 Soil Available Phosphorus

During all growth stages, available soil-P content was found highest in the case of untreated T_2 and lowest in the case of T_1 (control) (Table 3). Results showed that with the progression of soybean age, soil-P contents had decreased simultaneously in all treatments. Among bio-primed treatments, T_3 showed significantly higher soil-P status ($P < 0.05$) followed by T_4, T_6, and T_5.

3.6 Soil Organic Carbon

The highest soil organic C (%) was recorded from T_3 (Table 3). There were no significant differences ($P < 0.05$) among T_1, T_2, T_4, and T_6 at 30 DAS and 75 DAS, respectively. At 50 DAS, soil organic C (%) was found to follow the

Table 3 Oxidizable soil organic carbon, soil available phosphorus content, and rhizosphere fungal colonization in peak vegetative stage (30 DAS), pod development stage (50 DAS), and harvesting stage (75 DAS) of soybean (n = 3; mean ± SE)

Treatments	Soil organic C (%)			Soil available-P content (mg kg^{-1})			Rhizosphere colonization by *T. viride* (CFU g^{-1})		
	30 DAS	50 DAS	75 DAS	30 DAS	50 DAS	75 DAS	30 DAS	50 DAS	75 DAS
T_1	$0.31^b±0.005$	$0.294^c±0.004$	$0.286^b±0.005$	$15.412^f±0.056$	$15.264^f±0.075$	$12.537^f±0.036$	$3^c±0.58×10^3$	$2.33^d±1.2×10^3$	$2.33^d±0.33×10^3$
T_2	$0.305^b±0.002$	$0.29^c±0.004$	$0.289^b±0.003$	$23.261^a±0.03$	$22.457^a±0.096$	$18.592^a±0.099$	$5^c±1.16×10^3$	$3.67^d±0.67×10^3$	$4^d±0.58×10^3$
T_3	$0.354^a±0.005$	$0.326^a±0.003$	$0.319^a±0.006$	$21.665^b±0.163$	$20.109^b±0.075$	$17.843^b±0.022$	$26.33^a±1.2×10^3$	$43.67^a±2.03×10^3$	$48.33^a±1.76×10^3$
T_4	$0.313^b±0.002$	$0.31^b±0.001$	$0.294^b±0.005$	$19.794^c±0.084$	$19.336^c±0.081$	$16.149^c±0.156$	$20.33^b±1.45×10^3$	$35.33^{bc}±1.45×10^3$	$41^{bc}±2.52×10^3$
T_5	$0.28^c±0.003$	$0.274^d±0.001$	$0.251^c±0.012$	$17.224^e±0.086$	$16.595^e±0.071$	$14.605^e±0.138$	$22.67^{ab}±2.03×10^3$	$38.67^b±2.03×10^3$	$42.67^{ab}±1.45×10^3$
T_6	$0.308^b±0.002$	$0.303^b±0.001$	$0.288^b±0.002$	$19.192^d±0.1$	$18.291^d±0.046$	$15.252^d±0.078$	$20.67^b±2.03×10^3$	$31.67^c±1.76×10^3$	$36.33^c±3.18×10^3$
CD (0.05)	0.011	0.008	0.019	0.296	0.233	0.311	$4.613×10^3$	$4.926×10^3$	$5.915×10^3$

T_1: control; T_2: RDF (29 mg N kg^{-1}, 17.9 mg P kg^{-1}, and 17.9 mg K kg^{-1}) without *T. viride*; T_3, 90% RDF + *T. viride*; T_4, 80% RDF + *T. viride*; T_5, 75% RDF + *T. viride*; T_6, 70% RDF + *T. viride*. *CD* critical difference, *CFU* colony-forming unit, *DAS* days after sowing, *RDF* recommended doses of fertilizers. Different lower case letters on mean values are significantly different at 95% confidence limit within columns

order: $T_4 \sim T_6 > T_2 = T_1$ (Table 3). In every stage, however, T_5 was found to be the lowest (Table 3).

3.7 Soil Dehydrogenase Activity

Maximum dehydrogenase activities were recorded in T_3 (10.63, 16.79, and 18.16 μg TPF h^{-1} g^{-1} at 30, 50, and 75 DAS, respectively), whereas the lowest activities were recorded in control (Fig. 1d). Dehydrogenase activities of T_3 were about 2 (30 DAS), 1.76 (50 DAS), and 2.1 times (75 DAS) higher than T_1, but it was nearly 1.95 times higher than T_2 during harvesting of soybean. At 30 DAS, T_4, T_5, and T_6 were statistically at par with each other. Activities recorded in T_4 did not vary significantly from T_5 at 75 DAS. Soil dehydrogenase activities at 50 DAS followed an order of decrement: $T_3 > T_5 > T_4 > T_6 > T_2 > T_1$ (Fig. 1d).

3.8 Fungal Colonization

Data of *T. viride* counts (from TSM) from the rhizosphere are depicted in Table 3. The highest number of colonies in TSM was obtained from soils of T_3 (26.33×10^3, 43.67×10^3, and 48.33×10^3 CFU g^{-1} at 30, 50, and 75 DAS, respectively) which were significantly higher ($P < 0.05$) than all other treatments except for T_5 at 30 DAS (22.67×10^3 CFU g^{-1}) and 75 DAS (42.67×10^3 CFU g^{-1}). The number of colonies at 30 DAS showed the following trend, $T_3 > T_5 > T_6 = T_4 > T_2 > T_1$, while at 50 and 75 DAS, the trend in the treatment was as follows: $T_3 > T_5 > T_4 > T_6 > T_2 > T_1$ (Table 3).

In contrast to soil, root colonization showed higher ($P < 0.05$) fungal colony densities in bio-primed soybeans at 30 DAS and 50 DAS; however, at 75 DAS, T_4 was recorded with significantly ($P < 0.05$) lower fungal colonies (23.3×10^4 CFU g^{-1}) than other bio-primed treatments (Table 2). During all stages, T_1 and T_2 were found with significantly ($P < 0.05$) lower colonies of *T. viride*. Over time, fungal root colonizations got increased in each treatment and followed the order of decrement: $T_6 > T_5 > T_3 > T_4 > T_1 > T_2$ (Table 2). We have observed that root colonization in bio-primed treatments had been increased over time. Root colonization was found higher as compared to rhizospheric colonization (Tables 2 and 3).

3.9 Use Efficiency of Phosphorus

Data presented in Table 2 show the apparent P-recovery percentage of soybean seeds. The highest recovery was obtained from T_3 (11.767%). APR of all five treatments showed significant differences ($P < 0.05$) among themselves. Though T_4 received a graded dose of phosphorus, its seed-P recovery was statistically at par with T_2 (untreated). The APR percentages, calculated against T_1,

were found to follow the decreasing order: $T_3 > T_4 \sim T_2 > T_5 > T_6$ (Table 2).

4 Discussion

Measurement of fungal colonies (CFU g^{-1}) in TSM has given a fair idea about the dominance of *T. viride* in the rhizosphere and their colonization abilities in roots of soybean. In our study, we confirmed that seed bio-priming with *Trichoderma viride* BHU-2953 enhances fungal colonization. Higher colonizations were recorded from bio-primed treatments (both in roots and rhizospheres), and these values were way too higher than untreated ones, signifying increased fungal proliferation upon priming. Higher root exudation under low soil-P content might have triggered *T. viride* population in these soils (Krishnapriya and Pandey 2016). These released organic compounds help in the proliferation of *Trichoderma* in the rhizosphere (Lombardi et al. 2018). Although in control and RDF, there were still some fungal colonies of *Trichoderma* spp., developed on TSM. We suspect that some spores of native *Trichoderma* might have remained in the soil as we did not sterilize it. There was no confirmation about whether this native *Trichoderma* spp. was *T. viride* or not because we did not opt for the speciation of these native fungi. However, the colonies, formed, were very small in numbers as compared to bio-primed treatments (Table 3). Sureshrao et al. (2016) also reported that seed bio-priming enhances rhizospheric colonization of *T. viride* in rice. Over time, fungal colony formation inside the roots as well as in rhizospheres had increased in bio-primed treatments because of their higher sporulation ability. However, rhizospheres of untreated soybeans were recorded with a decrement in colony formation over time.

Similarly, the measurement of soil dehydrogenase activity is another fair index of rhizospheric microbial activity. Higher dehydrogenase activities in bio-primed treatments indicate more viable population of *T. viride* ($R^2 = 0.96$, 0.99, and 0.99 at 30, 50, and 75 DAS, respectively; $P < 0.05$) (Table 4). In the present study, untreated T_1 and T_2 were also recorded with a reasonable amount of dehydrogenase activities due to the presence of soybean roots and native soil microbes (Gai et al. 2017). However, the activities got reduced at 75 DAS in contrast to the soils of bio-primed soybeans (Fig. 1d). This might explain that dehydrogenase activity can be decreased with the initiation of plant senescence, and the result is in conformity with the observations made by Omara et al. (2017). Similarly, higher soil dehydrogenase activities were observed by Velmourougane et al. (2017) in chickpea, using *T. viride* and *A. chroococcum* as biofilm. Continuous depletion of native soil organic C over time (Table 3) can be attributed to decrement in root exudation with the progression of plant growth (Qiao et al. 2017). This might have forced *T. viride* (in rhizosphere) to shift its C nutrition from root exudation to native soil organic C which helped the fungus to maintain its increasing population.

Strong positive correlations were found between acid phosphatase activities and fungal counts in rhizospheres ($R^2 = 0.89$, 0.9, and 0.89 at 30, 50, and 75 DAS, respectively; $P < 0.05$; Table 4). It has been found that single inoculation of *T. viride* or in combination with other microbes had shown higher soil acid phosphatase activities (Yadav and Aggarwal 2015). Strong negative correlations, found between soil-pH and acid phosphatase activity ($R^2 = -0.92$, -0.93, and -0.96 at 30 DAS, 50 DAS, and 75 DAS; $P < 0.05$), explain inverse dependency of extracellular acid phosphatase with pH (Table 4). Similar results were also found by Nannipieri et al. (2011). Despite the lower soil-pH values, very strong correlations were found between soil alkaline phosphatase activities and rhizospheric *T. viride* populations at 30 DAS ($R^2 = 0.98$; $P < 0.05$), 50 DAS ($R^2 = 0.96$; $P < 0.05$), and 75 DAS ($R^2 = 0.97$; $P < 0.05$) (Table 4). This indicates that alkaline phosphatase activity was better associated with rhizospheric *T. viride* population than plant roots. Spohn and Kuzyakov (2013) suggested that soil alkaline phosphatase activities are mostly associated with the microbes present in soil, whereas the origin of soil acid phosphatase activities can be attributed to both plants and microbes. Progression of soybean growth, production of organic acids by *T. viride*, and application of urea and DAP (except control) might have lowered down the soil-pH which triggered acid phosphatase activities but restricted the alkaline one (Han et al. 2015; Tawaraya et al. 2014; Wang et al. 2006). Kunze et al. (2011) also confirmed negligible activities of alkaline phosphatase in strong acid soils which are in accordance with our finding.

Longer root lengths were observed in bio-primed soybeans, though these treatments received lower grades of NPK in comparison to untreated RDF (Table 2). Reasons might be attributed to *Trichoderma*-induced root elongation and more root-forking through the production of indole acetic acid derivatives (Björkman 2004; Druzhinina et al. 2011). The fungus can even interrupt the production of ethylene, a constraint for root elongation, and helps bio-primed soybean roots to elongate within soils (Druzhinina et al. 2011). Meena et al. (2016) found that priming with *Trichoderma* had induced higher root lengths in wheat. Velmourougane et al. (2017) also found higher root lengths in chickpea, treated with *Trichoderma viride* and *Trichoderma viride* + *Azotobacter chroococcum* as biofilm.

Concentrations of phosphorus have increased in plant vegetative parts with succeeding soybean growth stages

Table 4 Pearson's correlation coefficients (R^2) and corresponding *P* values for different paired parameters

	Dehydrogenase × *T. viride*	Acid phosphatase × *T. viride*	Alkaline phosphatase × *T. viride*	Soil-pH × acid phosphatase	Plant P × acid phosphatase	Plant P × alkaline phosphatase	Soil-P × P-fertilization	Root length × plant P	Root length × seed-P	Seed-P × acid phosphatase	Seed-P × P-fertilization
30 DAS											
Pearson co-efficient (R^2)	0.96	0.89	0.98	−0.92	0.96	0.91	0.85	–	–	–	–
P value	0.001456	0.00894	0.000469	0.004326	0.000962	0.005581	0.015437	–	–	–	–
50 DAS											
Pearson co-efficient (R^2)	0.99	0.9	0.96	−0.93	0.98	0.91	0.83	–	–	–	–
P value	2.075×10^{-5}	0.007536	0.001338	0.004039	0.000303	0.006417	0.02117	–	–	–	–
75 DAS											
Pearson co-efficient (R^2)	0.99	0.89	0.9	−0.96	0.98	0.81	0.89	0.85	0.9	0.87	0.87
P value	1.23×10^{-5}	0.009532	0.007233	0.001243	0.000222	0.025396	0.008451	0.01702	0.006957	0.012792	0.012973

Root length and seed-P were not measured at peak vegetative stage (30 DAS) and pod development stage (50 DAS)

(Table 2). Results indicate that acid phosphatase (R^2 = 0.96, 0.98, and 0.98 at 30, 50, and 75 DAS, respectively) and alkaline phosphatase activities (R^2 = 0.91, 0.91, and 0.9 at 30, 50, and 75 DAS, respectively) are significantly ($P < 0.05$) correlated with phosphorus contents in plant vegetative parts (Table 4). Similar strong relation was observed between seed-P and acid phosphatase activity ($R^2 = 0.87$; $P < 0.05$). This indicates solubilization of soil locked-P by phosphatase enzymes, activities of which were found much higher in bio-primed treatments. Saini et al. (2019) have also found similar results in chrysanthemum while applying *T. viride* in combination with AM fungi and *P. fluorescens*. Again, root length was also found to influence P-content in vegetative parts (R^2 = 0.85; $P < 0.05$) and seed-P ($R^2 = 0.9$; $P < 0.05$) positively through better soil exploration (Table 4). In an experiment, carried out by Tanwar et al. (2013), higher P-concentration in broccoli root was found to be associated with *Trichoderma viride* application. Though bio-primed soybeans received relatively low P-doses as compared to RDF, similar percentages of seed-P were found in treatments that received even 20% reduced doses (Table 2). This gives insight into the ability of soybean plant to partition its phosphorus from vegetative parts to reproductive ones (Bender et al. 2015). Fertilization also helped to maintain desired levels of phosphorus in seeds ($R^2 = 0.87$; $P < 0.05$; Table 4). Treatments that received 10% and 20% reduced P-doses (T_3 and T_4) along with higher fungal colony densities in the micro-rhizosphere might have triggered the acid phosphatase activity which resulted in efficient seed-P recovery in comparison to RDF. Results are in agreement with Krishnapriya and Pandey (2016).

We recorded soil available-P content (mg kg^{-1}) to cross-check the soil-P uptake by soybeans and found that soil-P availability was positively correlated with external P-application (R^2 = 0.85, 0.83, and 0.89 at 30, 50, and 75 DAS, respectively; $P < 0.05$; Table 4). Maximum P-fertilization without *T. viride* inoculation might be the sole reason for the highest available soil-P status in T_2, whereas increased soil-P uptake in T_5-plants had led to relatively lower phosphorus availability in T_5 soils as compared to T_6. Further, it was observed that rhizospheric acid phosphatase activity had been increased up to 50 DAS but got decreased at 75 DAS (Fig. 1a). This might be a consequence of soybean aging that had declined the crop-P need at the time of harvesting. The same is not true for alkaline soil phosphatase, activity of which had been decreased at the pod development stage followed by an increase in activity during harvesting (Fig. 1b). Higher doses of external P-application without seed bio-priming might also have contributed to a decrease in the soil phosphatase activities (especially alkaline phosphatase) in RDF rhizospheres, while higher N-dose had increased the acid phosphatase activities in RDF as compared to control

(Marklein and Houlton 2012; Zhou et al. 2016). This study showed that not only *T. viride* has a distinct influence on maintaining higher soil phosphatase activity; pH and plant age can also regulate these enzymes.

5 Conclusion

This study was able to establish that seed bio-priming of *Trichoderma viride* BHU-2953 along with graded fertilizer phosphorus doses significantly increased phosphorus content in soybean (cv. JS95-60) as compared to only phosphatic fertilizer application in the full recommended dose. Inoculation of *Trichoderma viride* had shown a dual effect on phosphorus acquisition in vegetative as well as in reproductive parts through (i) soybean root elongation and (ii) soil phosphorus solubilization by enhanced acid phosphatase activities. Our study has also reported a comparable seed phosphorus recovery in bio-primed soybeans up to 80% recommended phosphorus dose which emerged as an alternative to the full recommended dose of phosphorus without priming. These attributes make seed bio-priming with *Trichoderma viride* a viable option for minimizing the application of fertilizer phosphorus, maintaining soil sustainability and crop productivity in the integrated plant nutrition system. Another inference, drawn from this experiment, is that acid phosphatase activity was strongly influenced by low soil-pH as well as *Trichoderma viride* population, whereas alkaline phosphatase under acid soil was recorded to link better with the viable population of this fungus. In the future, further investigations should include organic phosphorus sources and the contribution of phytase in plant phosphorus nutrition to evaluate the effectiveness of *Trichoderma viride* priming.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no competing interests.

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