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Response of phosphorus levels to chickpea (*Cicer arietinum* L.) cultivars and soil microbial activity in an Alfisol

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ABSTRACT

A field experiment was carried out during winter seasons of 2012-13 and 2013-14 to study the effect of application of different levels of phosphorus (P) on the yield attributing characters, yield of chickpea (Cicer arietinum L.) and soil microbial diversity in an Alfisol at the Bidhan Chandra Krishi Viswavidyalaya, West Bengal, India. There were 15 treatments in the combination of 3 chickpea cultivars (Anuradha, Pusa1003 and DCP-92-3) and 5 rates of P_2O_5 (0, 20, 40, 60 and 80 kg P_2O_5 ha^{-1}) arranged in a split plot design. The cultivar DCP-92-3 showed the highest yield attributing characters and yield. Higher application of P significantly increased the seed yield and total P uptake but P recovery and agronomic efficiency decreased. The optimum and economic levels of P_2O_5 were 70.59 and 66.54 kg ha^{-1} respectively. Higher P fertilization increased the total bacterial population and microbial biomass carbon but had no effect on the diversity of ribotypes in the soil samples.

***Keywords*:** Chickpea, phosphorus, soil microbial diversity, yield

India is the largest producer, consumer and importer of pulses in the world. India accounts for about 33 percent of world area and about 22 percent of world production. In West Bengal from an area of 0.02 m ha about 0.03 m t of chickpea is produced with an average yield of 1110 kg ha^{-1} (Shalendra, 2012). There is a vast scope for increasing productivity of chickpea by optimizing the available resources. Legumes generally have higher P requirement because the process of symbiotic nitrogen (N) fixation consumes a lot of energy. Phosphorus stimulates early root development, leaf size, tillering, flowering, grain yield, hastens maturity and is essential for cell division, seed and fruit development. Phosphorus is the most difficult element to manage. The use efficiency of phosphorus is only 8–20 per cent. Phosphorus is present as mineral deposits, therefore, world resources of P are limited and should be used efficiently in order to conserve the resource base while sustaining agricultural productivity.

Soil microorganisms play an important role in the soil environment. Fertilizer applications to soil can change the soil microbial community directly or indirectly since they alter the soil physical, chemical and biological properties (Beauregard *et al*., 2010). Some studies have documented that fertilization had significant impacts on the population, composition and function of soil microorganisms, while other studies had reported relatively little or no effect on soil microbial diversity (Treseder, 2008). Numerous studies were conducted on the impact of nitrogen fertilizer, manure, and different management practices on soil microbial community but there are few works on the impact of P fertilizer (Rooney and Clipson, 2009).

This study was conducted with the objective to assess the P use efficiency and to optimize P use in chickpea through economically appropriate rates and to evaluate the changes, if any, in soil microbial diversity by conventional and molecular techniques.

MATERIALS AND METHODS

Field experimental trials were conducted on Gangetic alluvial soil (Alfisol) at the "AB" Block Farm of Bidhan Chandra Krishi Viswavidyalaya, Kalyani, Nadia, and West Bengal, India during winter (October to March) seasons of 2012-13 and 2013-14. The experimental area is situated at 23°5' N and 89°0' E in the new alluvial zone of West Bengal with an altitude of 9.75 m above mean sea level. The climate of the experimental site is subtropical humid. The soil of the experimental field was sandy clay loam in texture having a pH of 6.2, soil organic carbon of 0.56 %, total N of 0.08% N, available P_2O_5 of 45.6 kg ha^{-1} and available K_2O of 138 kg ha^{-1}. The experiment included 15 treatments which were the combination of 3 chickpea

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cultivars (Factor A) [*i.e.* Anuradha (V1), Pusa1003(V2), DCP-92-3 (V3)] and 5 treatments (Factor B) representing 5 rates of mineral P fertilizer, *i.e.* 0, 20, 40, 60 and 80 kg P_2O_5 ha^{-1}. The treatments were arranged in a split plot design where chickpea cultivars were allotted in the main plots (measuring 20 m^2) with 3 replicates. The phosphorus fertilizer treatments (Factor B) were allotted to the sub plots of size 3 × 4 m each. After the layout of plots according to the split plot design blanket doses of 20 kg N and 40 kg K_2O ha^{-1} were applied to all the plots supplemented through urea and muriate of potash respectively. Requisite quantities of single super phosphate were applied to the respective plots to supplement P_2O_5 according to the treatments. Seeds of the test chickpea cultivars were sown at 30 cm row spacing. The plant to plant distance was maintained at 15 cm by thinning at 10 days after sowing. The sowing dates were 9 and 26 November during 2012-13 and 2013-14, respectively. The crop was harvested at physiological maturity stage leaving 2 border rows and kept for 2-3 days for drying. Data on the yield attributing characters were recorded from 10 randomly selected plants from each plot at harvest. The threshed seed and haulm yields were recorded separately from the harvested area and the yields ha^{-1} were derived.

Plant samples were collected at harvest randomly from the net plot area of each plot according to the treatments and separated as seed and haulm. The samples were then washed in distilled water, oven dried (80^0 C to constant weight), grind in a Wiley mill and passed through a 40 mesh sieve. The P concentration in plant was estimated following standard procedures.

The agronomic efficiency (AE) which is the crop yield increase per unit kg grain or seed kg^{-1} nutrient was computed by the formula: $AE= (Y_P-Y_O)/A_P)$, where, Y_p=Yield of chickpea at applied P level; Y_o=Yield of chickpea without P application; A_p= amount of P application (Prasad *et al*., 2003). The per cent apparent phosphorus recovery of applied P was calculated by the formula $\{[(N_F - N_C)/P] \times 100\}$ where, N_F = Nutrient uptake in fertilized plot (kg ha^{-1}); N_C = Nutrient uptake in control plot; P = kgha^{-1} (Craswell, 1987).

The relationship between yield and fertilizer dosage was found to be defined by the quadratic model in the best way as: $Y = a + bx + cx^2$; where Y = seed yield (kg ha^{-1}); x = the dosage of the P applied (kg ha^{-1}) and a, b and c are the parameters of the model. Differentiating Y with respect to P doses of the regression model give the doses for maximum yield which is estimated by the equation $P_{max} = - b/2c$; where, b and c are constants from the regression model. The equation for economic dose for maximum profit (E) was calculated as : $E = 1/2c (Ps/P_{NU} – b)$; where, b and c are the constants, Ps and P_{NU} are prices of chickpea seed and P respectively (Colwell, 1994). The unit price of chickpea seed was taken as Rs 33 kg^{-1} as the average value of two years. The per unit price of P, averaged over two years, was Rs 43.9 kg^{-1}. The response to economic optimum dose (REOD) of P was computed by using the equation as suggested by Islam *et al*. (2012): $REOD= (Y_{opt.}-Y_{cont})/X_{opt}$; where,$Y_{opt}$= Yield computed at economic optimum dose; Y_{cont}=Yield in control plot ; X_{opt}= Economic optimum dose.

Enumeration of total aerobic bacterial count in soil was done by the method suggested by Clark (1967). Microbial biomass carbon (MBC) in soil was determined by fumigation-extraction method as described by Joergensen (1995).

Soil samples were collected from the 45 plots at 2 days after the chickpea harvest following the two successive years of cultivation in fixed plots during the year 2014 for determination of soil microbial biodiversity. Approximately 1 kg of soil was cut from the rhizosphere zone (20-25 cm) from each plot, according to the doses of the phosphorus fertilization treatments, at five randomly selected locations and then mixed as one sample for each dose, irrespective of the cultivars. Altogether five composite soil samples were stored at -20^0C temperature before DNA extraction. To get rid of humic and fulvic acid, soil was treated with the Tris buffer (pH 7.0) containing 50 mM $CaCl_2$. The DNA was extracted from the collected soil samples stored earlier with soil DNA isolation kit of XcelrisInc (Ahmedabad) with slight modification. Quality and quantity of soil DNA was determined by electrophoresis in 1% agarose gel.

The Amplified Fragment Length Polymorphism (AFLP) method involves four basic steps like restriction-digestion of soil- DNA, ligation of adoptors compatible to the two digested ends, selective amplification of ligated products by PCR and finally profiling in the gel (Vos *et al.*, 1995). About 2 µg DNA was double digested by the enzyme EcoRI and MseI making the final volume to 25 µl for restriction-digestion of soil DNA. Treated DNA was allowed to digest at 37^0C overnight for complete digestion. Reaction condition is as follows: DNA - 10 µl; 10 x NEB buffer 3- 2.5µl; EcoRI (20u/ µl) - 1 µl; MseI (20u/ µl) - 1 µl; Water - 10 µl. After the digestion, remaining enzymes were inactivated by incubating the digested product at 65^{o}C temperature for 20 minutes. Digestion of DNA was checked by 1% agarose gel. EcoRI (5'CTC GTA GAC TGC GTA CC 3' and 3'CAT CTG ACG CAT GG TTAA 5') and MseI (5' GACGATGAGTCCTGAG 3' and 3'TACTCAGGACTCAT-5') adoptors were ligated to the 10µl digested DNA in a total volume of 50 µl using 1 unit T4 DNA ligase in RL buffer plus ATP (1±2 mM) at 16^0C for an hour.

AFLP primers consist of three parts, a core sequence complementary to the adoptor sequence, an enzyme specific sequence and a selective extension. In pre-amplification, only one nucleotide is added extra after the enzyme specific sequence, like when A is added after EcoRI specific sequence, named as EcoA. Hence, number of digested product take part in amplification will be one fourth of the total digested fragments. So, pre-amplification was done following the amplification condition as: Ligated DNA, 5 µl; 10× Taq Pol Buffer, 2.5 µl; 2.5mM dNTP mix, 2.0 µl; EcoA pre-amplification primer (100ng μl^{-1}), 1.0 µl; MseA pre-amplification primer (100ng μl^{-1}), 1.0 µl; Taq DNA polymerase (3u μl^{-1}), 1.0 µl; water, 12.5 µl.

PCR cycle consisted of 94°C for 1 min and then 35 cycles of 95°C for 45 s, 55°C for 45 s, 72°C for 1.30 min and 72°C for 7 min followed by 4° C for 5 min. Amplified products were checked in 1% agarose gel. Pre-amplified PCR product was diluted ten folds and used for final PCR reaction by specific primers. Specific primers were generated by addition of one or two nucleotides more after pre-amplification primer. Reaction condition was same as that of pre-amplification reaction. Final PCR product was finally loaded in 1% agarose gel for getting the sample specific DNA profiling.

Statistical analysis of data for various characters studied in the present study was performed as per split plot design analyzed with the help of SPSS statistical software (ver 11.0). The Least Significant Difference (LSD) was used to compare means at 5 per cent probability level.

RESULTS AND DISCUSSION

There was remarkable difference between the cultivars in the yield attributing characters (Table 1). The V3 cultivar produced higher number of pods $plant^{-1}$ (60.16) than that of V2 (49.47) and V1 (40.63) cultivars). The number of pods $plant^{-1}$ increased with each successive increase in levels of P. The P_{80} treatment contributed to express the maximum number of pods $plant^{-1}$ (70.17) in contrast to the other P treatments. Statistically significant variation in number of seeds pod^{-1} was evident between (among) the three tested chickpea cultivars. The V3 cultivar had the (maximum) number of seeds pod^{-1}. The descending order of magnitude of seeds pod^{-1} was V3 (1.54), V2 (1.33) and V1 (1.07). There was statistically variation in number of seeds pod^{-1} between the P application treatments. The two years average data showed that the statistical ranking of the P application treatments was in the order of P_{80} (1.44)e"P_{60} (1.39)e"P_{40} (1.33) e"P_{20} (1.26)>P_0 (1.14). There was significant variation in thousand seeds weight between the cultivars and applied levels of P. The order of thousand seeds weight was P_{80} (161.29 g) e" P_{60} (152.98 g) e" P_{40} (149.22 g) > P_{20}(132.73 g) > P_0(127.89 g). This indicated that P nutrition is essential for increasing the grain weight.

Table 1: Yield components and yield of chickpea cultivars applied with different doses of P fertilizer

Treatment	No. of pods $plant^{-1}$			No of seed $spod^{-1}$			1000 seeds weight (g)			Haulm yield (kg ha^{-1})			Seed yield (kg ha^{-1})		
	1st Yr	2nd Yr	Mean	1st Yr	2nd Yr	Mean	1st Yr	2nd Yr	Mean	1st Yr	2nd Yr	Mean	1st Yr	2nd Yr	Mean
Cultivar (C)															
V1:Anuradha	37.0	44.3	40.6	1.1	1.1	1.1	123.8	128.1	126.0	2264.0	2452.0	2358.0	1050.0	1121.0	1086.0
V2:Pusa-1003	45.8	53.2	49.5	1.3	1.4	1.3	132.6	137.4	135.0	2597.0	2637.0	2617.0	1263.0	1295.0	1279.0
V3:DCP-92-3	55.5	64.8	60.2	1.5	1.5	1.5	167.3	171.7	169.5	2851.0	2913.0	2882.0	1381.0	1480.0	1430.0
LSD (0.05)	**2.4**	**1.0**	**1.4**	**0.0**	**0.2**	**0.1**	**3.6**	**2.6**	**2.1**	**75.7**	**87.5**	**71.2**	**58.7**	**17.1**	**24.7**
P levels (kg ha^{-1})															
P0	18.2	23.0	20.6	1.1	1.1	1.1	118.6	123.7	121.1	2020.0	2116.0	2068.0	939.0	971.0	955.0
P20	33.7	45.5	39.6	1.3	1.3	1.3	130.9	134.6	132.7	2334.0	2382.0	2358.0	1186.0	1230.0	1208.0
P40	50.3	62.5	56.4	1.3	1.4	1.3	145.4	153.0	149.2	2519.0	2571.0	2545.0	1294.0	1390.0	1342.0
P60	60.5	66.9	63.7	1.4	1.4	1.4	151.3	154.7	153.0	2806.0	3030.0	2918.0	1350.0	1440.0	1395.0
P80	67.8	72.6	70.2	1.4	1.4	1.4	160.0	162.5	161.3	3173.0	3237.0	3205.0	1388.0	1463.0	1425.0
LSD (0.05)	**5.1**	**2.9**	**3.8**	**0.1**	**0.2**	**0.1**	**8.2**	**3.7**	**5.2**	**71.0**	**52.3**	**47.9**	**44.2**	**14.7**	**22.5**

Distinct differences between the cultivars in haulm yield could be observed (Table 1). The significantly highest haulm yield was registered against the V3 cultivar (2882 kg ha^{-1}) followed by the V2 cultivar (2617 kg ha^{-1}) and the lowest in the V1 cultivar (2358).The P_{80} treatment produced the highest haulm yield (3205) followed by decreasing order of magnitude of haulm yield as: P_{60} (2918 kg ha^{-1}) > P_{40} (2545 kg ha^{-1}) > P_{20} (2358 kg ha^{-1}).The significantly lowest haulm yield was recorded against the control treatment *i.e.* P_0 (2068.2 kg ha^{-1}). Basir *et al.* (2008) reported that plots receiving 60 kg P_2O_5 ha^{-1} produced maximum haulm yield.

There was statistically significant difference in seed yield between the cultivars (Table 1). The V3

cultivar was the highest yielder producing 1430 kg ha^{-1} of seed yield which was significantly higher than the V2 (1279 kg ha^{-1}) and V1 cultivars (1086 kg ha^{-1}). There was significant increase in seed yield of chickpea with P application. The seed yield obtained with the P_{80} treatment (1425 kg ha^{-1}) was maximum which was at par with P_{60}. The P_{80}, P_{60}, P_{40} and P_{20} treatments increased the seed yield of chickpea by 49.2, 46.03, 40.46 and 26.45% respectively over the control treatment. The increment in seed yield of chickpea with increase in P levels might be attributed to the physiological role of P on the meristematic activity of plant tissues enhancing plant growth and also its function as a part of enzyme system play a vital role in the synthesis of other foods from carbohydrate.

Statistical variation in total P uptake was evident between the cultivars (Table 2).The highest total P uptake occurred in the V3 cultivar (6.94 $kgha^{-1}$) followed by the V2 cultivar (5.78 $kgha^{-1}$) and the V1 cultivar (4.61 $kgha^{-1}$). Irrespective of the cultivars, there was statistically significant variation in the P uptake between the P application treatments. Higher the P dose higher was the P uptake. The P80 treatment registered the highest P uptake than the other P application treatments. Singh and Singh (2012) found that higher P application enhanced the P uptake in chickpea. The apparent P recovery ranged from 6.03 to 7.70 % (Table 2). Although the P_{20} treatment showed the maximum apparent P recovery of 7.70 %, which was statistically similar to the P_{40} treatment (7.39 %). The P_{60} and the P_{80} treatments followed next in order registering apparent P recovery of 6.15 and 6.03 % respectively and were in the same statistical rank. The higher apparent recovery at lower P application rates in the present study was also in accordance to that reported by Islam *et al.* (2012).The agronomic efficiency (AE) varied from 5.88 to 12.63 kg seed kg P^{-1} applied (Table 2). The AE varied statistically and significantly between the P application treatments. The application of higher doses of P reduced the AE values. The maximum AE was observed in the P_{20} treatment (12.63) owing to greater grain production per unit of P applied. The other treatments could be ranked as P40 (9.66) > P_{60} (7.33) > P_{80} (5.88). According to a study conducted by Devi *et al*. (2012) the decline of AE with high level of P application might be due to fixation of P in soil.

Table 2: Total P uptake, apparent P recovery and agronomic efficiency by the application of P fertilizer to chickpea cultivars

Treatment	Total P uptake ($kgha^{-1}$)			Apparent P recovery (%)			Agronomic efficiency (kg seed P^{-1} applied)		
Cultivar	2012-13	2013-14	Mean	2012-13	2013-14	Mean	2012-13	2013-14	Mean
V1:Anuradha	4.47	4.76	4.62						
V2:Pusa-1003	5.57	6.00	5.78						
V3:DCP-92-3	6.70	7.19	6.95						
LSD (0.05)	**0.21**	**0.64**	**0.40**						
P levels (kg ha^{-1})									
P0	3.06	3.30	3.18	-	-	-	-	-	-
P20	4.59	4.85	4.72	7.65	7.74	7.70	12.32	12.95	12.63
P40	5.96	6.31	6.14	7.25	7.52	7.39	8.86	10.47	9.66
P60	6.68	7.06	6.87	6.04	6.27	6.15	6.84	7.81	7.33
P80	7.62	8.40	8.01	5.70	6.37	6.03	5.61	6.15	5.88
LSD (0.05)	0.47	0.71	0.35	1.89	1.37	0.89	0.95	1.18	0.83

Data obtained in this study was examined by analyzing the chickpea seed yield to ascertain the relationship between the two variables. The model which defines this relation between the applied P levels and yield of chickpea were obtained using second degree polynomial function. The derived functional equation was $Y=964.5+13.13x-0.093x^2$ (Table 3). By using the developed functions, the optimum and economic levels of P were 70.59 and 66.54 kg ha^{-1} respectively. The predicted yields of chickpea were 1428 and 1426 kg ha^{-1} against the optimum and economic levels of P. The response at economic optimum dose was 7.07 kg seed yield per kg P. Thus it appears that it is rational to decide the P amount to be applied to chickpea in the Gangetic alluvial soils between 65.63 and 67.68 kg ha^{-1} to achieve economically optimum yield. The highest yield can be obtained by using P fertilizer between 69.27 and 72.22 kg ha^{-1}.

Table 3: Response equation, optimum dose, economic dose and predicted seed yields of chickpea as a function of P

Response equation	R^2	Optimum Dose (kg ha^{-1})	Predicted yield at (kg ha^{-1})	Economic optimum dose (kg ha^{-1})	Predicted yield at EOD (kg ha^{-1})	Response at EOD (kg seed yield kg P^{-1})
$Y=964.5+13.13x-0.093X^2$	99.3	70.59	1428	66.54	1426	7.07

The cultivars did not have any significant bearing on the total bacterial count in soil (Table 4). However, the highest total bacterial count in soil could be detected against the V3 cultivar (1.94) followed by the V2 cultivar (1.78) and V1 cultivar (1.68). P fertilization significantly affected the total bacterial count in soil. Increase in the dose of P fertilization increased the total bacterial count in soil. The significantly highest bacterial count was recorded against the P_{80} treatment (2.13). This increase in total bacterial count could be attributed to the increase in C supply due to increased plant growth.

There was significant variation in soil microbial biomass carbon (SMBC) between the chickpea cultivars (Table 4). The SMBC registered under the V3 cultivar (308) remained *at par* with the V_2 cultivar (305) was higher than that under the V_1 cultivar (294). The SMBC increased with increases in the doses of P. The highest SMBC was observed with the P_{80} treatment (328). Zhong *et al*. (2010) inferred that application of fertilizers did not directly influence microbial parameters in soil, but did so indirectly by increasing the contents of certain critical plant nutrients, thus, promoting plant biomass and increasing soil organic accumulation and enhancing soil microbial biomass and activities (Islam *et al*., 2011).

Table 4: Total bacterial count and microbial biomass carbon of soil as affected by different levels phosphorus application to chickpea cultivars

Treatment	Total aerobic bacterial count (CFU g^{-1} of oven dry soil)	Soil microbial biomass carbon (ìg g^{-1}soil)
Cultivar		
V1:Anuradha	1.68	294
V2:Pusa-1003	1.78	305
V3:DCP-92-3	1.94	308
LSD (0.05)	**NS**	**5.48**
P levels (kg ha^{-1})		
P0	1.50	275
P20	1.67	291
P40	1.77	302
P60	1.93	315
P80	2.13	328
LSD (0.05)	**0.199**	**6.86**

Note: NS-Non significant

In essence, AFLP methods allow PCR amplification to detect polymorphisms of genomic restriction fragments. The AFLP-PCR is the most popular method belonging to the LM-PCR (Ligation mediated) group. AFLP markers were assessed for their usefulness in characterizing molecular diversity of the sampled soils from plots applied with different doses of P application to chickpea. Visible bands varied from 0.7kb to 2.5kb. Surprisingly, banding patterns in all the treatments were alike (Fig. 1). All the soil samples produced four discrete and reproducible bands. Microbial community in the root rhizosphere remained same for all the treatments. P fertilization treatments had no effect on the diversity of ribotypes, detected in the soil samples. Contradicting reports on the effect of phosphorus fertilizer application on soil microbial diversity and community structure are documented. Shi *et al*. (2013) concluded that size, activity, and structure

of the soil microbial community did not differ due to P fertilization.

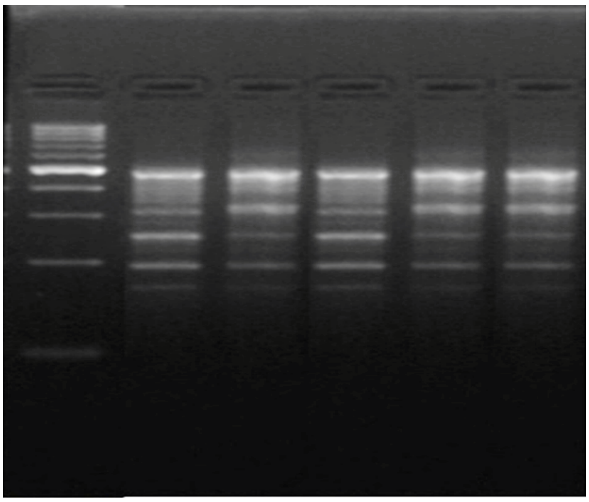

Fig. 1: AFLP pattern using Eco AGC and MseTGC primer pairs; L 500bp ladder, Lane 1-P 0kg ha^{-1}, Lane 2- P20 kg ha^{-1}, Lane 3- P40 kg ha^{-1}, Lane 4- P60 kg ha^{-1} and Lane 5- P 0 kg ha^{-1}

It can be recommended that the best chickpea cultivar would be to choose DCP-92-3 and fertilized with 80 kg P_2O_5 ha^{-1} for maximum yield. However, it would be economical to apply 65.63 and 67.68 kg P_2O_5 ha^{-1}. Unnecessary higher doses can be prevented which can economize the non renewable sources of P. Soil microbial diversity remains unaltered by short term P fertilization.

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