Pak. J. Bot., 45(3): 1017-1022, 2013.

DEGRADATION OF LEGUME PHYTATE IN SOIL USING FUNGAL PHYTASE

FAREEHA NAZ, ASMA MAQBOOL AND KAUSER ABDULLA MALIK*

Department of Biological Sciences, Forman Christian College (A Chartered University), Lahore, Pakistan
**Corresponding author e-mail: kausermalik@fccollege.edu.pk*

Abstract

Release of inorganic P was studied as a result of degradation of phytates during legume green manuring in soil. *Aspergillus niger* strain 419 was also inoculated for the production of phytase. Four sets of experiments were performed for maximum phytate degradation: (a) soil and legumes, (b) soil, legumes and phytase, (c) soil and phytase, (d) soil, legumes and *A. niger* strain 419. The soil in all of these samples was sterilized. Inorganic P was checked in each set after three different intervals of time (5 days, 8 days and 12 days) to estimate the degradation of phytates. The sample having *A. niger* strain 419 had highest content of free phosphorous after 5 days of incubation. The amount of free phosphorous reduced after 8 days in all samples except for the one containing soil, legumes and phytase. The amount of estimated inorganic P increased slightly in this sample. An increase in inorganic P was observed after 12 days in all samples except the first sample (soil and legumes). Estimated inorganic P further decreased in this sample. Same experiments were also performed with unsterilized soil. Inorganic P in all of the unsterilized samples decreased after 8 days and then increased after 12 days. The sample containing phytase, soil and legumes showed highest content of phosphorous after 12 days of interval, among unsterilized samples.

Introduction

Phosphorous (P) being an essential plant nutrient is needed to maintain and enhance plant growth. The deficiency of phosphorous in soil acts as a limiting factor in the growth of agricultural crops (Yasmin & Bano, 2011). In order to meet the requirements of crops, phosphatic fertilizers, in the form of diammonium phosphates (DAP), superphosphates, concentrated superphosphates (CSP), etc., are used. However, these fertilizers are not only expensive but also it is generally reported that around 20% of applied P fertilizer is used by plants and the rest of it is converted to inorganic and organic forms that are not readily available for plant uptake (Sanyal & De Datta, 1991). Inorganic form of P is present in soil as complexes with different forms of Al, Fe, Ca, Mg and other elements that are insoluble and thus remain inaccessible to plants. A large part of soil total P is organic which is considered a vital source for plant nutrition. However organic forms of P must be mineralized to make available to plants (Richardson *et al*., 2000; Khalid *et al*., 2011).

Major form of organic P is present as phytates in soil. Phytate is the salt of phytic acid (myo-inositol hexakis dihydrogen phosphate) (Jiang *et al*., 2001; George *et al*., 2006). Phytate is an important reservoir of phosphorous, mostly in seeds and grains. It also has a strong tendency to chelate metal ions like Ca, Mg, Fe, Zn, and Mn, because of its biochemical structure that is a myo-inositol ring with six reactive phosphate groups. A large portion of animal feed comprises of seeds and grains and monogastric animals lack enzymes to digest phytates. Therefore, undigested phytates from these animals become part of manure and remain concentrated in soil (Bilyeu *et al*., 2008).

Legumes that have been reported to contain the highest content of phytates are generally used for green manuring as well as fodder for live stock (Kaprelynts *et al*., 2003; *Achakzai et al.*, 2012). Phytate bound phosphorus in manure thus remains in the soil and is of no use to plants. As plants may not release phosphatases of their own, phosphorous cannot be liberated from the complexes like phytates. The two isomers of phytase (myo-inositol hexakisphosphate phosphohydrolase), namely 3-phytase (EC. 3.1.3.8) from microbes and 6-phytase (EC. 3.1.3.26) from plants have the ability to catalyze the conversion of phytate to inositol polyphosphates and free orthophosphoric acid (Wodzinski & Ullah, 1996). Three classes of enzyme phytase have been identified that initiate dephosphosphorylation of phytate at different positions on the inositol ring and generate different isomers of lower inositol phosphates (Bohn *et al*., 2008).

Phytase has been found in lower organisms like microbes including bacteria and fungi as well as in some plants. Plants, however, have been found to show only limited phytase activity to acquire phytate P form soil, on their own (Hayes *et al*., 2000). Phytase has been detected in some plant roots (Hubel & Beck, 1996; Hayes *et al*., 1999) but it is not certain if the enzyme is extracellular and can release P from phytate external to roots (Verwoerd *et al*., 1995; Brinch-Pedersen *et al*., 2000). On the other hand, microbes are considered the best source for phytase production, even on the commercial scale. The ability of plants to attain P from phytate is improved when the growth media is inoculated with the microbes that secrete phytase (Richardson *et al*., 2001). Various strains of bacteria yeast and fungi are used for phytase production but two species of *Aspergillus*, *A. niger* and *A. ficuum* are of great importance for enzyme production on a large scale (Kim *et al*., 1999; Ahmad *et al*., 2000).

It has been observed that *A. niger* produces a greater yield of phytase as compared to other microbial and plant sources. *A. niger* phytase is an extracellular glycoprotein (Han *et al*., 1999) and is specific for phytate (Sariyska *et al*., 2005). These characteristics make *A. niger* a suitable source for phytases.

As mentioned above, a large portion of phosphorus is concentrated in mature legumes as phytates; this phosphorus can be freed from bound phytates by the action of phytase and can then be used for plant P nutrition. The purpose of the current study is to inoculate soil containing legumes with *A. niger* and analyze the degradation of phytates in legumes. The phytates in

legumes can act as substrate for the enzyme released by *A. niger*. This could increase the availability of free phosphorous in soil and can therefore lead to easy absorption of P by plants.

Materials and Methods

Collection of fungal strain and soil samples: *Aspergillus niger* strain strain 419 culture was obtained from Institute of Agricultural Sciences, University of the Punjab, Lahore. The required soil samples were obtained from wheat fields on the University campus. The soil was silty clay loam having a pH of 8.2.

Growth of *Aspergillus niger* strain 419: *Aspergillus niger* strain strain 419 was maintained on 2% malt extract agar (MEA) for spore propagation for four days at 30ºC (Greiner *et al*., 2009). For estimating the enzyme activity, 0.1 ml of spore suspension having 2-3 x 10^7 spores was added to liquid starch medium containing starch 40g/dm^3, glucose 30g/dm^3, $NaNO_3$ 8.6 g/dm^3, K_2HPO_4 0.1 g/dm^3, $MgSO_4$ 0.5 g/dm^3, KCl 0.5 g/dm^3, $FeSO_4$ 0.1 g/dm^3. The pH was set to 5. It was incubated for 7 days at 30 ºC on a rotary shaker (220 rpm) (Sariyska *et al*., 2005).

Determination of phytase activity in *A. niger* strain 419 and soil: An aliquot of 10 ml of the culture grown on liquid starch medium for 7 days was extracted with 50 ml of sodium acetate (0.2 M, pH 5.5) buffer at 37 °C by putting on a shaker at 220 rpm for 1 h. Then the slurry was filtered through muslin cloth and was centrifuged at 14000 rpm for 10 min. The supernatant was then used for phytase assay (Gulati *et al*., 2007). Phytase activity of *A. niger* strain 419 was estimated using the method of Choi *et al*., (2001) and Gulati *et al*., (2007) with some modifications. In this method, 0.1 ml of enzyme solution was incubated with 0.9 ml of 0.5% w/w sodium phytate prepared in sodium acetate (0.2 M, pH 5.5) buffer. The enzyme reaction was carried out at 37°C for 30 min and the reaction was then stopped by addition of 0.75 ml of 5% trichloroacetic acid (TCA). The liberated phosphate ions were quantified by mixing 100 µl of assay mixture with 900 µl of color reagent (ammonium molybdate reagent) and taking the absorbance of this solution at 882 nm after 30 min. of color development.

Same procedure was repeated with soil samples to check the phytase activity in soil. One g of soil was added to 10 ml of sodium acetate buffer (0.2 M, pH5.5). The samples were placed on a shaker at 350 rpm and 37°C for 1 h. The samples were spun for 10 minutes at 4°C at 14000 rpm. The supernatant was then used for phytase assay as stated above. The concentration of phosphorous was calculated by drawing standard phosphorous calibration curve.

Phosphorous (P) content in soil

Organic P in soil: One g of soil was weighed in porcelain crucibles for ignition in a muffle furnace. Soil samples were ignited at 550°C for 1 h. The crucibles were allowed to cool and the ignited soils were transferred to a clean specimen container and in other container one g of uniginted soil was taken. Both samples were extracted by adding 50 ml of 1 N HCl and placed on a shaker for overnight at 150 rpm. Samples were centrifuged at 14000 rpm for 15 min. Supernatants were filtered through Whatman No.1 filter paper. Then 100 µl of each sample was allowed to react with 900 µl of color reagent and the absorbance was measured on spectrophotometer at 882 nm (Saunders & Williams, 1955). The experiment was performed with triplicate samples. The difference between ignited and unignited samples gave the value of organic P in soil.

Inorganic P in soil: One g of soil sample was weighed. Triplicates of each soil sample were extracted with 30 ml of 0.5 M sodium bicarbonate buffer for 1 h (Turner & Haygarth, 2003). The soil solutions were centrifuged at 14000 rpm for 10 min., filtered through filter paper and 100 µl of filtrate was allowed to react with 900µl of color reagent. After 30 min. of color development, absorbance of the samples was recorded using spectrophotometer at 882 nm. Inorganic P was calculated from the standard curve.

Determination of inorganic P for estimation of degradation of phytates: Four sets of experiments were designed. Three of them had 10 g of legumes (chick pea) in 100 g of soil. The soil and the legumes were sterilized by autoclaving at 15 psi and 120°C for 15 min. The first set (soil + legumes) acted as control. In second set 5 ml of phytase was added to the triplicate samples (soil + legumes + phytase). The phytase enzyme used had enzyme activity of 1.518 U/min/100 µl. In third set, all triplicates were inoculated with *A. niger* strain 419 spores (soil + legumes + *A. niger* strain 419). Inoculation of *A. niger* spores was done by transferring spores from culture plate with the help of inoculating needle to 1 ml of sterile water in an eppendorf tube. The spores suspended in water were then added to the soil sample. The fourth set that did not have any legumes, was also treated with 5 ml of phytase (soil + phytase). Inorganic P was estimated in all samples after 5, 8 and 12 days of incubation at 30°C according to Turner & Haygarth (2003) method. Same procedure was also repeated with unsterilized soil.

Statistical analysis: The data represented here is the mean of triplicates. The two ways ANOVA was applied at a confidence level of 95% using SPSS version 17.0. Duration and the type of sample were taken as factors whereas absorbance was taken as response. Pair wise comparison was also conducted using Tukey's HSD test.

Results

Determination of phytase activity in *A. niger* strain 419 and soil: *A. niger* strain 419 culture were grown on liquid starch media for 7 days at 30 °C. After harvesting with sodium acetate buffer (0.2 M, pH 5.5) phytase activity of *A. niger* strain 419 was estimated. The phytase activity in fermented slurry of fungus and soil sample was calculated using standard phosphorous calibration curve.

Phytase assay was performed for *A. niger* strain 419 and soil samples. The absorbance of the samples taken was used to calculate phosphorous liberated by the action of enzyme and thus phytase activity against sodium phytate was determined (Table 1). The results showed that 0.884 µmol of phosphorous was released by *A. niger* strain 419 phytase solution and 0.656 µmol of phosphorus was detected by soil sample assayed for phytase. Since 1 unit of enzyme activity is defined as 1 µmol of phosphorous released per min. under assay conditions (Choi *et al*., 2001), the enzyme activity observed in *A. niger* strain 419 fermented slurry was 0.884 units while 0.656 units were estimated in soil sample.

Table 1. Phytase activity in *A. niger* strain 419 and soil sample.

Samples for phytase activity	Concentration liberated P (µmol)	Phytase activity (U/min/100µl)
A. niger strain 419	0.884*	0.884*
Soil	0.656^{+}	0.656^{+}

Concentration of liberated P was calculated in order to estimate phytase activity in triplicate samples. Values are the mean of three samples with standard error *± 0.075 and $^{+}$± 0.049

Phosphorous (P) content in soil

Organic P in soil: Organic P in triplicate set of soil samples was determined by estimating P content in unignited and ignited soil samples. P content was calculated using standard phosphorus calibration curve.

Spectrophotometric detection of P in unignited and ignited soil samples was done and concentration of P was calculated. The ignited soil sample had 20.182 µg of P/g and unignited soil sample had 13.816 µg P/g. The calculated organic P was found to be 6.366 µg (20.182 µg – 13.816 µg) in 1 g of soil tested.

Inorganic P in soil: Inorganic P in triplicate set of soil samples was determined by recording the absorbance of the sample after colorimetric reaction. The amount of inorganic P estimated using was 0.488 ± 0.019 µg in 1 g of soil sample.

Determination of inorganic P for estimation of degradation of phytates in sterilized soil: The amount of phytate degraded in sterilized soil sample was estimated on the basis of released inorganic P (Table 2).

Table 2. Comparison of estimated inorganic P after different time intervals in sterilized samples.

Samples	Inorganic P after 5 days (µg)	Inorganic P after 8 days (µg)	Inorganic P after 12 days (µg)
Soil + legumes	0.794 ± 0.124	0.537 ± 0.218	0.478 ± 0.184
Soil + Phytase	1.185 ± 0.097	0.498 ± 0.079	1.383 ± 0.047
Soil + legumes + Phytase	1.452 ± 0.496	1.788 ± 0.493	3.495 ± 0.501
Soil + legumes + *A. niger*strain 419	2.461 ± 0.296	1.482 ± 0.521	5.369 ± 0.042

After 5 days of incubation, the sample containing *A. niger* strain 419 was found to have the highest amount of inorganic P i.e. 2.461 µg. The samples containing soil, legumes and phytase had 1.452 µg whereas 1.185 µg of inorganic P was estimated in the samples having soil and phytase only. The samples that contained no phytase or source of phytase was reported to have the lowest amount of inorganic P i.e. 0.794 µg.

Therefore, the amount of inorganic P estimated after 5 days of incubation in different sets of experiments was in following order:

Soil + legumes + *A. niger* strain 419 > Soil + legumes + phytase > Soil + phytase > Soil + legumes

After 8 days of incubation, there was a decrease in inorganic P in all the samples except for the one containing soil, legumes and phytase. The highest amount of inorganic P (1.788 µg) was observed in the samples containing soil, legumes and phytase and the least (0.537 µg) in samples having soil and legumes in them.

The results indicated that afer 12 days of incubation, estimated inorganic P again increased in all samples except for the treatment containing soil and legumes and no source of phytase. The samples containing *A. niger* strain 419 in them had highest quantity of inorganic P i.e. 5.369 µg.

Results of two way ANOVA showed that the effect of different samples on absorbance was significantly different (p=0.000).Tukey HSD test showed that differnce between treatments after 5 and 12 days, and 8 and 12 days was significant (p=0.000) while difference between 5 and 8 days was non significant (p=0.229).

Determination of inorganic P for estimation of degradation of phytates in unsterilized soil: The estimation of inorganic P in unsterilized soil sample (Table 3) indicated that the samples containig soil and legumes had 3.208 µg of inorgnic P after 5 days of incubation. Highest amount of inorganic P was estimated in samples containing phytase which was 4.869 µg. The samples that were inoculated with *A. niger* strain 419, showed 3.144 µg of inorganic P whereas least amount was observed in the samples containing soil and phytase only i.e. 0.537 µg. Thus, the amount of inorganic P estimated after 5 days of incubation in different sets of experiments was in following order:

Soil + legumes + phytase > Soil + legumes > Soil + legumes + *A. niger* strain 419 > Soil + phytase

It was observed that after 8 days of incubation, inorganic P content in all samples was reduced. After 12 days of incubation an increase was seen in inorganic P content of all the samples. Highest amount was found in samples containing soil, legumes and phytase (4.118 µg) and the lowest was noticed in soil and phytase samples (1.536 µg).

Results of two way ANOVA showed that the effect of different samples on absorbance was significantly different (p=0.000).Tukey HSD test showed that differnce between treatments after 5 & 8 days and 8 & 12 days was significant (p=0.003; p=0.000) while between 5 and 12 days was non significant (p=0.113).

Discussion

Estimation of organic and inorganic P in untreated soil in our study has indicated that the amount of organic P was greater than the inorganic P. The amount of organic P estimated in 1 g of soil sample was 6.366 µg while 0.498 µg of inorganic P was found in soil. Other studies have also shown that a major part of P in soil is in organic form (Turner *et al.*, 2002). Phytase assay of unsterilized soil samples indicated the presence of phytase activity that may be due to soil microorganisms inhabiting the soil environment as shown by Richardson *et al*., (2001).

A greater part of total P form complexes with phytates in legumes (Steiner *et al*., 2007). Therefore, more P was liberated in the sample containing legumes and phytase (or source of phytase).

These observations suggest that presence of *A. niger* strain 419 in soil had improved the concentration of inorganic P in soil showing that *A. niger* strain 419 had possibly secreted the phytase enzyme which was active after 5 days. Inorganic P was again checked after 8 days of incubation and a decrease in estimated inorganic P was observed in all treatments except for the one containing soil, phytase and legumes. However, a marked increase in inorganic P was observed after 12 days in all samples except the one without phytase. It has been reported that *A. niger* in the presence of P source in soil improves plant growth (Vassilev *et al*., 1996). Zayed and Abel-Motaa (2005) also observed increased amount of soluble P in composts having *A. niger*, *T. viride* and/ or manure from farm yard.

The possible reason for decrease in P content after 8 days of incubation could be adsorption of phytase enzyme to soil. George *et al*., (2005) had reported that phytase activity was maintained in solution for at least 8 days at neutral pH. Immobilization of enzyme by increasing pH has also been observed by Quiquampoix & Ratcliffe (1992). However, adsorption of phytase to soil particles varies with type of soil. These patterns at different pH differ according to the nature of soil and enzyme (Blackburn *et al*., 2011). Adsorption may not deactivate the enzyme as there was increase in P after 12 days. It showed recovery of enzyme. Adsorption can be effective for enzyme as it is protected from the effect of proteinases in soil. But this is not the case with all phosphatases as some alkaline phosphatases were found to exhibit less stability on adsorption (Carrasco *et al*., 1995).

The results with unsterilized soil showed the same trend as observed in sterilized soil i.e. inorganic P decreased after 8 days and increased again after 12 days. Contrary to above results, it was observed that the soil having legumes and no external phytase or *A. niger* strain 419 inoculation, had higher amount of inorganic P and followed the same trend as the samples having external source of phytase. Some soil microorganisms play an important part in nutrient cycling in soil. Some of them are also involved in mobilization of P from its bound state and make it available for the plant roots (Richardson, 2000). These results support the idea that other microbes were also involved in phytate hydrolysis.

Another explanation of decrease in inorganic P in all samples after interval of 8 days could be immobilization of inorganic P by the soil microorganisms. Different phosphorous solubilizing bacteria have been reported to immobilize P in soil and then make it available for plants by liberating from their cells (Bünemann *et al*., 2004; Khan *et al*., 2009). All unsterilized samples had followed the same pattern i.e. the reduction in inorganic P content after 8 days and then an increase in estimated inorganic P after 12 days. The microbial population in these samples could have trapped the free P in soil and later released it from the cells.

The samples having soil and phytase showed lesser release of inorganic P in sterilized and unsterilized soils. This may be because there was no external phytate added to the soil. Therefore, substrate availability is also important to make maximum P available for plants. This observation coincides with the study made by Adams and Pate (1992) that showed decreased hydrolysis by phytase due to less substrate.

Table 3. Comparison of estimated inorganic P after different time intervals in unsterilized samples.

Samples	Inorganic P after 5 days (µg)	Inorganic P after 8 days (µg)	Inorganic P after 12 days (µg)
Soil + legumes	3.208 ±0.167	2.907 ±0.510	3.534 ±0.051
Soil + Phytase	0.537 ±0.052	0.290 ±0.021	1.536 ±0.044
Soil + legumes + Phytase	4.869 ±0.401	2.432 ±0.545	4.968 ±0.244
Soil + legumes + *A. niger* strain 419	3.144 ±0.297	1.823 ±0.383	4.118 ±0.153

Conclusion

Phosphorous requirements of plants can be met by allowing degradation of organic P in the form of phytates in the legumes which are generally used for green manuring. For this purpose inoculation of phytase producing microorganisms such as *A. niger* can help in degrading phytates thus releasing inorganic P. It has been shown that phytase from *A. niger* strain 419 was released in soil which acted on phytates thus increasing free phosphorous in soil. It will result in increased P uptake by plants thus reducing dependence on expensive phosphatic fertilizers.

Acknowledgements

We gratefully acknowledge the partial financial support from the Pakistan Science Foundation for a project on "Increasing phosphorus use efficiency in wheat through genetic engineering" Project No. PSF/NSLP/P-FCCU(121). The results presented here are part of an M. Phil thesis of Miss. Fareeha Naz.

References

Achakzai, A.K.K., B.H. Shah and M.A. Wahid. 2012. Effect of nitrogen fertilizer on the growth of mungbean [*Vigna radiata* (L.) Wilczek] grown in Quetta. *Pak. J. Bot*., 44(3): 981-987.

Adams, M.A. and J.S. Pate. 1992. Availability of organic and inorganic forms of phosphorous to lupins (*Lupinus* spp.). *Plant Soil*, 145: 107-113.

Ahmad, T., S. Rasool, M. Sarwar, A.U. Haq and Z.U. Hasan. 2000. Effect of microbial phytase produced from a fungus *Aspergillus niger* on bioavailability of phosphorus and calcium in broiler chickens. *Anim. Feed Sci. Technol*., 83(2): 103-114.

Bilyeu, K.D., P. Zeng, P. Coello, Z.J. Zhang, H.B. Krishnan, A. Bailey, P.R. Beuselinck and J.C. Polacco. 2008. Quantitative conversion of phytate to inorganic phosphorus in soybean seeds expressing a bacterial phytase. *Plant Physiol*., 146(2): 468-477.

Blackburn, M.D., M. Jorquera, L. Gianfreda, M. Rao, R. Greiner, E. Garrido and M.L. Mora. 2011. Activity stabilization of *Aspergillus niger* and *Escherichia coli* phytases immobilized on allophanic synthetic compounds and montmorillonite nanoclays. *Bioresour. Technol*., 102(20): 9360-9367.

Bohn, L., A.S. Meyer and S.K. Rasmussen. 2008. Phytate: impact on environment and human nutrition. A challenge for molecular breeding. *J. Zhejiang Univ. Sci. B*., 9(3): 165-191.

Brinch-Pedersen, H., A. Olesen, S.K. Rasmussen and P.B. Holm. 2000. Generation of transgenic wheat (*Triticumaestivum* L.) for constitutive accumulation of an *Aspergillus* phytase. *Mol. Breed*., 6:195-206.

Bünemann, E.K., D.A. Bossio, P.C. Smithson, E. Frossard and A. Oberson. 2004. Microbial community composition and substrate use in a highly weathered soil as affected by crop rotation and P fertilization. *Soil Biol. Biochem*., 36: 889-901.

Carrasco, M.S., J.C. Rad and S. Gonzalez-Carcedo. 1995. Immobilization of alkaline phosphatase by sorption on Na-sepiolite. *Bioresour. Technol*., 51: 175-181.

Choi, Y.M., H.J. Suh and J.M. Kim. 2001. Purification and properties of extracellular phytase from *Bacillus* sp. KHU-10. *J. Prot. Chem*., 20(4): 287-292.

George, T.S., A.E. Richardson and R.J. Simpson. 2005. Behavior of plant-derived extracellular phytase upon addition to soil. *Soil Biol. Biochem*., 37: 977-988.

George, T.S., B.L. Turner, P.J. Gregory, B.J. Cade-menun and A.E. Richardson. 2006. Depletion of organic phosphorus from Oxisols in relation to phosphatase activities in the rhizosphere. *E. J. Soil Sci.*, 57: 47-57.

Greiner, R., L. Gomes da Silva and S. Couri. 2009. Purification and characterization of an extracellular phytase from *Aspergillus niger* 11T53A9. *Braz. J. Microbiol*., 40: 795-807.

Gulati, H.K.., B.S. Chadha and H.S. Saini. 2007. Production, purification and characterization of thermostable phytase from thermophilic fungus *Thermomyces lanuginosus* TL-7. *Acta Microbiol et Immunol. Hung*., 54(2): 121-138.

Han, Y., D.B. Wilson and X.G. Lei. 1999. Expression of an *Aspergillus niger* phytase gene (*phyA*) in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol*., 65(5): 1915-1918.

Hayes, J.E., A.E Richardson and R.J. Simpson. 1999. Phytase and acid phosphatase activities in extracts from roots of temperate pasture grass and legume seedlings. *Aust. J. Plant Physiol*., 26: 801-809.

Hayes, J.E., R.J. Simpson and A.E. Richardson. 2000. The growth and phosphorous utilization of plants in sterile media when supplied with inositol hexaphosphate, glucose-1-phosphate or inorganic phosphate. *Plant Soil*, 220: 165-174.

Hubel, F. and E. Beck. 1996. Maize root phytase. Purification, characterization and localization of enzyme activity and its putative substrate. *Plant Physiol*., 112: 1429-1436.

Jiang, L., T.E. Phillips, C.A. Hamm, Y.M. Drozdowicz, P.A. Rea, M. Maeshima, S.W. Rogers and J.C. Rogers. 2001. The protein storage vacuole: a unique compound organelle. *J. Cell Biol.*, 155: 991-1002.

Kaprelynts, L.V., S.V. Kisilev and E.G. Lorgachova. 2003. Soybean isoflavones and prospects of their therapeutic application. *Voprosy Pitaniya*, 72: 36-41.

Khalid, R., K.S. Khan, Z. Akram, R. Qureshi and M. Gulfraz. 2011. Relationship of plant available sulphur with soil characteristics,rainfall and yield levels of oilseed crops in Pothwar Pakistan. *Pak. J. Bot*., 43(6): 2929-2935.

Khan, A.A., G. Jilani, M.S. Akhtar and S.M.S. Naqvi and M. Rasheed. 2009. Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. *J. Agric. Biol. Sci*., 1(1): 48-58.

Kim, D.S., J.S. Godber and H.R. Kim. 1999. Culture conditions for a new phytase-producing fungus. *Biotechnol. Lett*., 21: 1077-1081.

Quiquampoix, H. and R.G. Ratcliffe. 1992. A ^{13}P NMR study of the adsorption of bovine serum albumin on montmorillonite using phosphate and the paramagnetic cation Mn^{2+}: modification of conformation with pH. *Colloid Surface A*., 75: 85-93.

Richardson, A.E. 2000. Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *Aust. J. Plant Physiol*., 28(9): 897-906.

Richardson, A.E., P.A. Hadobas and J.E. Hayes. 2000. Acid phosphomonoesterase and phytase activities of wheat (*Triticum astivum* L.) roots and utilization of organic phosphorous substrates by seedlings grown in sterile culture. *Plant Cell Environ*., 23: 397-405.

Richardson, A.E., P.A. Hadobas, J.E. Hayes, C.O. O'Hara and R.J. Simpson. 2001. Utilization of phosphorous by pasture plants supplied with myo-inositolhexaphosphate is enhanced by the presence of soil microorganisms. *Plant Soil*, 229: 47-56.

Sanyal, S.K. and S.K. De Datta. 1991. Chemistry of phosphorus transformations in soil. *Adv. Soil Sci*., 16: 1-120.

Sariyska, M.V., S.A Gargova, L.A. Koleva and A.I. Angelov. 2005. *Aspergillus niger* phytase: Purification and characterization. *Biotechnol. Biotech. Eq*., 19(3): 98-105.

Saunders, W.M.H. and E.G. Williams. 1955. Observations on the determination of total organic phosphorus in soils. *J. Soil Sci*., 6: 254-267.

Steiner, T., R. Mosenthin, B. Zimmermannb, R. Greiner and S. Roth. 2007. Distribution of phytase activity, total phosphorus and phytate phosphorus in legume seeds, cereals and cereal by-products as influenced by harvest year and cultivar. *Anim. Feed Sci. Tech*., 133: 320-334.

Turner, B.L. and P.M. Haygarth. 2003. Changes in bicarbonate-extractable inorganic and organic phosphorus by drying pasture soils. *Soil Sci. Soc. Am. J*., 67: 344-350.

Turner, B.L., M.J. Papházy, P.M. Haygarth and I.D. McKelvie. 2002. Inositol phosphates in the environment. *Philos. Trans. R. Soc. Lond. B Biol. Sci*., 357: 449-469.

Vassilev, N., I. Franco, M. Vassileva and R. Azcon. 1996. Improved plant growth with rock phosphate solubilized by *Aspergillus niger* grown on sugar-beet waste. *Bioresource Technol*., 55(3): 237-241.

Verwoerd, T.C., P.A. van Paridon, A.J. van Ooyen, J.W. van Lent, A. Hoekema and J. Pen. 1995. Stable accumulation of *Aspergillus niger* phytase in transgenic tobacco leaves. *Plant Physiol*., 109: 1199-1205.

Wodzinski, R.J. and A.H.J. Ullah. 1996. Phytase. *Adv. Appl. Microbiol*., 42: 263-302.

Zayed, G. and H. Abel-Motaa. 2005. Bio-active composts from rice straw enriched with rock phosphate and their effect on the phosphorous nutrition and microbial community in rhizosphere of cowpea. *Bioresource Technol*., 96(8): 929-935.

Yasmin, H. and A. Bano. 2011. Isolation and characterization of phosphate solubilizing bacteria from rhizosphere soil of weeds of khewra salt range and Attock. *Pak. J. Bot*., 43(3): 1663-1668.

(Received for publication 28 January 2012)