academicJournals

Vol. 4(5), pp. 52-57, July 2013
DOI: 10.5897/JYFR2013.0120
ISSN 2141-2413 ©2013 Academic Journals
http://www.academicjournals.org/JYFR

Journal of Yeast and Fungal Research

Full Length Research Paper

Comparative study on solubilization of tri-calcium phosphate (TCP) by phosphate solubilizing fungi (PSF) isolated from Nsukka pepper plant rhizosphere and root free soil

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Accepted July 26, 2013

Phosphate solubilizing fungi (PSF) possessing the ability to solubilize insoluble phosphate was isolated from the rhizospheric soil of Nsukka pepper plant. Twelve (12) fungal strains were isolated from both root-free soil (non-rhizosphere) and rhizosphere of Nsukka pepper plant. Of the 12 fungi isolated, 6 strains were from Nsukka pepper plant rhizosphere whereas the other six were from root-free soil. PF isolates were designated to be isolates from the rhizosphere of Nsukka pepper whereas NF isolates were designated to be isolates from non-rhizosphere soil. The former isolates were able to solubilize insoluble phosphate, both in PVK agar and NBRIP broth, whereas the latter isolates were unable to solubilize insoluble phosphate on Pikovskaya (PVK) agar medium but solubilize slightly on NBRIP broth. Fungal phosphate solubilization was analyzed by determining the quantity solubilized using NBRIP broth and calculation of the phosphate solubilization efficiency (E) on PVK agar was estimated using E = solubilization diameter/growth diameter x 100. The highest phosphate solubilization efficiency was demonstrated by PF7 isolate as 240.0% followed by PF2 isolate as 137.5% with 4.12 and 4.06 mg/ml, respectively, as the NBRIP broth analysis, whereas the non-rhizosphere isolates showed no solubilization effect on PVK agar medium but slight solubilization on NBRIP.

Key words: Phosphate solubilizing fungi, Pikovskaya, phosphate solubilization efficiency, rhizosphere.

INTRODUCTION

Phosphate fertilizers might increase phosphate availability initially, but will promote the formation of insoluble phosphate compounds making phosphorus unavailable to plants. Therefore, phosphate solubilizing microorganisms may be a choice for maintaining the steady supply of plant available phosphate (Xie, 2008). Root exudates are important for microbial attraction and fungal establishment on roots rhizosphere. Some of the metabolites known to excrete as the exudates from plants are the sugars and the amino acids which serve as required nutrients

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Abbreviations: PSF, Phosphate solubilizing fungi; **PSE,** phosphate solubilization efficiency; **pNPP,** para-nitro phenyl phosphate; **NBRIP-BPB,** National Botanical Research Institute's Phosphate-Bromo Phenol Blue; **NF,** non-rhizospheric fungi; **PF,** pepper rhizospheric fungi.

for growth of microorganisms (Odunfa, 1979). From the root exudates of pepper plants, twelve amino acids and seven sugars were detected (Naqvi and Chauhan, 1980). The exudation of carbon-containing metabolites and amino acids from the roots into the soil matrix serves as substrates for the fungi, resulting in an increased microbial biomass which gives rise to organic acid production (Rogier et al., 2012). Root exudates serve as an important source of nutrients for microorganisms in the rhizosphere and induce an aggregation of microbes around the root region, this forms a mutual relationship between the plant and the microbe, whereby the plant provides nutrients to the microbes and the microbes solubilize phosphate for plant uptake (Shukla et al., 2011). The presence of total organic acid (TOA) posed the concept of phosphate solubilization by the process of acidification and chelation (Akintokun et al., 2007; Khan et al., 2009). Microorganisms are known to solubilize insoluble phosphate (for example, tri-calcium phosphate) through the production of organic acids (Jayandra et al., 1999) and these acids lower the pH of the medium and bring about the dissolution of bound forms of phosphate (Sharma et al., 2012). Phosphorus deficiency is a major constraint for crop production (Aadarsh, 2011) and many soils are deficient in readily available forms of phosphorus for plant uptake, therefore in order to circumvent phosphorus deficiency, phosphate solubilizing microorganism could play an important role in supplying phosphate to plants in a more environmentally-friendly and sustainable manner. Since the indiscriminate and excessive application of chemical fertilizers has led to high accumulation of insoluble phosphate, unhealthy nature and environmentally unfriendly nature of soil, we were desperate to find an alternative strategy by exploring the potentials in Nsukka pepper plants rhizosphere.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from rhizosphere of pepper plants and non rhizosphere region, at University of Nigeria, Nsukka agriculture farm. The soil samples were taken within 1 and 10 cm radius by 5 to 10 cm depths for rhizosphere collection and 500 to 1000 cm by 5 to 10 cm depth away from the rhizosphere.

Isolation of indigenous rhizospheric and non rhizospheric fungi

From each soil sample, 10 g were transferred to 250 mL Erlenmeyer flask containing 90 ml of sterile distilled water. The flasks were shaken for about 20 minand allowed settling for few minutes. A 1 ml of the suspension were transferred to 9 ml of distilled water in test tube and serially diluted. The appropriate dilution was plated out using 0.1 ml aliquot on potato dextrose agar (PDA) medium. The soil samples were also sprinkled directly on some plates and incubated.

Inoculums size determination

A haemocytometer (Improved Neubauer counting chamber) and a cover glass were thoroughly cleaned with moist cloth. The chamber was placed on a flat horizontal surface and the cover glass, slides into position using firm pressure. A 0.1ml cell suspension of a 5 day culture was transferred to the chamber using pasteur pipette held at 45°C. The filled chamber was placed on the microscope and the cells were counted using 40x objective lens. The area counted (A), depth of the counting chamber (D), average number of cell counted (N) and the dilution factor (DF) of cells per ml was calculated as Cell/mL =N ‘x’ DF ‘x’ 10^5/ A ‘x’ D.

Composition of PVK medium (Pikovskaya, 1948), supplemented with bromo-phenol-blue

Glucose 10.0 g, $Ca_3(PO_4)_2$ 5.0 g*, $(NH_4)_2SO_4$ 0.5 g, NaCl 0.2 g, $MgSO_4.7H_2O$ 0.1 g, KCl 0.2 g, yeast extract 0.5 g, $MnSO_4.H_2O$ 0.002 g, $FeSO_4.7H_2O$ 0.002 g, Agar 15.0 g, distilled H_2O 1000 ml, bromo-phenol-blue (BPB) 0.025 g* and pH 7.0 were used.

Isolation of phosphate solubilizing fungi (PSF)

Each fungal isolate were aseptically transferred onto Pikovskaya (1948) medium (PVK) supplemented with bromo-phenol-blue (BPB) and tri-calcium phosphate (TCP) using point inoculation and incubated at 28°C for 7 days. The solubilizations of phosphate were observed as a zone of clearance with a diameter that was measured in millimeters. The phosphate solubilization ability of the fungi was analyzed by determining the phosphate solubilization efficiency (E) of each isolate [E = solubilization diameter / growth diameter x 100] (Nguyen et al., 1992) sited by Qurban (2012). After confirming the phosphate solubilizing ability on solid medium, the phosphate solubilization were also carried out using National Botanical Research Institute’s Phosphate - Bromo Phenol Blue (NBRIP-BPB) broth (Pradhan and Sukla, 2005).

Composition of NBRIP-BPB liquid medium

NBRIP-BPB liquid medium consist of $(NH_4)_2SO_4$(0.1 g/l), $Ca_3(PO_4)_2$ (5.0 g/l), $MgSO_4.H_2O$ (0.25 g/l), $MgCl_2.6H_2O$ (5.0 g/l), KCl (0.2 g/l), BPB (0.025 g/l), glucose (10 g/l) (Nautiyal, 1999) inoculated with a 1% (v/v) inoculums pre-culture grown in the same medium. The phosphate solubilization activity of each of the isolates was determined by growing the isolates in NBRIP medium containing a pH indicator (bromophenol blue) for 12 days (taking reading at 4 days intervals) at 29°C. At the end of the incubation period, spectrophotometric readings were taken at OD_{600} and the final values were subtracted from the initial values (control) (Nautiyal, 1999).

Quantification of total organic acids produced by each isolate

The broth cultures of the PSF isolates were used for this assay. Titrable acidity was estimated by titrating one milliliter of the culture supernatant against 0.1 M NaOH in the presence of phenolphthalein as an indicator (Whitelaw, 2000). The titrable acidity was expressed as milliliter (ml) of 0.1 M NaOH consumed per 1.0 ml of culture filtrate.

Statistical analysis

All experiments were conducted in triplicates and mean of the values reported. Means were calculated using GENSTAT statistical package. Significant difference was set at $P < 0.05$ level.

RESULTS

Isolation and enumeration of phosphate solubilizing fungi from the rhizosphere and non rhizosphere soil

Out of the twelve (12) fungi strains isolated from both soil samples, only six (6) showed significant zone of phosphate solubilization on PVK-BPB agar medium. Clear halos were formed around the colonies after 5 to 7 days of incubation on solidified PVK medium supplemented with TCP, indicating phosphate-solubilizing ability of the fungal isolates. The phosphate solubilizing fungi strains are PF2, PF3, PF4, PF5, PF6 and PF7, with PF7 (Figure 3) showing the highest percentage of phosphate solubilization efficiency. The non-rhizospheric fungi (NF) isolates showed no significant zone of phosphate solubilization on PVK-BPB agar medium as shown in Table 1.

Effect of cell size on phosphate solubilization

Higher fungal populations were found in rhizospheric region of Nsukka pepper plant when compared with non-rhizosphere soil (Table 2) and may contribute to its solubilization effect. Quantitative assays conducted on efficiency of the phosphate solubilizing fungi were based on the lowering of pH, owing to production of organic acids into the surrounding medium. The highest amount of insoluble phosphate solubilized was found to be 4.12 mg/ml by isolate PF7 (cell-size 1.74×10^8), followed by 4.06 mg/ml solubilized by isolate PF2 (cell-size1.34×10^8) and 4.04 mg/ml solubilized by isolate PF6 (cell-size 9.80×10^7), whereas the least amount of insoluble phosphate solubilized was 0.28 mg/ml by NF20 (cell-size1.54×10^3).

Effect of solubilization with time

As shown in Figure 1, none of the isolates was able to solubilize insoluble phosphate on the day of inoculation (day zero) because no metabolites have been produced, but as the growth phase entered its exponential phase, they utilize the available nutrient thereby excreting metabolites (eg. organic acid) for insoluble phosphate solubilization. When compared with Figure 2, decrease in pH increased the solubilization, with the isolates highest solubilization of insoluble phosphate on the 12^{th} day of incubation.

Reduction in pH, as a result of total organic acid produced by PSF

Increases in titrable acidity were observed to correlate with reduction in pH of the medium as shown in Figure 2. Results indicated that all the fungi isolates that produced organic acid also solubilizes insoluble phosphate and quantity solubilized is commensurate to organic acid produced. These pH reductions are due to secretion of organic acids excreted by PSF (Sharma, 2012).

DISCUSSION

The result obtained in Table 1 highlights the existence of phosphate solubilizing fungi in the rhizospheric soils of Nsukka pepper plants.

Baby et al. (2001) sited by Sharma et al. (2012) carried out an investigation on microbial dynamics in the rhizosphere of tea plants and reported that there were significant difference on the population level of PSB in different seedlings of tea. Higher PSB populations were found in rhizosphere other than non-rhizosphere soil (Qurban et al., 2012) and Lynch (1986) in his work also observed a high percentage of PSB population concentrates in the rhizosphere of plants. All these findings supported our work, which explained the high microbial level in the rhizospheric region of Nsukka pepper plants.

The production of halos around the colony of the organism is an indication of the presence of phosphate solubilizing organisms. Owing to confirmation on agar solubilization, NBRIP broth modification medium were used, where BPB produces a blue-colored dye that decolorizes to light yellow, due to a drop in pH of the medium. In the course of our work, NBRIP-BPB broth medium at pH 7, dropped to pH 2.42 by the action of PF7 isolate, which showed the maximum phosphate solubilization of 4.12 mg/ml while NF20 isolate dropped slightly to pH 6.25 showing the least phosphate solubilization of 0.28 mg/ml, although the NF-isolates did not give any detectable solubilization zone (halos) in plate PVK agar assay.

Laboratory study reviewed by Kucey et al. (1989) and sited by Ramachandran et al. (2003) have shown that the microbial solubilization of insoluble phosphate in liquid medium has been due to the excretion of diffusible organic acids as a result of which a decrease in pH were obtained. This is consistent with our work where, all the PF isolates, NF11 and NF14 were able to produce organic acids that solubilize insoluble phosphate. But contradictory results were produced when isolates NF17, NF18, NF19 and NF20 slightly solubilizes insoluble phosphate in NBRIP broth without organic acid production. The slight variation in some NF-isolates' phosphate solubilization without organic acid production, may be as a result of phosphatase activity that directly affects the decomposition of soil organic and inorganic insoluble phosphate (DaWei et al., 2011). The solubilization strength of the isolates might be attributed to microbial population size, pH and soil enzyme activity Ponmurugan and Gopi, 2006).

The results that were obtained in this study suggested the existence of phosphate solubilizing fungi in the rhizospheric region of Nsukka pepper plants, which might be organism to produce more organic acid, thereby decreasing the pH of the medium to an extent that causes solubilization of insoluble phosphate. Varsha et al. (2010) reported that the production of organic acids by some fungi brings about the drop in pH and dissociation of insoluble phosphate, termed phosphate solubilization Figure 2 is consistent with Pradhan and Sukla (2005) and

Table 1. Phosphate solubilization efficiency using PVK agar.

Isolate	Solubilization diameter [SD] (mm)	Growth diameter [GD] (mm)	Solubilization efficiency [SE]
Control	0.0	0	0.0
PF2	66.0	48.0	137.5
PF3	43.0	32.0	134.4
PF4	43.0	35.0	122.9
PF5	24.0	22.0	109.1
PF6	41.0	31.0	132.3
PF7	60.0	25.0	240.0
NF11	0.0	16.0	0.0
NF14	0.0	11.0	0.0
NFI7	0.0	13.0	0.0
NF18	0.0	06.0	0.0
NF19	0.0	15.0	0.0
NF20	0.0	14.0	0.0

Table 2. Comparative analysis of cell numbers to solubilization.

Isolate	Solubilization efficiency	Cell numbers (cell/ml/)	pH value	Quantity solubilized (mg/ml)
Control	0.0	0.0	7.00	0.0000
PF2	137.5	$1.34x10^8$	2.42	4.0587
PF3	134.4	$6.41x10^7$	2.57	3.6897
PF4	122.9	$7.10x10^7$	3.60	3.6933
PF5	109.1	$3.50x10^7$	4.17	3.1067
PF6	132.3	$9.80x10^7$	2.46	4.0433
PF7	240.0	$1.74x10^8$	2.42	4.1233
NF11	0.0	$4.22x10^4$	4.75	1.4433
NF14	0.0	$2.63x10^4$	4.24	2.8633
NF17	0.0	$4.61x10^3$	6.80	0.9690
NF18	0.0	$6.20x10^3$	6.02	0.8033
NF19	0.0	$2.21x10^3$	5.97	0.7633
NF20	0.0	$1.54x10^3$	6.25	0.2833

Varsha et al. (2010) where decrease in pH increases quantities of insoluble phosphate solubilized.

The result of the phosphate solubilization activity showed that PF-isolates from Nsukka pepper plant rhizosphere had higher solubilizing activity than the NF-isolates from the non-rhizosphere region. However, there is a positive correlation between phosphate solubilizing capacity and organic acid activity (Figure 2).

Based on the results of this study, Nsukka pepper plant exudates contributes greatly to the conglomeration of phosphate solubilizing fungi as shown in Table 2, and therefore can be suggested as plant booster for phosphate solubilization, making soluble phosphate available for plants uptake and improvement of agricultural sustainability.

ACKNOWLEDGEMENT

The authors are grateful to Director of South East Zonal Biotechnology Centre, University of Nigeria, Nsukka, Prof. C. E. A. Okezie for his support.

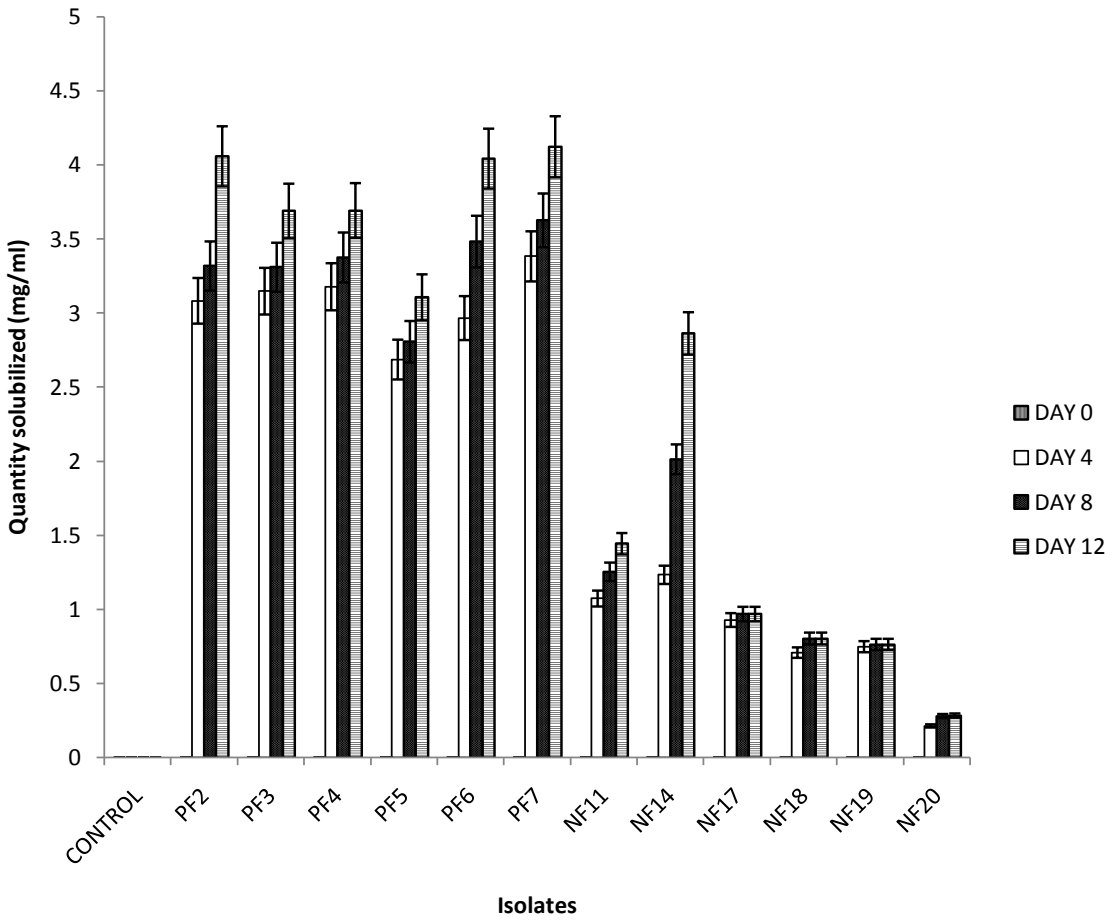

Figure 1. Quantity of phosphate solubilized (mg/ml) with time, using NBRIP-BPB broth.

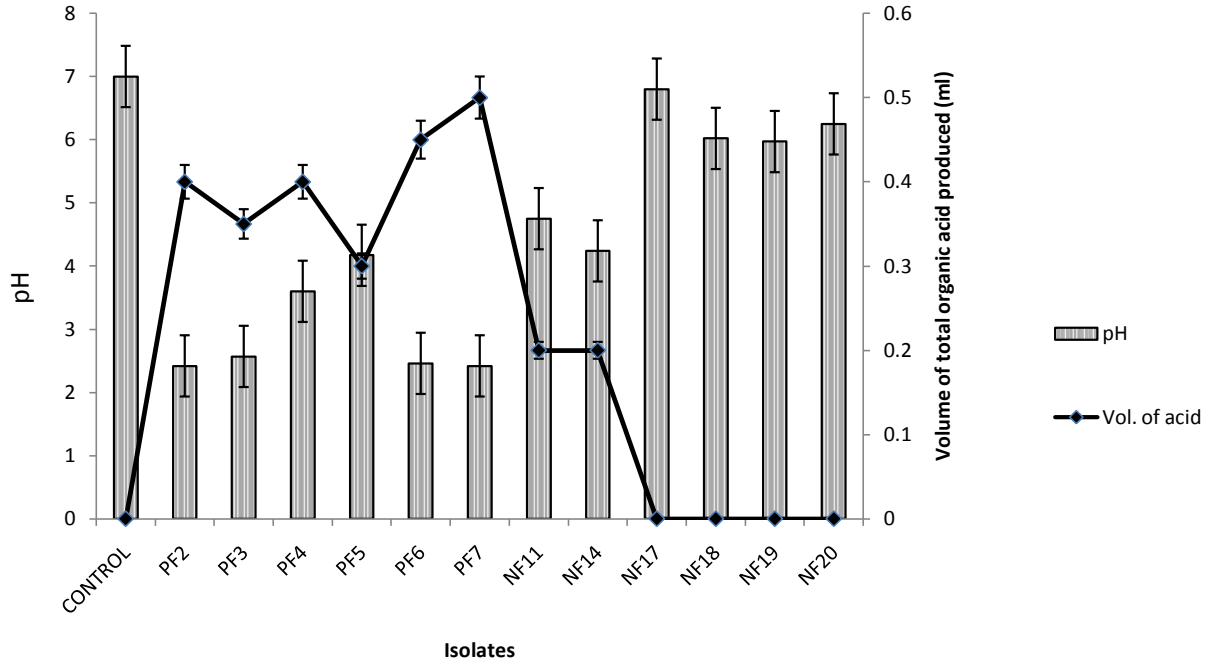

Figure 2. Comparative assay of total organic acids and pH of each isolate.

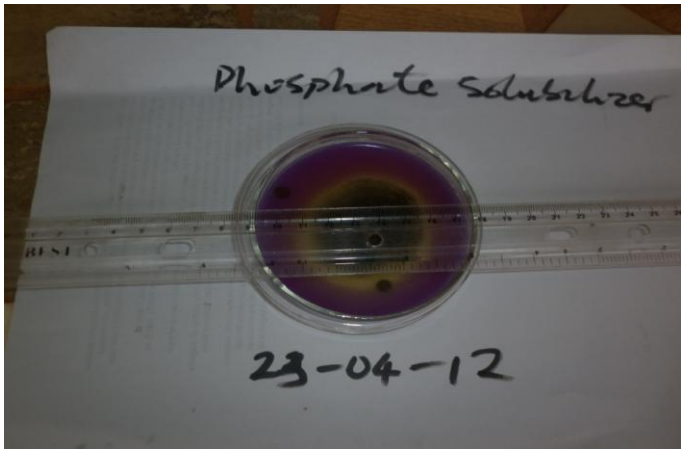

Figure 3. Formation of halo on PVK-BPB'S medium by the PF7 isolate.

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