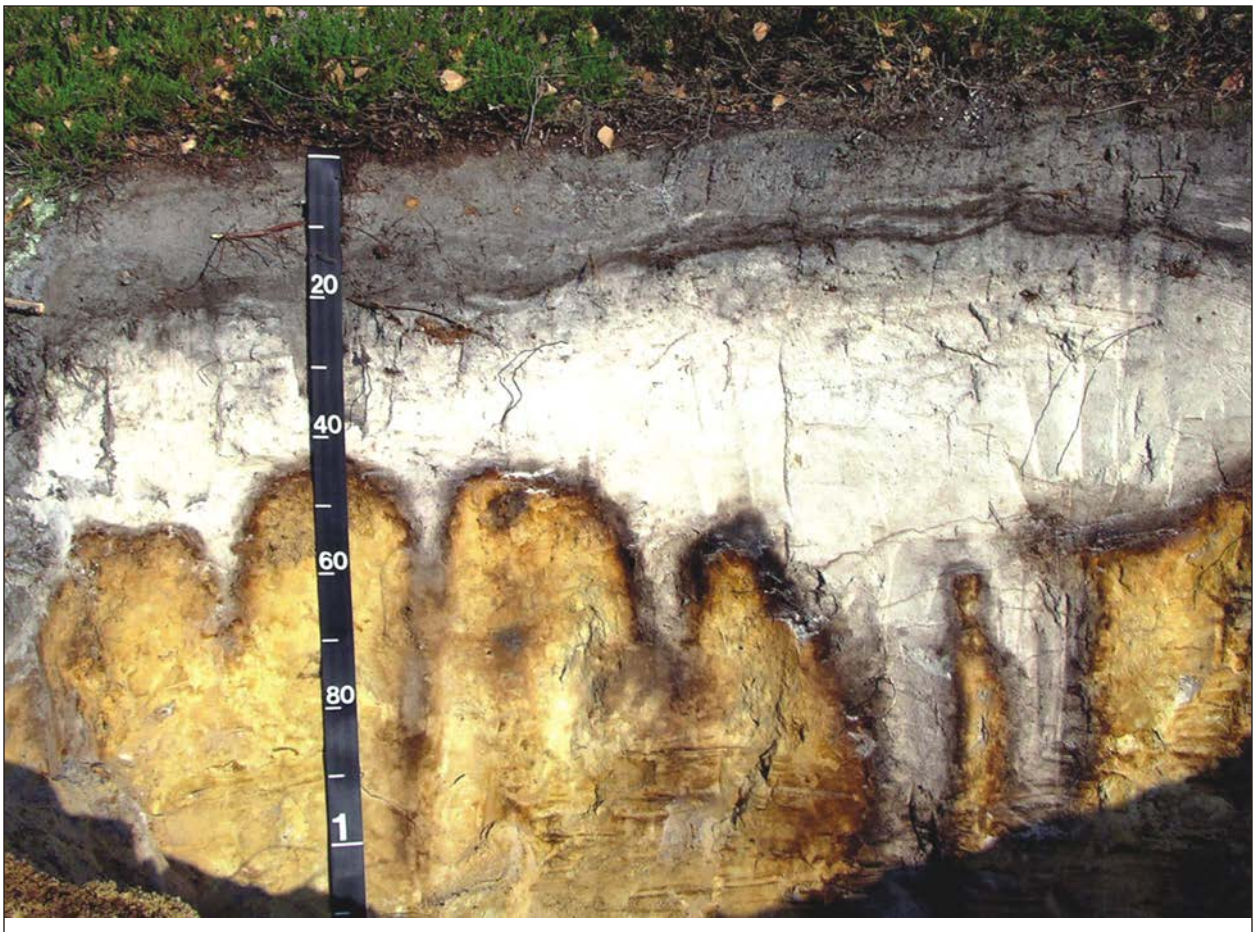

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Solubilization of Insoluble Phosphate by Organic Acid-Producing Fungi Isolated from Nigerian Soil

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Abstract: The ability of thirty-one fungal strains isolated from Nigerian cultivated farmland to solubilize rock phosphate and tri-calcium phosphate (TCP) was investigated. pH, titratable acidity, available phosphorus, total phosphorus and organic acid released were analysed as a measure of solubilization ability in liquid based medium containing rock phosphate and TCP. Isolated fungal species belong mainly to the genera of *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, *Mucor*, *Ovularopsis*, *Tritirachium* and *Geotrichum*. Apart from *Geotrichum*, all the isolates were able to solubilize phosphate rock and TCP. Phosphate solubilization was accompanied by a decrease in the pH of the medium by all the strains; however, this decrease differed significantly among isolates ($p<0.05$). The production of fumaric, acetic, gluconic, lactic and succinic acids accompanied solubilization of TCP, while citric, fumaric, malic and tartaric acid were detected in extracts of phosphate rock medium. Significant differences ($p<0.05$) were observed in the type and amount of organic acids produced by the fungi species *Aspergillus terreus* produced the highest amount of fumaric acid (264.45 mg/100 mL in TCP medium, while *A. niger* produced the highest amount of malic acid (18.20 mg/100 mL) in rock phosphate medium Succinic acid was the least produced of the acids.

Key words: Rock phosphate, solubilization, fungi, organic acids

INTRODUCTION

Apart from nitrogen, phosphorous is one major nutrient required for healthy growth by plants. Phosphorous is naturally present in soil but not all of it is readily available for plant use as some of it is transformed into insoluble complexes with soil constituents and therefore reducing the overall availability and efficiency of soil phosphorous (Vassilev, 2002). As such, in order to maintain the amount of phosphorous available in soil for plant use, large amount of phosphorous based fertilizer is added to soil (Omar, 1998), often, the bulk of which could also be converted to insoluble form. There is therefore a need for frequent application of soluble forms of inorganic phosphorous to soil. In Nigeria, inorganic fertilizer is not within the reach of peasant farmers as they are very expensive. In view of the recent interest in environmental friendly approach to agricultural techniques, more attention is being given to bio sourcing for plant nutrients and the use of rock phosphate as a source of phosphorous for plants is receiving attention.

Natural rock phosphate has been reported to be a source of phosphorous in a wide range of soil types (Chien and Meon, 1995). The rock phosphate has to be dissolved to be available for plant use. Many soil microorganisms have been reported to solubilize inorganic phosphates (Asea *et al*., 1988; Illmer and Schimer, 1992; Richardson, 2001; Nahas, 2004; Chuang *et al*., 2006; Alikhani *et al*., 2006).

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In fact, rock phosphate dissolution by microorganisms directly affects soil fertility (Reyes *et al*., 2002). Solubilization of inorganic phosphate by microorganisms involve a wide range of processes involving the secretion of organic acids, lowering of pH as a result of acid production, ion chelation and exchange reactions (Molla and Chowdhary, 1984) which are considered to be part of the phosphorous cycle.

Several species of microorganisms isolated from soil particularly; fungi, bacteria and yeasts and actinomycetes have been isolated and studied for their phosphorous solubilization abilities in vitro (Whitelaw, 2000). Species of fungi particularly *Aspergillus*, *Penicillum* and some yeasts have been reported to be involved in the solubilization of inorganic phosphates. These fungal species are capable of producing citric acid and form non-ionizable association with calcium. These fungal species have been reported to possess better solubilizing abilities than bacteria (Nahas, 1996).

In practical terms, application of phosphorous solubilization microorganisms in the field has been reported to increase crop yield (Akinrinde *et al*., 2003).

Although studies on solubilization of inorganic phosphates have been reported for different soil types in different parts of the world, there are no reports on soil microorganisms native to Nigerian soils. This study was therefore carried out to investigate fungal species isolated from soils in south western Nigeria capable of solubilizing insoluble phosphates.

MATERIALS AND METHODS

Phosphate Sources

Ogun phosphate rock used in this study was collected from an uncultivated farmland in Oja-Odan, Ogun State, southwestern Nigeria, while tri-calcium phosphate ($Ca_3(PO_4)_5$) was obtained from the soil science laboratory of the University of Agriculture, Abeokuta, Nigeria. Ogun rock phosphate was pulverized into fine powder and further sieved in a 63 mm mesh to remove coarse particles.

Isolation of Soil Fungi

Fungal species used for the experiment were isolated from Teaching and Research farms of the University of Agriculture, Abeokuta after serial dilution of soil solution; pour plate technique was used for the isolation using a basal medium comprising of 10 g glucose, 0.5 g $(NH_4)_2SO_4$; 0.2 g NaCl; 0.1 g $MgSO_4$, $7H_2O$; 0.2 g KCl; 0.5 g yeast extract; 0.2 g $FeSO_4.7H_2O$; 3 g $Ca_3(PO_4)_2$; 0.2 g $MnSO_4$ and 15 g agar in 1 L distilled water. Plates were incubated at room temperature for 2-5 days. Colonies were purified by repeated sub-culturing; pure cultures were maintained on Potato Dextrose Agar (PDA) slants at 5±2°C. Fungi isolates were identified by their colony characteristics and microscopic features particularly their vegetative and reproductive structures including conidia and sporangia with reference to Bernett (1960) and Gilman (1975). The nine most effective fungi isolates that solubilized phosphorus in both phosphate rock and tri-calcium were used to assay for the organic acids.

In vitro Phosphate Solubilizing Test

The isolates were tested for phosphate solubilizing ability using the basal medium in which the phosphorus source was replaced by 2 g phosphate rock or 10 g tri-calcium phosphate. An aliquot of 100 mL of the basal medium were measured into 250 mL Erlenmeyer flasks. NaOH was added to adjust the pH to 7 prior to sterilization at 121°C for 15 min. The flasks were inoculated with 4 mm mycelial discs of actively growing fungal isolate. Control flasks were not inoculated. The flasks were incubated at 20°C in a shaker incubator for 14 days. After the incubation period, the cultures were harvested by filtration. The filtrates were analysed for pH, titratable acidity (Nahas *et al*., 1990), soluble phosphate (Ames, 1966), total phosphorus (Singh and Amberger, 1991). Organic acids were determined by spectrophotometric method as described by AOAC (1990). The experiments were done in triplicates.

Data Analysis

Anova and Duncan's multiple Range tests were employed for comparing means using SAS version 6.0. (SAS, 1989).

RESULTS

The thirty one fungi species isolated and used for the experiments are mainly species of *Aspergillus*, *Penicillium*, *Mucor*, *Trichoderma*, *Fusarium*, *Ovulariopsis*, *Tritirachium* and *Geotrichum* (Table 1). Compared to the control, pH of inoculated media decreased, this is an indication of acid production, there were however significant differences in the pH recorded for the different fungal species. Differences were also observed on the pH, titratable acidity, soluble phosphorous and total phosphorous in media supplemented with phosphate rock and tri-calcium phosphate of the test isolates (Table 1 and 2). *Fusarium oxysporium* (CL) effected the least pH change (3.0) in phosphate rock medium, while *Penicillium digitatum* effected the least pH change (2.23) in tri-calcium phosphate medium. The titratable acidity recorded in rock phosphate ranged between 0.40-18.30%, while in tri-calcium phosphate it ranged between 0.63-26.73%.

Table 1: Final pH, Titratable Acidity (TA), Soluble Phosphorus (SP) and Total Phosphorus (TP) of the culture media supplemented with rock phosphates and inoculated with fungi isolated from soil

	Phosphate rock			
Fungi isolate	pH	TA (%)	SP (mg/100 mL)	TP (mg/100 mL)
Control	6.9b	0.23r	0.04o	4.50j
Ovulariopsis species (AGL)	3.6t	3.07o	0.50o	5.10h
Ovulariopsis species (GL)	4.58l	2.77o	0.65o	4.50j
Mucor hiemalis	3.03w	6.67m	2.80f	9.00b
Mucor racemosus	5.10g	1.70p	0.80o	6.80c
Aspergillus candidus (RS)	5.34c	1.17p	0.80o	7.00c
Aspergillus candidus (RP1)	4.41m	3.73o	0.00o	2.40n
Aspergillus candidus (RP2)	3.87q	15.70c	4.00c	8.20p
Aspergillus flavus (FL)	3.95q	14.77f	1.80n	8.60i
Aspergillus flavus (RS)	5.01g	3.13q	1.00o	6.25p
Aspergillus flavus (AGL)	3.28o	18.50b	3.50d	6.80c
Aspergillus flavus (RP)	3.30c	4.47m	0.30o	5.30g
Aspergillus niger (RP1)	3.88q	15.70c	4.20c	4.88bc
Aspergillus niger (RP2)	3.14o	16.67c	2.30j	9.95a
Aspergillus wentii	3.55t	15.97b	2.10i	8.20p
Aspergillus flavipes	3.62t	6.83m	0.80o	4.50j
Aspergillus terreus	4.00p	9.70j	2.10i	6.25c
Aspergillus awamori	3.63c	8.30j	1.60n	6.60c
Aspergillus ochraceus	5.18d	3.10o	0.00o	4.90c
Penicillium chemesiniuin	3.79q	8.30k	2.30i	7.00c
Penicillium claviforme	4.39m	13.80g	2.10k	6.40c
Penicillium digitatum (GL)	5.42c	6.73j	0.50o	5.30a
Penicillium digitatum (RS)	4.25n	8.47j	3.30d	6.10d
Trichoderma isridae	4.89h	9.80j	2.00i	9.00b
Trichoderma harzinum	5.08f	3.27o	0.05o	6.40c
Tritirachium species (CL)	4.21n	9.73j	2.10k	7.20c
Tritirachium species (CL)	4.27n	4.67m	2.00m	4.70j
Fusarium udam	4.13a	1.77p	O.00o	5.10h
Fusarium oxysporum (CL)	3.00w	10.23i	3.30e	6.80c
Fusarium oxysporum (GL1)	3.00w	10.23i	3.30e	6.60c
Fusarium oxysporum (GL2)	5.42c	6.73m	0.00o	5.30g
Geotrichum species	6.98b	0.40r	0.00o	5.10h

Values followed by different alphabets within columns are significantly different at $p<0.05$

Table 2: Final pH, Titratable Acidity (TA), Soluble Phosphorus (SP) and Total Phosphorus (TP) of the culture media supplemented with Tri-calcium phosphates (TCP) and inoculated with fungi isolated from soil

	Tri-calcium phosphate			
Fungi isolate	pH	TA (%)	SP (mg/100 mL)	TP (mg/100 mL)
Control	7.06b	0.30r	0.05p	1.90
Ovulariopsis species (AGL)	5.28d	8.00k	2.30j	3.10m
Ovulariopsis species (GL)	5.21d	6.67m	2.10i	3.00n
Mucor hiemalis	3.78r	10.67i	3.30d	5.10h
Mucor racemosus	4.75k	9.67j	3.15e	4.10i
Aspergillus candidus (RS)	4.03o	7.77l	3.15e	4.90i
Aspergillus candidus (RP1)	5.13e	8.77j	0.65o	2.40n
Aspergillus candidus (RP2)	3.37u	18.70b	5.55a	6.40p
Aspergillus flavus (FL)	4.24n	19.77b	3.50d	4.90i
Aspergillus flavus (RS)	4.74k	8.87j	2.00i	2.80p
Aspergillus flavus (AGL)	5.54c	9.63j	0.00o	2.60n
Aspergillus flavus (RP)	4.87l	6.67m	1.60n	3.00n
Aspergillus niger (RP1)	3.33v	26.73a	5.55a	6.25c
Aspergillus niger (RP2)	3.65s	25.70a	3.30d	4.70l
Aspergillus wentii	4.32n	25.17a	3.70d	6.60p
Aspergillus flavipes	4.41m	8.83j	2.65g	2.60n
Aspergillus terreus	4.79j	16.70c	3.30d	4.30k
Aspergillus awamori	5.40c	15.73e	2.30j	2.60n
Aspergillus ochraceus	3.03u	7.83k	1.60n	2.60n
Penicillium chemesiniuin	4.04o	12.73h	2.50h	3.20n
Penicillium claviforme	4.27o	19.60b	3.00f	4.00p
Penicillium digitatum (GL)	2.23o	13.73a	1.60n	4.50j
Penicillium digitatum (RS)	4.23n	11.03l	4.90b	5.90e
Trichoderma isridae	4.23n	20.03b	4.00d	5.10h
Trichoderma harzinum	4.26n	9.10j	2.00m	4.70j
Tritirachium species (CL)	4.13n	14.77f	2.03j	2.40o
Tritirachium species (CL)	5.23u	8.67j	2.50g	2.80n
Fusarium udam	4.48l	6.73m	2.30j	3.00n
Fusarium oxysporum (CL)	3.26v	17.80b	4.90b	5.90f
Fusarium oxysporum (GL1)	3.26v	17.83b	4.90b	5.90f
Fusarium oxysporum (GL2)	4.27n	13.73g	1.60n	4.50j
Geotrichum species	6.90b	0.63r	0.00o	5.10h

Values followed by different alphabets within columns are significantly different at $p<0.05$

Table 3: Organic acid produced by the most solubilized soil fungi in basal medium modified with Phosphate rock (R) at 14 days of incubation

Fungi isolate		Acetic acid (mg/100 mL)	Citric acid (mg/100 mL)	Fumaric acid (mg/100 mL)	Gluconic acid (mg/100 mL)	Glutaric acid (mg/100 mL)
Control	R	0.00k	0.05i	1.78a	0.00m	0.20e
Aspergillus flavus	R	4.82g	19.32g	19.00a	12.50l	5.00d
Aspergillus candidus	R	1.85j	27.05f	22.78a	28.75h	7.00b
Aspergillus niger	R	6.30f	57.96c	25.00a	20.00i	0.00e
Aspergillus terreus	R	5.56g	54.09c	28.89a	033.75g	0.00e
Aspergillus wentii	R	5.93f	69.80a	26.67a	17.50j	0.00e
Fusarium oxysporum	R	3.34i	50.23d	18.34a	26.25h	0.00e
Penicillium chernestinum	R	4.10h	34.78e	21.11a	15.00k	0.00e
Trichoderma isridae	R	7.41e	11.59h	16.11a	18.75j	0.00e
Tritirachum species	R	11.85b	54.09c	25.56a	22.50i	0.00e

Fungi isolate		Lactic acid (mg/100 mL)	Maleic acid (mg/100 mL)	Malic acid (mg/100 mL)	Succinic acid (mg/100 mL)	Tartaric acid (mg/100 mL)
Control	R	0.00g	0.05m	0.15p	0.00i	0.35n
Aspergillus flavus	R	3.26e	97.18f	75.00h	0.00i	22.12b
Aspergillus candidus	R	2.83e	128.60a	130.30b	0.00i	13.46f
Aspergillus niger	R	4.35d	111.40d	138.20a	0.00i	24.04a
Aspergillus terreus	R	3.91d	105.70e	106.60c	0.00i	11.54h
Aspergillus wentii	R	3.05e	114.30c	102.60e	0.00i	25.96a
Fusarium oxysporum	R	2.58f	120.00b	110.60c	0.00i	20.19c
Penicillium chernestinum	R	3.70d	102.90e	98.69f	0.00i	17.30d
Trichoderma isridae	R	3.26e	82.86h	82.86g	0.00i	16.35e
Tritirachum species	R	4.57d	117.20b	122.20c	0.00i	12.50g

`Values followed by different alphabet within columns are significantly different at $p< 0.05$

Results of the study showed that soluble phosphate levels in culture medium was higher in medium containing tri-calcium (1.6-5.55 mg 100 mL^{-1}) compared to rock phosphate medium (0.30-4.20 mg 100 mL^{-1}. Some of the fungi species did not release soluble phosphorous rather; they utilized available phosphate. There were significant differences in the organic acids produced by the fungal species in the modified media. More acetic acids, gluconic, glutaric, lactic and succinic acids were produced by the fungi in tri-calcium modified medium, while more citric, fumaric, maleic, malic and tartaric acids were produced by the fungal species in phosphate rock medium. *Aspergillus niger* produced the highest malic acid (138.20 mg 100 mL^{-1}) in phosphate rock medium and *Aspergillus terreus* produced the highest fumaric acid (264.45 mg 100 mL^{-1}) in tri-calcium medium (Table 3).

DISCUSSION

With the exception of *Geotricum* sp. all the fungal isolates tested were able to solubilize phosphate rock and tri-calcium phosphate *in vitro*. Kucey and Paul (1982) recognised and reported the ability of several species of *Aspergillus* and *Penicillium* to solubilize insoluble phosphate. However, it is pertinent to note that other soil microorganisms are capable of solubilizing insoluble soil phosphate. In addition to some bacteria species (Nahas, 2006), soil chytrids have been reported to possess solubilizing ability (Midgley *et al*., 2006). Also, Rhizobia were reported to possess the ability to solubilize soil phosphate (Alikhani *et al*., 2006)

Significant decrease in pH accompanied the release of phosphorous from TCP in culture supernatants. This confirms the implication of organic acid production in phosphorous solubilization by fungi. The increase observed in titratable acid is responsible for the observed decrease in pH. This effect was pronounced in media supplemented with tri-calcium phosphate. This is an indication that solubilization was more on tri-calcium phosphate medium.

Observations on the type of organic acids produced by the nine selected fungal isolates (Table 4) showed that more acids were produced in tri-calcium medium than in medium supplemented with phosphate rock. Succinic acid was not produced by fungal isolates on phosphate based medium

Table 4: Organic acid produced by the most solubilized soil fungi in basal medium modified with tri-calcium Phosphate (TCP) at 14 days of incubation

Fungi isolate		Acetic acid (mg/100 mL)	Citric acid (mg/100 mL)	Fumaric acid (mg/100 mL)	Gluconic acid (mg/100 mL)	Glutaric acid (mg/100 mL)
Control	C	0.00k	0.00j	1.11a	0.00m	0.00e
Aspergillus flavus	C	10.74c	0.00j	12.78a	36.50f	0.00e
Aspergillus candidus	C	15.56a	0.00j	13.89a	36.50f	7.00b
Aspergillus niger	C	8.52d	0.00j	13.34a	46.25c	4.67d
Aspergillus terreus	C	13.71a	0.00j	264.45a	47.50c	8.00a
Aspergillus wentii	C	7.04f	0.00j	9.45a	51.25b	9.00a
Fusarium oxysporum	C	12.60b	7.73i	11.67a	43.75d	7.67a
Penicillium chernestinum	C	5.93f	0.00j	10.56a	40.00e	5.67c
Trichoderma isridae	C	10.00c	0.00j	8.34a	38.75e	4.34d
Tritirachum species	C	11.85b	0.00j	15.56a	56.25a	0.00e

Fungi isolate		Lactic acid (mg/100 mL)	Maleic acid (mg/100 mL)	Malic acid (mg/100 mL)	Succinic acid (mg/100 mL)	Tartaric acid (mg/100 mL)
Control	C	0.10g	0.00m	0.00p	0.00i	0.00n
Aspergillus flavus	C	5.00d	54.30j	51.32m	0.14g	15.39e
Aspergillus candidus	C	4.36d	51.43j	55.27l	0.19f	10.58h
Aspergillus niger	C	7.39b	48.57k	82.90g	0.48a	4.82l
Aspergillus terreus	C	5.87c	65.72i	71.06h	0.34c	5.77k
Aspergillus wentii	C	9.13b	88.57g	67.11i	0.40b	8.66i
Fusarium oxysporum	C	4.57d	62.86i	63.16j	0.38b	8.66i
Penicillium chernestinum	C	11.31a	42.86l	47.37n	0.29d	7.69j
Trichoderma isridae	C	2.83e	40.00l	59.21k	0.10i	2.89m
Tritirachum species	C	3.06e	54.29j	35.55o	0.25e	5.77k

Values followed by different alphabets within columns are significantly different at $p< 0.05$

but some amount of the acid was produced on tri-calcium based medium. Perhaps this is an indication that solubilizing ability may have a relationship with the type of organic acids produced by the fungi species rather than the quality of acid (Singh and Amberger, 1998).

In view of the fact that natural soil environment is more complex than *in vitro* setting, it is possible that several other factors may be at play in determining solubilizing ability of a fungi species. Pradhan and Sukla (2005) observed diverse levels of phosphate solubilization activity in the presence of various carbon and nitrogen sources.

Generally, within organisms a significant strain effect was observed, indicating that the ability of strains to solubilize phosphate varies significantly. Based on observations in this study a relationship seems to be established between the pH values, titratable acidity, the type of fungi species and solubilizing ability. This may imply that these parameters constitute the factors that are likely to affect solubilization of insoluble phosphates by Nigerian soil fungi.

As earlier reports have established that calcium in soil are dissolved by acidification. It is possible that strains of fungi that exhibit good ability to acidify its external medium as observed in this study will show some degree of phosphorous solubilization.

In conclusion, results of this study have shown that several naturally occurring fungi species isolated from Nigerian soil are capable of producing organic acids that aid the solubilization of insoluble phosphates. As this is a preliminary report, investigations are on going on other details of the solubilizing ability of the fungal species.

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