Plant Archives Vol. 16 No. 2, 2016 pp. 925-930 *ISSN 0972-5210*

PHOSPHATE SOLUBILISING AND INDOLE ACETIC ACID PRODUCING POTENTIAL OF MYCOFLORA ASSOCIATED WITH TRADITIONAL LIVESTOCK MANURE IN INDIAN HIMALAYAN REGION

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Abstract

Livestock manure is a key component of hill agro-ecosystem, especially farmers holding low lands of Indian Himalayan region. Manure is prepared from the bovine excreta mixed with bedding material and collected outside the house in the form of a heap. In the present investigation, we isolated the dominant plant growth promoting fungal genera during the decomposition process of Livestock manure and assayed for their Plant growth promoting potential (PGPP) *viz*. Phosphate solubilization and Indole acetic acid (IAA) production. In order to get isolation of fungi from manure, a 180 days experiment was conducted using approximately 1.5t Livestock manure, near Chauras Campus of HNB Garhwal University (560 amsl).

A total of 8 dominant fungi belonging to 2 genera (*Aspergillus* and *Penicillium*) were tested for their *in-vitro* Phosphate solubilization (Solubilization index in solid medium and spectrophotometrically in broth medium) using pikovskaya medium containing tri-calcium phosphate (TCP) as a sole phosphorus source and for Indole production efficiency. The highest amount of phosphate solubilization showed by *Aspergillus niger* after 6 days of incubation period (280 µg/ml) followed by *Penicillium citrinum* (228 µg/ml) and *Penicillium* sp.1 (205 µg/ml) with corresponding pH value of 3.1, 4.3 and 4.8 respectively at an incubation temperature of 30±2°C. IAA production estimated maximum by *A. niger* (82 µg/ml) followed by *A.* sp.1 (73 µg/ml) and *A. flavus* (76 µg/ml), while least value of indole production was reflected by *P. Funiculosum* (42 µg/ml) at incubation temperature of 30±2°C.

Key words : PGPP, IAA, solubilization index, Tricalcium phosphate, Spectrophotometrically.

Introduction

In the past few decades, the traditional knowledge and practices of farming have almost been eroded from many parts of India due to influx of modern technologies (Gopinath *et al*., 2009). However, for farmers (especially small and marginal) in Indian Himalaya, the purchase of manufactured fertilizers and pesticides is and will continue to be constrained by their high costs and unavailability. Furthermore, the use of locally available natural resources and traditional knowledge of farmers are far more likely to meet the needs and aspirations of resource-poor farmers than those that require costly external inputs (Parrott *et al*., 2006). In this traditional method of manure preparation, bovine excreta mixed with bedding material (Forest litter) is collected and dumped outside the house in the form of a heap. It not only maintains the quality of soil but crop production is also better in terms of quality and quantity subject to other climatic factors. The open heap system vary somewhat to close pit compost system in terms of aeration, temperature, pH and moisture etc, which invite both aerobic as well as anaerobic microbes to take part in decomposition process (Ivon *et al*., 2007). This leads to the higher microbial diversity of bacteria and fungi during the decomposition process. The addition of Livestock manure enhances the density of microorganisms in soil due to the high load of residential microflora (Saison *et al*., 2006), which are subsequently responsible for the higher nutrient bio-transformation.

Phosphorus is the most important element in the nutrition of plants, next to nitrogen (N) which limits the agricultural production. Although, P is abundant in soils in both inorganic and organic forms, it is a major limiting factor for plant growth as it is in an unavailable form for

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root uptake. The application of P in soil as a fertilizer is utilized by plants (1%) and rest is rapidly converted into insoluble complexes (Calcium Phosphate, Iron phosphate, Aluminum phosphate etc.) in soil. This loss of P in soil leads to the need of frequent P application as P-fertilizers, but the use of regular basis has become costly and environmentally undesirable. Therefore, composted organic matter is an important reservoir of immobilized P that accounts for 20–80% of P in soils (Richardson, 1994). Only 0.1% of the total P exists in available form for plant uptake (Zhou *et al*., 1992) because of its fixation into an unavailable form. Fungi are the more potent solubilizer of inorganic phosphate than bacteria through the production of strong organic acids. Filamentous fungi are generally used as a producer of organic acids (*Aspergillus* and *Penicillium*) all over the world and also tested for the solubilization of inorganic phosphate (Richa *et al*., 2007).

Microorganisms (bacteria and fungi) and plants are able to produce IAA (Gopinathan *et al*., 1992; Jameson, 2000; Reineke *et al*., 2008). The role of microbial mediated IAA production in plant-microbe interactions has recently received increasing attention (Spaepen *et al*., 2011). Diverse soil microorganisms including bacteria, fungi and algae are capable of producing physiologically active quantities of auxins, which may exert pronounced effects on plant growth and establishment (Stein *et al*., 1990). Microorganism used plant exudates (*i.e* Tryptophan) which are secreted into the soil as a precursor molecule for the biosynthesis of growth hormone IAA (Kamilova *et al*., 2006).

Use of composted livestock manure in agriculture practices has gained popularity in recent decades as public concern over the environmental impact of synthetic inputs in agriculture has increased. Organic manure application has been associated not only with improvements of physical and chemical properties of soil but various biological parameters *i.e* soil enzymatic activity, microbial mediated nutrient transformation, are also affected by it**.** With an emphasis on the mycoflora of Livestock manure, this study was aimed to investigate the P-solubilizing and Indole acetic acid producing efficiency of fungi associated with livestock manure during decomposition process, under laboratory conditions.

Materials and Methods

Manure preparation and sample collection

Traditional method of manure preparation was followed for this study; a heap was formed using 1.5t livestock manure. Composite samples were collected periodically from different depths of manure heap at an intervals of 30 days up to 180 days. 10 gm of manure sample aseptically transferred to 250 ml Erlenmeyer flask using 90 ml sterile double distilled water and hand shaken thoroughly for about 15-20 minute. Serial dilution was prepared as described by Timonin (1940). 1 ml of sample was poured into each of media using Potato dextrose agar media (Potatoes, infusion from 200 gm; Dextrose 20 gm; Agar 15 gm per 1000ml of distilled water; pH 5.8), Czaper-dox media (Sucrose 30 gm; Sodium nitrate 2 gm; Dipotassium phosphate 1 gm; Magnesium sulphate 0.50 gm; Potassium chloride 0.50 gm; Ferrous sulphate 0.01 gm; Agar 15 gm per 1000ml of distilled water; pH 6) and Pikovskaya media. Plates were incubated for 5-7 days at 30±2°C and pure culture was maintained on the basis of colony color, growth, morphology etc for further analysis.

Identification of fungal isolates was done with the help of conventional techniques as described in various literature *i.e* A Manual of soil fungi-by Gilman *et al*. (1956), Illustrated Genera of Imperfect Fungi- by Barnet *et al*. (1972), Monographic contribution on Trichoderma- by Nagamani *et al*. (2002). The Genus Aspergillus, by Raper and Fennel (1973).

Analysis of plant growth promoting activity

Phosphate solubilization

For qualitative estimation of phosphate solubilization, sterilized Pikovskaya's medium was poured into petri plates and pin point inoculation was made under aseptic condition. The plates were incubated at 30±2°C for 6 to 8 days. Phosphate solubilizing efficiency of isolates were calculated as solubilizing index using formula, SI= Colony diameter + Halozone diameter / colony diameter (Edi *et al*., 1996).

For quantitative analysis of Phosphate solubilization, fungus culture was transferred to Pikovskaya's broth medium and incubated for 2, 4 and 6 days with continuous shaking (150 rpm) at 30°C. A 10 ml broth culture was transferred to centrifuge tube and centrifuged at 8000 rpm for 15 to 20 minute. Phosphorus in solution was extracted by AB-DTPA method (Soultanpour and Workman, 1979). Available P in broth was analyzed by Ascorbic acid method (Watanabe and Olsen, 1965). 1 ml of extracted broth was taken in 50 ml flask and 9 ml of distilled water + 2.5 ml of prepared color reagent (12 gm ammonium molybdate + 250 ml distilled water and 0.29 mg antimony potassium tartrate in 1000 ml of 5N H_2SO_4. Both the solution were mixed and volume was raised up to 2 L. 140 ml of this mixture was added to 0.74 gm ascorbic acid and shaken gently. The optical density due

to blue color was measured after 20 minute at 880 nm by spectrophotometer and the corresponding concentration of available P was calculated against standard phosphorus curve (KH_2PO_4).

pH change

Fungal culture were inoculated in Pikovskaya's broth medium in 250 ml flask and incubated at 30ºC for 2, 4 and 6 days. A change in medium pH was determined by digital pH meter after 2, 4 and 6 days of incubation period.

Indole acetic acid (IAA) production

The quantitative estimation of IAA in Czapeck-dox broth was analyzed by the method suggested by Bric *et al.* (1991). Fungal mycelia were inoculated in 100 ml Czapek-dox medium amended with 1000 µg/ml L-tryptophan and incubate at 30°C on shaker. After an interval of 2 days, 5 ml of culture was centrifuged at 8000 rpm for 15 minute. 1 ml of supernatant was mixed with 2 ml of salkowski's reagent (2% 0.5 M $FeCl_3$ in 35% Percloric acid). This mixture is left for 20 minute at room temperature and absorbance was measured at 540 nm spectrophotometrically. A standard curve was drawn for comparison to determine IAA production by isolate.

Results

Samples of manure were collected from developing manure heap, and isolation of fungi was done by serial dilution method on selective medium. The phosphate solubilizing and Indole acetic acid producing fungi screened out from isolated fungal flora for further quantitative analysis of plant growth promoting activities. A total of 8 fungal species were identified with the help of using conventional techniques *i.e* Macroscopic (Colony morphology, color, growth pattern) and Microscopic characters (Reproductive structures, mycelial organization) described in various literatures.

After the pin point inoculation of pure culture into Pikovskaya's medium containing tricalcium phosphate, all strains produced halozone around the colonies, indicating the solubilization of phosphate source used. Solubilization index (SI) was measured on the basis of colony diameter and halozone formed by fungi is depicted in table 1. Results showed that among all the isolates, highest SI value exhibited by *A. niger* (2.3) followed by *P. citrinum* (1.7) and *P. funiculosum* (1.6) after 6 days of incubation period at growing temperature of 30±2°C. The least value of phosphate solubilization after 6 days of incubation period, in terms of SI was observed in culture of *Penicillium* sp.2 (1.3), *Aspergillus* sp.1 (1.3) and *Penicillium* sp.3 (1.4).

Similarly, quantitative estimation of phosphate solubilization was done in broth medium containing tricalcium phosphate and the results has been shown in table 2. The results of P-solubilization in liquid medium by fungal strains showed that the *A. niger* (170, 215 and 280 µg/ml) and *P. citrinum* (132, 172 and 228 µg/ml) was the most potent P-solubilizer in broth medium, after 2, 4 and 6 days of incubation period at 30±2°C, respectively. The least observation for P-solubilization was recorded in broth culture of *Penicillium* sp.2 (153 µg/ml) and *Penicillium* sp.3 (167 µg/ml) after 6 days of incubation period. pH of the medium measured simultaneously after an interval of 2 days up to 6 days (Fig.1 to 3), the pattern of pH among all cultures was observed in decreasing trend due to the production of various organic acids (*i.e* Citric, gluconic, oxalic, succinic, fumaric etc.). The lowest pH value has recorded in *A. niger* (5.1, 4.3 and 3.1) culture broth followed by *A.* sp.1 (5.8, 4.7 and 4.1) and *P. citrinum* (5.6, 4.9 and 4.3), while highest value was observed in culture of *P. funiculosum* (6.5, 5.8 and 5.1) followed by *P.* sp.3 (6.4, 5.8 and 4.9) and *P.* sp.1 (6.4, 5.5 and 4.8) after 2, 4 and 6 days of incubation, respectively.

Assay of Plant growth hormone IAA production was done by tryptophan mediated IAA synthesis by fungal strains (table 3). The results of this study demonstrated the highest efficiency of *A. niger* (82 µg/ml) to synthesize IAA under *in-vitro* conditions followed by *A.* sp.1 (73 µg/ml) and *A. flavus* (67 µg/ml). *Aspergillus* was observed with higher efficiency to synthesize IAA as compare to *Penicillium*. The least value of IAA was observed in culture of *P. funiculosum* (42 µg/ml) followed by *P.* sp.2 (46 µg/ml), *P.* sp.1 (53 µg/ml).

Discussion

In the present investigation, dominant P-solubilizing and IAA producing residential mycoflora of decomposing Livestock manure were isolated, in which *A. niger* observed with highest P-solubilzing capability in solid and as well as in liquid medium followed by *P. citrinum* and *P.* sp.1. These findings are in accordance with the findings of Mittel *et al*. (2008) and Yadav *et al*. (2011). The fungi secretes various organic acids such as citric, oxalic, gluconic, succinic, fumaric etc. (Mendes *et al*., 2013; Reyes *et al*., 2001) into their surrounding medium which are responsible for the solubilization of inorganic P to available or soluble form. The maximum P-solubilization index was showed by *A. niger* (2.3) followed by *P. citrinum* (1.7) and *P. funiculosum* (1.6) after 6 days of incubation period. In general, P-solubilization index of fungi increased with the increasing colony diameter with time, however the solubilization pattern of P is somewhat dependent on the types of medium used. Some fungi

Table 1 : Solubilization index of isolated fungal strain from decomposing Livestock manure heap at different time intervals.

Strain	Solubilization Index		
	2 Days	4 Days	6 Days
A. niger	1.0	1.1	2.3
A. flavus	0.7	1.0	1.5
A. sp.1	0.5	0.8	1.3
P. citrinum	0.8	1.2	1.7
P. funiculosum	0.9	1.4	1.6
P. sp.1	1.0	1.3	1.5
P. sp.2	0.9	1.1	1.3
P. sp.3	0.6	0.9	1.4

Table 2 : Phosphate solubilisation efficiency of fungal isolates at different time intervals, isolated from manure heap.

Strains	P-solubilization (µg/ml)/Days		
	2 Days	4Days	6 Days
A. niger	170±5	215±6	280±8
A. flavus	110±3	143±5	177±4
A. sp.1	121±4	164±6	193±6
P. citrinum	132±6	172±4	228±9
P. funiculosum	115±3	183±5	180±5
P. sp.1	126±4	192±6	205±6
P. sp.2	87±3	135±5	153±4
P. sp.3	92±3	120±3	167±8

All values are based on the mean of three replicates.

Table 3 : *in-vitro* Indole acetic acid producing efficiency of isolated strains.

Strain	IAA µg/ml*
A. niger	82±5
A. flavus	67±4
A. sp.1	73±6
P. citrinum	61±5
P. funiculosum	42±4
P. sp.1	53±3
P. sp.2	46±3
P. sp.3	57±4

Values on the basis of three replicates.
*IAA at 1000 µg/ml Tryptophan in broth culture supplemented.

showed higher capability of P-solubilization in broth culture as compared to solid medium, additionally some fungi lost their ability to solubilize P due to repeated sub-culturing (Chabot *et al*., 1993; Nahas, 1996; Kucey *et al*., 1989).

Previous work on P-solubilizing microbes, suggests that the principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid

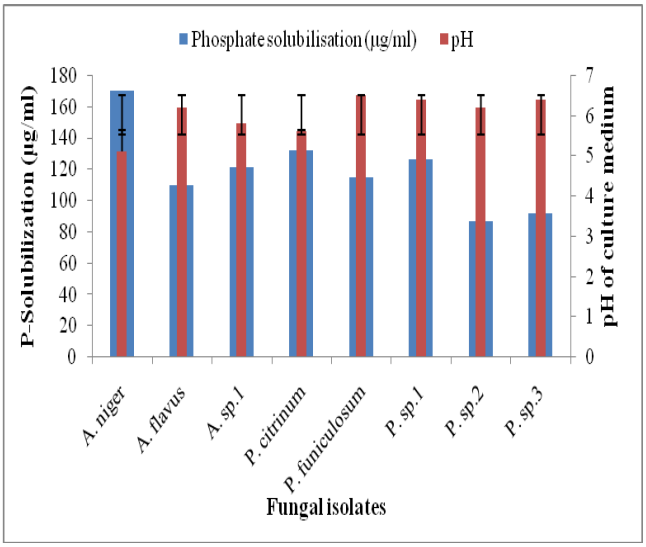

Fig. 1 : Concentration of Soluble-P in culture broth with corresponding pH value after 2 days of incubation (pH of broth culture is 7 initially).

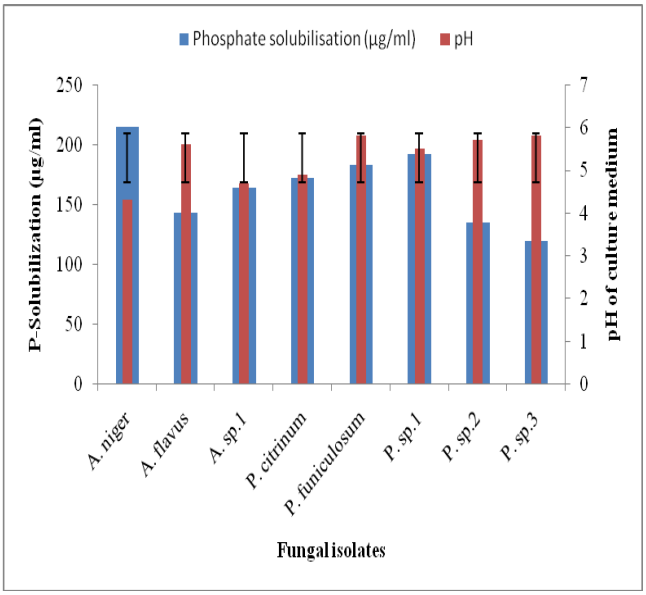

Fig. 2 : Concentration of Soluble-P in culture broth with corresponding pH value after 4 days of incubation (pH of broth culture is 7 initially).

phosphatases by Phosphate solubilizing microbes (PSM), which play a major role in the mineralization of organic phosphorous in soil (Thakuria *et al*., 2004). A high population density of phosphate solubilising bacteria in Livestock compost has been reported by Wickramatilake *et al*. (2011).

Culture filtrate of *A. niger* and *P. citrinum* observed with marked drop in pH values due to the production of strong acids which subsequently solubilize more P. The lowest pH value was recorded in broth culture of *A. niger* (5.1, 4.3 and 3.1) followed by *A.* sp.1 (5.8, 4.7 and 4.1)

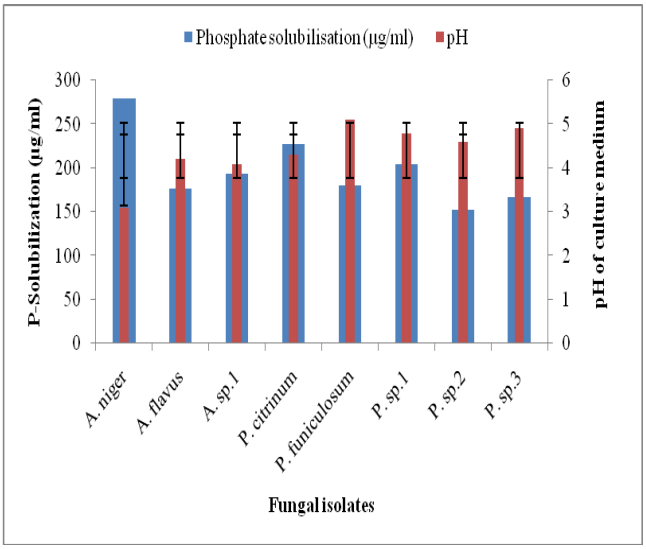

Fig. 3 : Concentration of Soluble-P in culture broth with corresponding pH value after 6 days of incubation (pH of broth culture is 7 initially).

and *P. citrinum* (5.6, 4.9, 4.3) after 2, 4 and 6 days of incubation period, respectively. These findings are consistence with the findings of Mittal *et al.* (2008). pH value of culture directly reflects the concentration or available-P or soluble-P in culture, lower the value of pH, higher will be the amount of solubilized-P and vice-versa. This up to some extend depend upon production of organic acids. Therefore, the less drops in pH value of culture filtrate was observed in *P. funiculosum* (6.5, 5.8 and 5.1) followed by *P.* sp.3 (6.4, 5.8 and 4.9) and *P.*sp.1 (6.4, 5.5 and 4.8) with the corresponding P-solubilization of *P. funiculosum* (115, 183, 180 µg/ml), *P.* ps.3 (92, 120, 167µg/ml), *P.* sp.1 (126, 192, 205 µg/ml) after 2, 4 and 6 days of incubation period.

Tryptophan is a naturally occurring chemical substance released by plants root. Soil microflora synthesize growth hormone with the help of this precursor substance which ultimately stimulate the growth of plants (Kamilova *et al*., 2006). In the present investigation, IAA producing capability of isolated fungi was determined, in which *A. niger* was observed most efficient strain among all the isolates with the production of 82 µg/ml IAA. This is followed by *A.* sp.1 (73 µg/ml) and *A. flavus* (67 µg/ml), furthermore, as compared to *Aspergillus*, *Penicillium* showed less IAA production (Yadav *et al*., 2011). Similar findings were observed in broth culture of *A. niger* supplemented with Tryphtophan with the highest IAA production after 6 days at incubation temperature of 30ºC (Bilkay *et al.,* 2010). The least IAA production was observed in culture filtrate of *P. funiculosum* (42 µg/ml) followed by *P.* sp.2 (46 µg/ml) and *P.* sp.1 (53µg/ml). The production of IAA also depends on the incubation temperature of culture, this was earlier demonstrated by various workers that maximum production of IAA was found at 28-30ºC (Gunasekaran, 1978; Hasan, 2002). The current study clearly demonstrated the importance of livestock manure, which can directly affect the rate of nutrients bio-transformation and enhancement of soil fertility of agricultural land.

Conclusion

The use of livestock manure can greatly affect the amount of microbial mediated availability of phosphorus in soil. Experimental findings reflected the plant growth promoting capability of residential mycoflora of manure under laboratory conditions in terms of Phosphate solubilization and IAA production. The genus *Aspergillus* exhibited efficient P-soulizing and IAA production capability as compared to *Penicillium*. The frequent application of Live-stock manure into soil could be an effective substitute for long term sustainability of soil and for better crop yields.

Acknowledgment

The corresponding author is thankful to Head, Department of Botany and Microbiology, HNB Garhwal University, Srinagar for providing necessary facilities, and Dr. S. K. Saini, Herbarium Division, BSIP Lucknow for culture identification. Financial assistance provided by the UGC, New Delhi to AU is highly acknowledged.

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