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ASPERGILLUS CARBONARIUS AS A POTENTIAL PHOSPHATE SOLUBILIZER

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Abstract – In this study, *Aspergillus carbonarius* was isolated, identified based on morphological and molecular characterization as 18s rRNA sequence and was deposited at Gen Bank, NCBI (MN904861). For the study of Phosphate (P) solubilization capacity of the fungus, different soil samples from different rhizosphere of medicinal plants were collected. Totally 37 P solubilized colonies of fungi were isolated after serial dilution using Pikovskaya's medium and one of the fungus with high P solubilization was selected and tested P solubilization efficiency under laboratory condition. The fungus showed good P solubilization results, Solubilization index (SI) was found 3.54, % Solubilization efficiency (SE) was measured 254, pH of culture filtrate after growth of fungus decreased from 6.89 to 3.6. The colour change from blue to yellow on agar plate and yellow from red in broth reduces to acidic condition due to growth of the fungus. Titrable acidity was found 37.76g/L and estimation of Phosphate in culture broth by Vanado – Molybdate method was found 30µg. The fungus showed positive result for siderophore and Indole Acetic Acid (IAA) production. Due to the phosphate solubilization capacity of the fungus, *A. carbonarius* can be recommended as Phosphate solubilizer in agricultural field.

INTRODUCTION

Next to Nitrogen Phosphorus (P) is one of 17 major plant nutrient, which is required for growth and development of plants and microorganisms. It is necessary to supply through fertilizers in agricultural field, but major portion of applied P gets fixed in the soil and unavailable for growth of plant (Naik *et al*., 2013). Phosphorus shows important role in growth of plant and it is the plant growth limiting nutrient element despite its abundance in the soils both organic forms and inorganic forms. Greater part of the soil, P approximately 95 – 99% is present in insoluble form of phosphates and cannot utilized these insoluble forms by the plant (Naik *et al.,* 2013). This unavailability is due to P-fixation, either it is adsorbed on the soil minerals or get precipitated by free Al^{3+} and Fe^{3+} in soil solutions (Anand *et al.,* 2016). There is a myriad of microorganisms, especially presented phosphate solubilizing microbes in the rhizosphere soil and play a significant role in the insoluble phosphate solubilization in the soil (Anand *et al.,* 2016). The phosphate solubilizing microbes convert these insoluble forms through special mechanisms, they carry out the process of acidification, chelation, exchange reaction (Gulati *et al*., 2010; Chung *et al*., 2005) and organic acids and inorganic acids produced by microbes (Mahamuni, 2012). The microorganisms produced organic and inorganic acids which convert tricalcium phosphate into di- and monobasic phosphates (Walpola and Yoon, 2012) with the net result of an enhanced availability of the element to the plants. The organic acid type produced by microbes and thire amounts differ with different organisunt (Mahamuni, 2012).

Most of the phosphate solubilizing microorganisms (PSM) produce organic acids as their waste products, these acids decreases the pH that is responsible for solubilization of the insoluble phosphates (Nelofer *et al.,* 2016). Both the group of microorganisms such as phosphate solubilizing bacteria (PSB) and phosphate solubilizing fungi (PSF) are equally important to enhance plant growth by means of solubilization mechanism and their acquisition to plant production through the synthesis of organic acids and plant growth

promoting substance (Anand *et al.,* 2016). They are also known to produce amino acid, vitamins, plant growth promoting substance like indole – 3 – acetic acid (IAA) and gibberlic acid which helps in better growth of plants (Nenwani *et al.,* 2010).

The medicinal plants are used as a source of medicine since ancient times, their rhizosphere inhabitant various microbes having greater activity. *Brassica* sp. is known to possess certain bioactive compounds and also have medicinal properties. The fungi have been isolated from the rhizosphere soil of medicinal plant *Brassica* sp. and studied their phosphate solubilization capacity. In this present study, *A. carbonarius* was isolated from rhizosphere soil of medicinal plant with Phosphorus solubilizing capacity. The P solubilizing capacity of *A. carbonarius* was studied by using Pikovskaya's medium containing tricalcium phosphate as P source. *A. carbonarius* produces organic acids which involved in phosphate solubilization mechanism. The fungus therefore, may be used as potential P solubilizer in agricultural field.

MATERIALS AND METHODS

Rhizosphere soil sample collection

The rhizosphere soil samples were collected at 10 – 15cm depth of roots of different medicinal plants around Malnad areas of Shivamogga (D). The collected samples were brought into the laboratory in sterilized polythene covers aseptically and maintained at 4 ^{0}C for further use.

Isolation and Molecular characterization of Phosphate Solubilizing Fungi

About 1g of soil suspended in 9 mL of sterilized 0.84% saline and serially diluted. Then the dilutions were plated on Pikovskaya's agar medium (containig insoluble tricalcium phosphate 5g, Glucose 10g, Ammonium sulphate 0.5g, Potassium chloride 0.2g, $MgSO_4$ $7H_2O$ 0.1g, $MnSO_4$ $7H_20$ trace, Ferrous sulphate trace, Yeast extract 0.5g, agar 20g, pH adjusted to 7.2 and dissolved in 1L of distilled water) and plates were incubated at room temperature for 7 days. Plates after incubation were examined for solubilization zone around fungal colonies, such solubilizing zoned colonies were picked and sub cultured for further use (Jain and Singh, 2015). For the fungal identification microscopic observation was done using standard manuals (Aneja, 2009; Subramanian, 1983; Barnett, 1975; Booth, 1971) and 18s rRNA sequencing was done for molecular identification.

Solubilization Index (SI)

The potential of phosphate solubilization by isolates were checked on the Pikovskaya's agar medium. Solubilization index is based on solubilization zone diameter and colony diameter of phosphate solubilizing fungi was calculated. The solubilization index was calculated after 7 days growth of point inoculation isolates on Pikovskaya's agar plate at room temperature. Solubilization index was determined by using the following formula (Yasser *et al.,* 2014).

$$SI = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

% Solubilization Efficiency (SE)

Solidified Pikovskaya's agar plates were point inoculated with isolates and incubated for 7days at room temperature. Then the % Solubilization Efficiency (SE) was measured using following formula (Saxena *et al.,* 2015; Raj 2014).

$$\% SE = \frac{\text{Solubilization zone}}{\text{Diameter of the colony}} \times 100$$

Measurement of pH

Initially culture filtrates of each PSF were collected and subjected to centrifugation at 1000rpm for 10min for obtaining supernatant. The pH of the culture supernatant was measured by pH meter before inoculation and after the period 7days incubation. Uninoculated broth used as control (Jain and Singh, 2015; Kumari *et al.,* 2010).

Qualitative acid production assay

Qualitative acid production on solid media: Pikovskaya's agar media supplemented with Bromophenol blue indicator and sterilized. The media was poured in to sterile plate. Then these solidified plates were point inoculated with each PSF cultures and incubated for 7 days at room temperature (Chadha *et al.,* 2015).

Qualitative acid production in broth: Sterile Pikovskaya's broth containing Bromo cresol purple indicator was inoculated with PSF culture and incubated for 7 days at room temperature (Khan and Gupta 2015).

Quantitative acid production assay (Titrable acidity)

The production of various organic acids from the

PSF cultures results in acidification of the microbial cell and its surroundings leads to solubilize the insoluble phosphate and the amount acid production was estimated by titration using alkali. Culture supernatant of each PSF cultures were collected by centrifugation at 1000 rpm for 10min. 50 mL of culture supernatant was titrated against 0.1N NaOH solution with few drops of phenolphthalein indicator. The titrable acidity was expressed in g/L (Khan and Gupta, 2015).

Estimation of Phosphate

Culture supernatant from PSF was used to estimate the Phosphate. PSF culture was inoculated in Pikovskaya's broth and incubated for 7 days at rotary shaker of 100rpm. Then culture filtrate was collected and centrifuged at 10000rpm for 30min. Estimation of phosphate in the supernatant by Vanado – molybdate method using vanadomolybdate reagent and it was expressed in µg/mL. The amount of phosphate was calculated from standard curve of KH_2PO_4. Absorbance of the developing yellow colour was measured at 420 nm (Verma and Ekka 2015; Sahoo and Gupta 2014).

Screening of siderophore production by PSF

Siderophore production by microorganisms was screened by using chrome azurol S (CAS) agar medium. The medium contains an iron CAS-HDTMA (Hexadecyltrimethyl ammonium bromide) complex which is blue coloured. The presence of Siderophore is indicated by decolourization of the blue coloured ferric-dye complex, resulting in a yellow to orange halo around the colonies.

About 60.5mg of Chrome azurol S was dissolved in 50ml of distilled water and mixed with 10 mL of Iron solution (1mM Ferric chloride in 10mM Hydrochloric acid). While constantly stirring this solution was slowly added to HDTMA solution (72.9mg of HDTMA dissolved in 40 mL of distilled water) and sterilized. The resultant dark purple liquid was added to sterile Pikovskaya's medium containing without Tricalcium phosphate to make CAS agar. Then the CAS agar plates were spot inoculated with each PSF culture and incubated at room temperature for 7 days.

Estimation of Indole Acetic Acid production by PSF

PSF culture was grown in potato dextrose broth supplemented with Tryptophan (1%). After complete growth, the culture filtrate was collected and at 1000 rpm for 10 min. About 2 mL of supernatant was mixed with 2 drops of orthophosphoric acid and 4 mL of Salkwoski reagent (50 mL of 35% perchloric acid, 1 mL of Ferric chloride solution) and kept for incubation. Development of pink colour after 2h incubation at room temperature indicates indole acetic acid (IAA) production (Nenwani *et al.,* 2010). Concentration of IAA production was estimated by standard graph taking concentration of standard IAA on X – axis and Optical Density (530nm) on Y – axis (Pant and Agrawal, 2014).

RESULTS AND DISCUSSION

Isolation and molecular characterization

Rhizosphere soil samples were collected from different medicinal plants in Malnad areas of Shivamogga (D). Among the soil sample, totally 37 fungal colonies were isolated by serial dilution method using Pikovskaya's medium (Table 1), ten colonies were showed halo zone and one fungal colony was selected with high P solubilization zone. Then the fungal colony was point inoculated on fresh medium to make pure culture of the fungus for further studies of phosphate solubilization. The fungus was identified as *A. carbonarius* based on microscopic observation using standard manual (Fig 1) and molecular characterization 18s rRNA sequencing (Fig. 2) and *A. carbonarius* (MN904861) was deposited at Gen Bank, NCBI. Nelofer *et al.,* (2016) have isolated bacterial and fungal colonies from soil were screened for phosphorus solubilization. One fungal colony was found to be

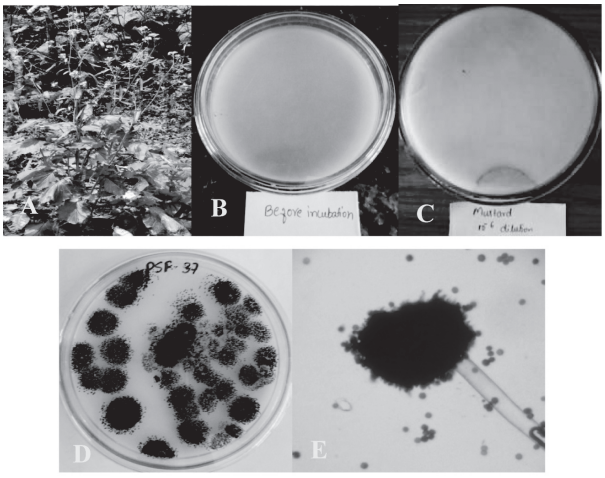

Fig. 1. A. Medicinal plant - *Brassica* sp., B and C: isolation of PSF (before and after incubation), D: *Aspergillus carbonarius* (Culture plate) and E: Microscopic view 40X

Table 1. Isolation of PSF Rhizosphere soil sample from medicinal plants

Sl No.	Plant name	Culture code	Sl No.	Plant name	Culture code
1.	*Datura fastuosa*	PSF 1	19	*Eucalyptus* sp.	PSF 19
2.	*Leucus aspera*	PSF 2	20	*Pongamia glabra*	PSF 20
3.	*Phyllanthus acidus*	PSF 3	21	*Vinca rosea*	PSF 21
4.	*Argemone mexicana*	PSF 4	22	*Ocimum sanctum*	PSF 22
5.	*Achyranthus aspera*	PSF 5	23	*Phyllanthus emblica*	PSF 23
6.	*Centella asiatica*	PSF 6	24	*Amaranthus viridis*	PSF 24
7.	*Asparagus racemosus*	PSF 7	25	*Alternanthera sessilis*	PSF 25
8.	*Gymnema sylvestres*	PSF 8	26	*Euphorbia hirta*	PSF 26
9.	*Tinospora cordifolia*	PSF 9	27	*Euphorbia heterophylla*	PSF 27
10.	*Costus ingneus*	PSF 10	28	*Ixora coccinea*	PSF 28
11.	*Saraca asoca*	PSF 11	29	*Mimosa pudica*	PSF 29
12.	*Calotropis* sp.	PSF 12	30	*Cassia occidentalis*	PSF 30
13.	*Vitex nigundo*	PSF 13	31	*Asclepias curassavica*	PSF 31
14.	*Holorrhena antidysenterica*	PSF 14	32	*Bauhinia purpurea*	PSF 32
15.	*Clitoria ternatea*	PSF 15	33	*Momordica charantia*	PSF 33
16.	*Wrightia tinctoria*	PSF 16	34	*Solanum xanthocarpum*	PSF 34
17.	*Santalum album*	PSF 17	35	*Eclipta prostrata*	PSF 35
				PSF 36	
18.	*Azadirachta indica*	PSF 18	36	*Brassica* sp.	PSF 37

efficient phosphorus solubilizer in Pikovskaya medium. The selected colony was identied as *Aspergillus niger* based on morphological and microscopic studies. Padmavathi (2015) have isolate *Aspergillus niger* from the soil showed extensive solubilization of Tri-calcium phosphate.

CCAGAAGGGCGGGGTCCTTTGGGCCAACC
TCCCACCCGTGTCTATTGTACCTGTTGCTTCG
GCGGGCCCGCCGCTTGTCGGCCGCCGG GGGG
GCATCTCTG CCCCT CGGGCCCGTGCCCGCC
GGAGATACCAACACGAACACTGTCTGAAAT
CGTGAAGTCTGAGTCGATTGTTTTCAATCAGT
TAAAACTTTCAACAATGGATCTCTTGGTTCC
GGCATCGATGAAGAACGCAGCGAAATGCGAT
AACTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAGTCTTTGAACGCACATTGCGCCCCCT
GGTATTCCG

Solubilization Index (SI)

The qualitative analysis of phosphate solubilization potential of *A. carbonarius* was measured *in vitro*. The *A. carbonarius* showed solubilization index (SI) of 3.54, (Fig. 3 and Table 2), this result was correlated

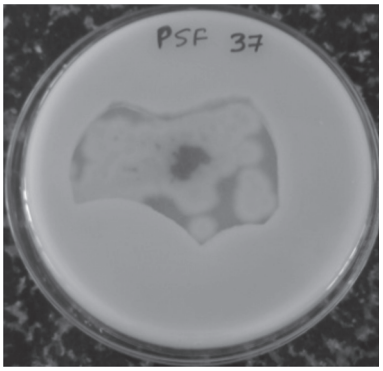

Fig. 3. Solubilization index (SI) of *Aspergillus carbonarius*

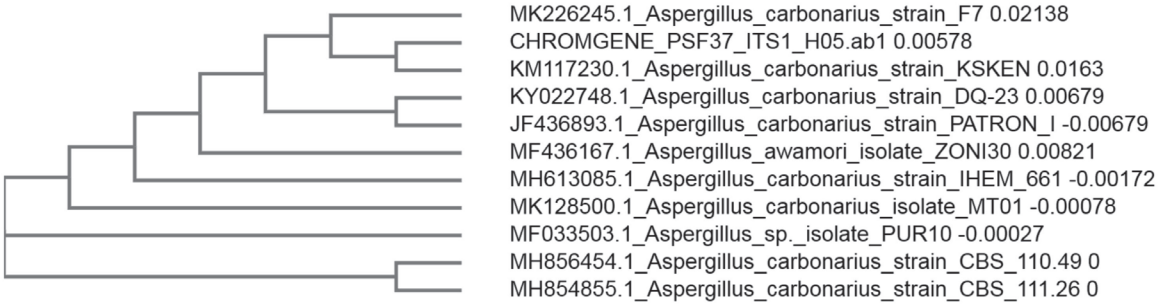

Fig. 2. Molecular characterization of *A. carbonarius* (18s r RNA sequence)

with previous studies of Verma and Ekka (2015) reported SI of 18 fungal culture strains ranged from 1.06 to 3.34, Yasser *et al.* (2014) reported SI of fungal cultures ranging from 1.05 to 1.45, while Mahamuni *et al.* (2012) reported SI fungal culture ranging from 1.13 to 1.59.

% Solubilization Efficiency (SE)

Measurement of % Solubilization efficiency (SE) of the *A. carbonarius* was recorded as 254 (Table 2), this result was correlated with earlier finding of Joseph and Jisha (2008) reported 100 to 575.

Measurement of pH

Decreased pH from 6.89 to 3.6 (Table 2) was recorded in the culture filtrate, after the period of incubation due to production of organic acids. These result was correlated with earlier findings of Yasser *et al.,* (2014) reduced pH recorded 4.80 – 5.4.

Qualitative acid production assay

Due to production of organic acids by the *A. carbonarius* showed reduction of pH in the culture broth was observed. This was observed by using colour indicators, the colour change was observed in the media during the growth of fungus. It showed colour change from blue to yellow on agar plate when using Bromophenal Blue (Fig 4), same result was observed in earlier findings of Chadha *et al.* (2015) and Promwee *et al.* (2014) and in the broth colour change from red to yellow was observed while using Bromocresol purple, same result was observed in earlier findings of Khan and Gupta (2015) (Fig. 4).

Quantitative acid production assay (Titrable acidity)

The measure of amount of acid present in the culture broth was titrated and was recorded 37.76g/L (Table 2), these result was correlated with earlier findings of Balaiah *et al*. (2015) and Reena et al. (2013) were also used 0.1N NaOH as alkali.

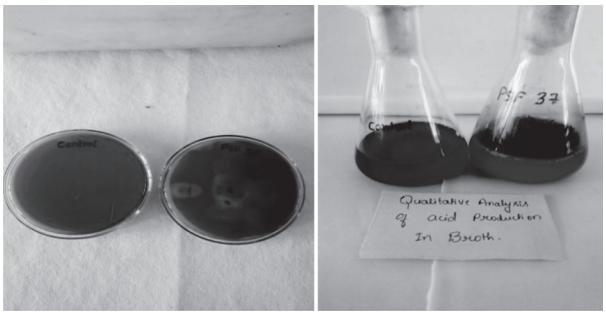

Fig. 4. Qualitative acid production on solid media (a) and in broth (b)

Estimation of Phosphate

The concentration of the phosphate in the broth was determined 30µg (Table 2), result was correlated with earlier findings of Verma and Ekka (2015), Sahoo and Gupta (2014) and Sagervanshi *et al.* (2012) were used Vanado – molybdate method.

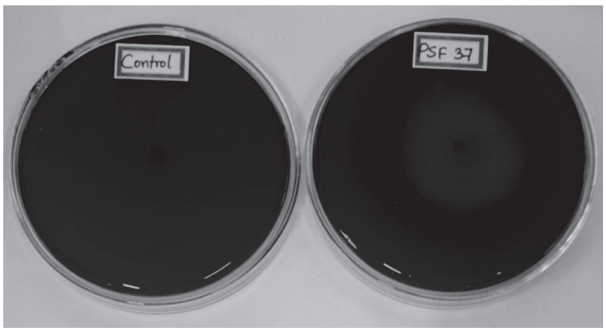

Fig. 5. Siderophore production by *Aspergillus carbonarius*

Table 2. Phosphate Solubilization tests by *A. carbonarius.*

Sl. No	Plant name	Culture Code	SI	SE	pH	TA (g/L)	Conc. of P (µg)	Siderophore production	Conc. of IAA (µg)
1	*Datura fastuosa*	PSF 1	2.47	147.1	4.8	23.2	145	+	400
2	*Phyllanthus acidus*	PSF 3	2.16	116	5.0	8.72	185	+	370
3	*Asparagus racemosus*	PSF 7	3.08	208	4.0	22.4	60	+	100
4	*Saraca asoca*	PSF 11	2.71	171.2	4.6	21.04	100	+	410
5	*Holorrhena antidysenterica*	PSF 14	2.60	160.5	4.87	20.88	120	+	290
6	*Clitoria ternatea*	PSF 15	1.08	8.8	5.5	13.6	290	+	210
7	*Pongamia glabra*	PSF 20	2.49	149.2	4.7	21.36	140	+	220
8	*Alternanthera sessilis*	PSF 25	1.35	35.7	5.38	12.56	255	-	150
9	*Bauhinia purpurea*	PSF 32	3.10	210	4.0	16.8	55	+	40
10	*Brassica* sp.	PSF 37	3.54	254	3.6	37.76	30	+++	80

Screening for siderophore production

Formation of pink halo around the colony on blue CAS agar plate was observed after the period of 7days incubation indicates siderophore production by Phosphate solubilizing fungus *A. carbonarius* (Fig 5).

Estimation of IAA

After incubation development of pink colour was observed, this indicates the production of IAA by the *A. carbonarius*. Then the amount of IAA produced by the *A. carbonarius* was estimated as 330µg (Table 2 and Fig 6) by comparing with the standard calibration curve. Pant and Agrawal (2014) reported production and estimation of IAA by plant growth promoting rhizobacteria. Nenwani, et al. (2010), report the PSF was produce phytohormone indole acetic acid (IAA), isolate F1 was found to produce 11.45 $\mu g ml^{-1}$ of IAA which is significantly high.

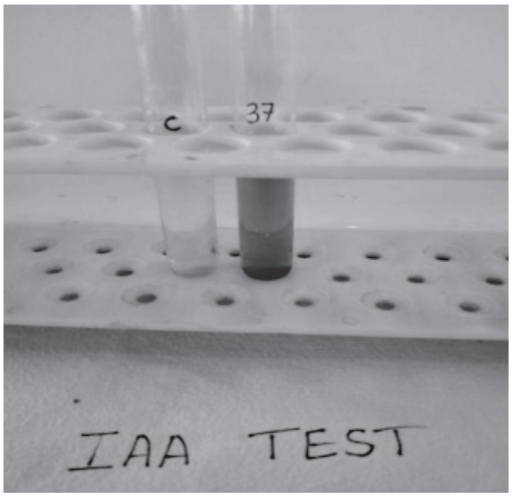

Fig. 6. IAA production by *A. carbonarius*

CONCLUSION

The present study deals with the isolation of typical fungus from rhizosphere soil and identified as *A. carbonarius* by microscopic observation, on molecular identification 18s rRNA sequencing. Due to their Phosphate solubilization capacity under laboratory condition *A. carbonarius* could be used for bio inoculums preparation as phosphate solubilizer in an eco-friendly and profitable manner.

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