



BIOSYNTHESIS OF SILVER NANOPARTICLES AND PHOSPHATE SOLUBILIZING ACTIVITY OF *PENICILLIUM OXALICUM*

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Abstract

The present study aimed on the isolation, molecular characterisation, check the phosphate (P) solubilisation and biosynthesis of silver nanoparticles and their antimicrobial activity of the fungus *Penicillium oxalicum* under laboratory condition. Totally 41 fungi were isolated from rhizosphere soil samples of different medicinal plants by serial dilution method. Target fungus was selected with high P solubilization zone and on the basis of microscopic observation and molecular identification, the fungus was identified as *P. oxalicum*. The fungus showed good results in different P solubilization tests, Solubilization Index was found 3.86, Solubilization Efficiency was measured 286, pH of the culture filtrate after the growth of fungus decreased from 6.89 to 3.7 due to production of organic acids. The colour change from blue to yellow on agar plate and red to yellow in broth due to acidic condition by the growth of fungus. Titrable acidity was measured 37.6g/L and 25µg of Phosphate was estimated in culture broth by Vanado – Molybdate method. The fungus showed positive for siderophore production and production of IAA was estimated and found 90µg. Silver nanoparticle (SNPs) was synthesized by culture filtrate of *P. oxalicum*, were visually observed for colour change and confirmed by UV – spectroscopy. Anti microbial activity of SNPs against pathogens were carried out by well diffusion assay. The diameter of the zone of inhibition was found *Psuedomonas syringae* (1.65cm) *P. aeruginosa* (1.7cm), *Klebsiella pneumonia* (1.66cm) *Xanthomonas compestris* (1.45cm) and *E. coli*, (2.1cm), *E. coli* was more susceptible. The antifungal property of SNPs reveals the diameter of the zone of inhibition was found *Curvularia* sp. (1.27cm), *Aspergillus niger* (1.22cm), *Fusarium* sp. (0.9cm) and *Alternaria* sp. no inhibition was observed. *Curvularia* sp. and *A. niger* were more susceptible to SNPs. Thus, the study concludes that due to the phosphate solubilization capacity of the fungus *P. oxalicum*, can be recommended as P solubilizer in agricultural field and as the biogenic and eco – friendly route for synthesizing SNPs with antimicrobial activity against pathogens.

Key words: P solubilization, organic acid, siderophore, silver nanoparticles, antimicrobial activity

Introduction

Phosphorus (P) is one of the major plant nutrients next to the Nitrogen, which is required for growth and development of plants and microorganisms. Phosphorus plays an important role in the plant growth and is the plant growth limiting nutrient despite its abundance in soils both organic and inorganic forms (Anand *et al.*, 2016). Phosphate solubilizing microorganisms (PSM) are more effective approach for providing balanced nutrition (Gupta *et al.*, 1998; Martins *et al.*, 2004) and recently, these phosphate solubilizing microorganisms have attracted the attention of agriculturalists as soil inoculums to improve the plant growth and yield (Yung, 1994; Yung *et al.*, 1998; Goldstein *et al.*, 1999; Fasim *et al.*, 2002). A greater part of soil, P approximately 95–99% is present in the form of

insoluble phosphate and cannot be utilized 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 the soil solutions (Anand *et al.*, 2016). There is a myriad of microorganisms, especially phosphate solubilizing microbes present in the rhizosphere soil and play a significant role in the solubilization of insoluble phosphate 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 and production of organic and inorganic acids (Mahamuni, 2012). 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

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solubilization mechanism and their acquisition to plant production through the synthesis of organic acids and plant growth promoting substance (Anand *et al.*, 2016).

In other way, Silver nanoparticles (SNPs) produced by phosphate solubilizing microbes have become the subject of immense research interest in the recent years due to their wide range of application as antimicrobial agents, water purification system, medical device and other consumer products (Wijnhoven *et al.*, 2009; Vishwanatha *et al.*, 2018). For synthesis of silver nanoparticles, biological entities and its metabolites were employed for safe, cost – effective and eco – friendly process (Li WR *et al.*, 2011). The initial isolation of phosphate solubilizers is made by using a medium suspended with insoluble phosphate such as tri–calcium phosphate. The production of solubilization zone around the colonies of the organisms is an indication of the presence of P-solubilizing organisms. Such cultures are isolated, identified and the extend of solubilization determined quantitatively. It has been observed that the capacity of PSM isolated to solubilize phosphate depends on the zone of their origin (Sharma 2007). Major mechanism of mineral phosphate solubilization is the action of organic acid synthesized by soil microorganisms. Production of these organic acids results in acidification of the microbial cell and surroundings (Nisha *et al.*, 2014).

Materials and Methods

Collection of rhizosphere soil samples

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

Isolation and Molecular characterization of phosphate solubilizing fungi

About 1g of soil samples were suspended in 9ml of sterilized 0.84% saline and serially diluted. Then dilutions were plated on Pikovskaya's agar plates and incubated at room temperature for 7days. Plates were examined for solubilization zone around fungal colonies were selected and sub cultured for further use (Parul and Dharmendra, 2015; Naik *et al.*, 2013). 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)

0.1ml of PSF culture in sterile distilled water was

placed on Pikovskaya's agar plate and incubated for 7days. Solubilization index (SI) was measured using following formula (Yasser *et al.*, 2014).

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

% Solubilization Efficiency (SE)

0.1ml of PSF culture in sterile distilled water was placed on Pikovskaya's agar plate and incubated for 7days. % Solubilization Efficiency (SE) was measured using following formula (Joseph and Jisha, 2008).

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

Measurement of pH

PSF culture filtrate was centrifuged at 1000rpm for 10min and supernatant was collected. The pH of the culture filtrate was measured by pH meter before inoculation and after the period of 7days incubation. Uninoculated broth served as control (Yasser *et al.*, 2014).

Qualitative acid production assay

Qualitative acid production on solid media

0.1ml of PSF culture preserved in sterile distilled water was placed on Pikovskaya's agar plates containing Bromophenol blue indicator and incubated for 7 days (Chadha *et al.*, 2015).

Qualitative acid production in broth

Sterile Pikovskaya's broth containing Bromocresol purple indicator was inoculated with PSF culture and incubated for 7days (Khan and Gupta, 2015).

Quantitative acid production assay (Titrable acidity)

PSF culture filtrate was centrifuged at 1000rpm for 10min and supernatant was collected. 50ml of supernatant was added with few drops of phenolphthalein indicator and titrated against 0.1N NaOH solution. The titrable acidity was expressed in g/ml (Khan and Gupta, 2015).

Estimation of Phosphate

PSF culture filtrate was centrifuged at 12000rpm for 20min, then phosphate in the supernatant was estimated by Vanado – molybdate method and it was expressed in terms of µg/ml phosphorus released in culture medium (Verma and Ekka, 2015).

Screening for siderophore production

Siderophore production was detected by using chrome azurol S (CAS) assay. The medium contains an

iron CAS-HDTMA (Hexadecyltrimethyl ammonium bromide) complex which is blue coloured. The presence of iron chelator (Siderophore) is indicated by decolourization of the blue coloured ferric-dye complex, resulting in a yellow to orange halo around the colonies. 60.5mg of Chrome azurol S was dissolved in 50ml of distilled water and mixed with 10ml 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 40ml 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 IAA

PSF culture was grown in potato dextrose broth supplemented with Tryptophan (1%). After growth, culture filtrate was centrifuged at 1000rpm for 10min and supernatant was collected. Supernatant (2ml) was mixed with 2 drops of orthophosphoric acid and 4ml of Salkowski reagent (50ml of 35% per chloric acid, 1ml of Ferric chloride solution) and allowed to stand in dark. 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).

Biosynthesis of Silver Nanoparticles

PSF culture was grown in Potato dextrose broth at room temperature for 14days. After the growth, culture filtrate was centrifuged at 1000rpm for 10min and supernatant was collected. Equal volume of supernatant and 5mM silver nitrate solution was mixed and kept for incubation at 24h. A colour change in the reaction mixture from colourless to yellowish brown indicates the formation of SNPs (Chandra and Singh 2018). The UV – spectroscopic analysis was carried out on the reaction mixture at a wavelength of 200 – 600nm (Zhao *et al.*,

2019).

Anti – microbial activity of SNPs

The SNPs synthesized from *P. oxalicum* were tested for antibacterial activity. The antibacterial activity determined by using the well diffusion method against pathogenic bacteria such as *Pseudomonas aeruginosa*, *P. syringae*, *Xanthomonas sp.*, *Klebsiella sp.* and *E. coli*. The culture suspension was swabbed on solidified Nutrient agar plates and wells were made by using sterile cork borer. Wells filled with SNPs mixture care should be taken that the mixture could not be over flow. Ciprofloxacin a standard antibiotic of concentration 10mg/ml was used as control. The plates were incubated at 37°C for 24h. After incubation anti – bacterial property of SNPs was determined by measuring the zone of inhibition around the well in diameter (mm) (Vishwanatha *et al.*, 2018).

The antifungal activity determined by using the well diffusion method against pathogenic fungi such as *Fusarium sp.*, *A. niger*, *Alternaria sp.*, *Curvularia sp.* The spore suspension was mixed with PDA media and poured into plate, allowed for solidification. Wells were made by using sterile cork borer and filled with SNPs mixture care should be taken that the mixture could not be over flow. Flucanazole a standard antifungal agent of concentration 10mg/ml was used as control. The plates were incubated at room temperature for 7days. After incubation, anti-fungal property of SNPs was determined by measuring the zone of inhibition around the well in diameter (mm) (Mussin *et al.*, 2019).

Results and Discussion

Isolation and molecular characterization:

Rhizosphere soil samples were collected from different medicinal plants around the Malnad areas of Shivamogga district. Among the different soil sample, 41 fungi were isolated by serial dilution method using Pikovskaya's medium table 1 and one fungal colony with high solubilization zone was selected for further P solubilization test. Then the fungal colony was point inoculated on fresh medium to make pure culture of the

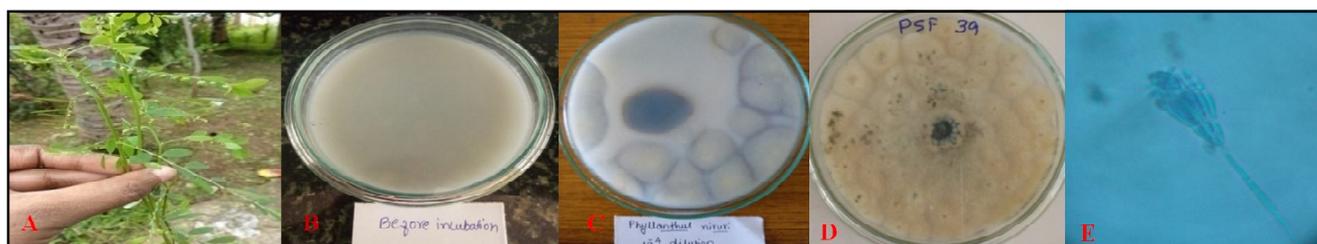


Fig. 1: Medicinal plant (*Phyllanthus niruri*) (A), isolation of PSF (before and after incubation) (B and C), *Penicillium oxalicum* (Culture plate) (D) and Microscopic view 40X (E).

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

SI No.	Plant name	Culture code	SI	SI No.	Plant name	Culture code	SI
1	Datura fastuosa	PSF 1	2.47	22	Ocimum sanctum	PSF 22	3.03
2	Leucus aspera	PSF 2	2.70	23	Phyllanthus emblica	PSF 23	2.37
3	Phyllanthus acidus	PSF 3	2.16	24	Amaranthus viridis	PSF 24	1.78
4	Argemone mexicana	PSF 4	1.29	25	Alternanthera sessilis	PSF 25	1.35
5	Achyranthus aspera	PSF 5	3.15	26	Euphorbia hirta	PSF 26	2.20
6	Centella asiatica	PSF 6	2.51	27	Euphorbia heterophylla	PSF 27	2.87
7	Asparagus racemosus	PSF 7	3.08	28	Ixora coccinea	PSF 28	2.53
8	Gymnema sylvestres	PSF 8	3.21	29	Mimosa pudica	PSF 29	2.86
9	Tinospora cordifolia	PSF 9	1.60	30	Cassia occidentalis	PSF 30	2.65
10	Costus ingneus	PSF 10	3.02	31	Asclepias curassavica	PSF 31	1.34
11	Saraca asoca	PSF 11	2.71	32	Bauhinia purpurea	PSF 32	3.10
12	Calotropis sp.	PSF 12	1.49	33	Momordica charantia	PSF 33	3.07
13	Vitex nigundo	PSF 13	1.33	34	Solanum xanthocarpum	PSF 34	3.01
14	Holorrhena antidysenterica	PSF 14	2.60	35	Eclipta prostrata	PSF 35	3.42
15	Clitoria ternatea	PSF 15	1.08	36		PSF 36	3.05
16	Wrightia tinctoria	PSF 16	-	37	Brassica sp.	PSF 37	-
17	Santalum album	PSF 17	2.36	38	Phyllanthus niruri	PSF 38	-
18	Azadirachta indica	PSF 18	1.27			PSF 39	3.86
19	Eucalyptus sp.	PSF 19	1.24	39	Phyllanthus sp.	PSF 40	-
20	Pongamia glabra	PSF 20	2.49	40	Acalypha indica	PSF 41	-
21	Vinca rosea	PSF 21	2.17				

fungus for further studies of phosphate solubilization. The fungus was identified as *Penicillium oxalicum* based on microscopic observation using standard manual Fig. 1 and molecular characterization, 18s rRNA sequencing Fig. 2 and *Penicillium oxalicum* (MN733168) was deposited at Gen Bank, NCBI.

Fasta Sequence:

>CHROMGENE_PSF39_ITS4_C08.ab1

GGGGAACCCTACCTGGATCCGAGGTCAACC
 TGGTTAAGATTGATGGTGTTCGCCGGCGGGCGC
 CGGCCGGCCTACAGAGCGGGTGACGAAGCCCC
 ATACGCTCGAGGACCGGACGCGGTGCCGCCGCT
 GCCTTTCGGGCCCCCCCCCGGAAGCGGGGGG
 GAGAGCCCAACACACAAGCCGTGCTTGAGGGCA
 GCAATGACGCTCGGACAGGCATGCCCCCCGAA

TACCAGGGGGCGCAATGTGCGTTCAAAGACTCG
 ATGATTCACCTGAATTCTGCAATTCACATTACTT
 ATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGA
 ACCAAGAGATCCGTTGTTGAAAGTTTTAACTGA
 TTTAGTCAAGTACTCACACTGCAATCTTCAGAC
 AAGAGTTCGTTTGTGTGTCTTCGGCGGGCGCGG
 GCCCGGGGCCCCCTCCGCCCGGAAGGGCGGCC
 CGGCCCCCCCCCCCGCGGAAAAAAAAAACCACCGG
 GGGGGGA.

Identified species (BLASTn comparison):

Top BLAST Hit 1: *Penicillium oxalicum* strain A95 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

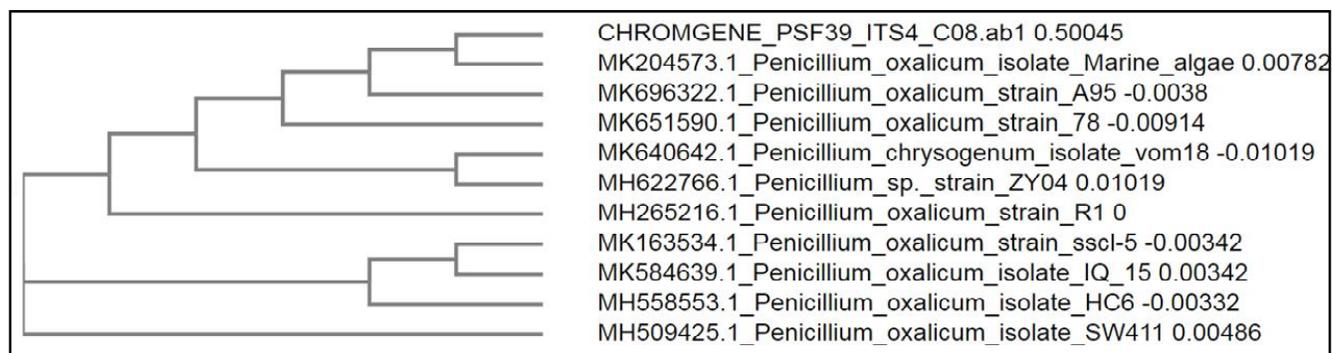


Fig. 2: Molecular characterisation of *Penicillium oxalicum*.

Identity: 99.53%

Coverage: 85%

E-value: 0.0

Accession No: MK696322.1.

Comparison with top 10 nearest species:

Solubilization Index (SI)

The qualitative analysis of phosphate solubilization potential of *P.oxalicum* was measured *in vitro*. The fungus *P. oxalicum* showed solubilization index (SI) of 3.86 (Fig 3), this result was correlated with previous studies of Verma and Ekka (2015) who have reported SI of 18 fungal culture strains ranging from 1.06 to 3.34. Yasser *et al.*, (2014) have reported SI of fungal cultures ranging from 1.05 to 1.45, while Mahamuni *et al.*, (2012) have reported SI fungal culture ranging from 1.13 to 1.59.

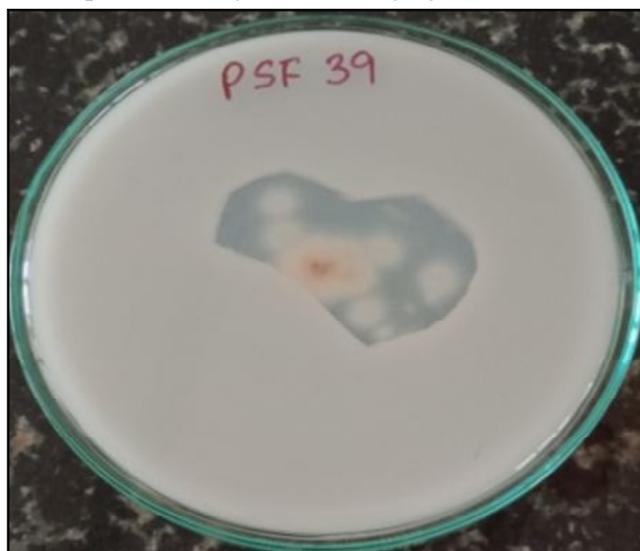


Fig 3: Solubilization index of *Penicillium oxalicum*.

% Solubilization Efficiency (SE)

Measurement of % Solubilization efficiency (SE) of the fungus *P. oxalicum* was recorded as 286 table 2, this result was correlated with earlier finding of Joseph and Jisha (2008) who have reported 100 to 575.

Measurement of pH

Decreased pH from 6.89 to 3.7 was recorded in the culture filtrate, after the period of incubation due to production of organic acids. This result was correlated with earlier findings of Yasser *et al.*, (2014) who have reported reduced pH 4.80 to 5.4.

Qualitative acid production assay:

Due to production of organic acids by the *P. oxalicum* 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 Bromophenol Blue Fig. 4, same results were 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.

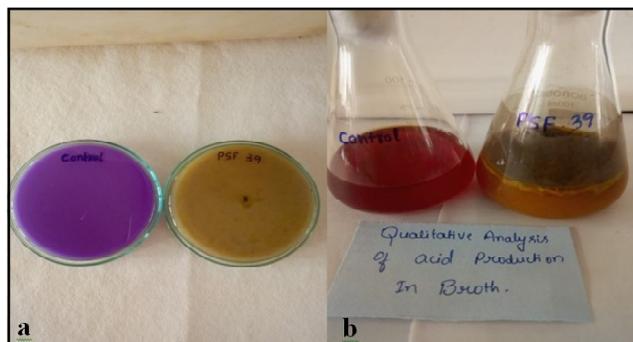


Fig 4: Qualitative acid production assay on solid media (a) and in broth (b)

Quantitative acid production assay (Titrable acidity):

The measure of amount of acid present in the culture broth was titrated and was recorded 37.6g/L table 2. This result was correlated with earlier findings of Balaiah *et al.*, (2016) and Reena *et al.*, (2013).

Estimation of Phosphate:

The concentration of the P solubilization in the broth was determined as 25µg table 2 in culture broth, the result was correlated with earlier findings of Verma and Ekka (2015), Sahoo and Gupta, (2014) and Sagervanshi *et al.*, (2012) have used Vanado – molybdate method to determine the solubilized phosphate in broth.

Screening for siderophore production

Formation of pink halo around the colony on blue CAS agar plate was observed after the period of 7 days incubation indicates siderophore production by Phosphate solubilizing fungus *P. oxalicum*.

Estimation of IAA

Development of pink colour was observed after the

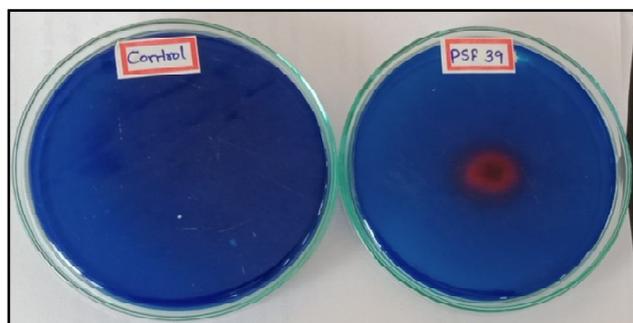


Fig 5: Production of Siderophore by *P. oxalicum*.

period incubation and the concentration of produced IAA was determined, 90µg table 2 and Fig. 5. Pant and Agrawal (2014) have isolated 6 bacterial cultures and estimated IAA by standard calibration curve. Nenwani, *et al.*, (2010), reported that PSF was able produce phytohormone indole acetic acid (IAA), isolate F1 was found to produce 11.45 µgml⁻¹ of IAA which is significantly

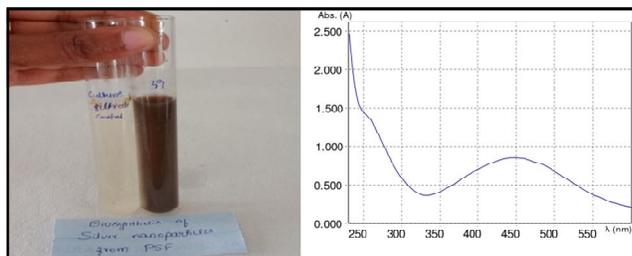


Fig 7: Biosynthesis of silver nanoparticles by *P. oxalicum* high.

Biosynthesis of Silver Nanoparticles

The synthesis of silver nanoparticles was initially observed with change of colour from pale yellow to yellowish brown with 5mM silver nitrate solution after 24h incubation Fig. 6, there was no colour change in control. Further confirmation of silver nanoparticles was carried out by UV – spectroscopic analysis with absorbance peak emerging between 200 – 600nm. Previous works on SNPs, Vishwanatha *et al.*, (2018) reported that the change in colour was observed due to synthesis of SNPs by *Aspergillus awamori* and further conformation carried out by UV- visible spectroscopic analysis.

Anti-microbial activity of SNPs

The antibacterial property of SNPs reveals the



Fig 6: Production of Indole Acetic Acid by *P. oxalicum*

Table 2: Phosphate solubilization tests of *P. oxalicum*.

Sl. No.	Culture	SI	% SE	pH	TA (g/L)	Siderophore production	Conc. of P (µg)	Conc. of IAA (µg)
1	<i>Penicillium oxalicum</i> (PSF 39)	3.86	286	3.79	37.6	+	25	90

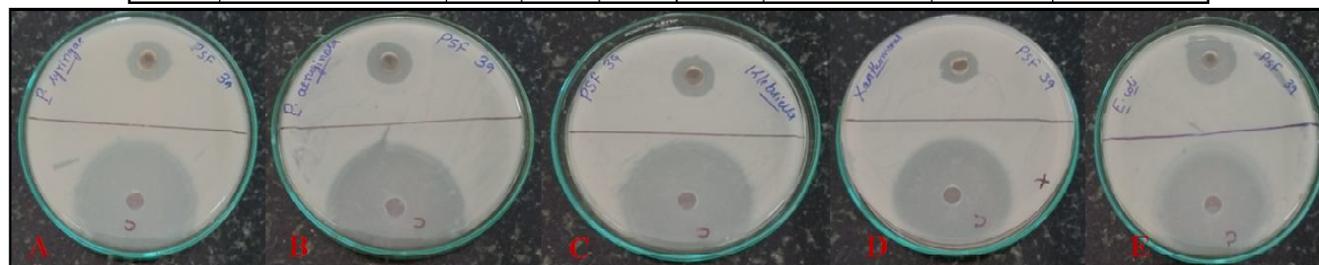


Fig 8: Anti-bacterial activity of Synthesized SNPs against plant pathogens *Psuedomonas syringe* (A), *P. aeruginosa* (B), *Klebsiella pneumoniae* (C), *Xanthomonas compestris* (D) and *E. coli* (E)

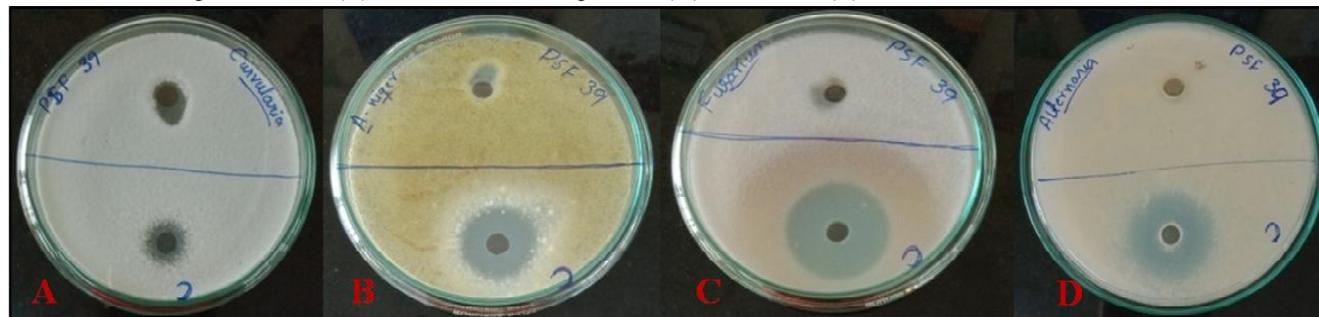


Fig 9: Anti-fungal activity of Synthesized SNPs against plant pathogens *Curvularia sp.* (A), *Aspergillus niger* (B), *Fusarium sp.* (C) and *Alternaria sp.* (D).

diameter of the zone of inhibition of 1.65cm, 1.7cm, 1.66cm, 1.45cm and 2.1cm for *Psuedomonas syringae*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Xanthomonas compestris* and *E. coli* respectively, control ciprofloxacin showed zone of inhibition 5.11cm, 5.03cm, 5.04cm, 4.61cm and 4.76cm for *Psuedomonas syringae*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Xanthomonas compestris* and *E. coli* respectively. *E. coli* was more susceptible to SNPs followed by *P. aeruginosa* and less susceptible to *Xanthomonas compestris*. These results correlated with the previous work carried out by Vishwanatha *et al.*, (2018) who have reported 17mm, 15mm and 17mm for *P. aeruginosa*, *K. pneumoniae* and *B. cereus* respectively, chloramphenicol used as control Fig. 8.

The antifungal property of SNPs reveals the diameter of the zone of inhibition of 1.27cm, 1.22cm, 0.9cm and no inhibition for *Curvularia* sp. *Aspergillus niger*, *Fusarium* sp. and *Alternaria* sp. respectively. *Curvularia* sp. and *A. niger* were more susceptible to SNPs. Control Flucanazole (10mg/ml) showed zone of inhibition 1.45cm, 2.67cm, 3.3cm, and 2.47cm respectively. Mussin *et al.*, (2019) reported that the antifungal activity of SNPs and ketoconazole against *M. furfur* evaluated by broth micro dilution method showed *in vitro* inhibitory activity against all isolates of *M. furfur* Fig. 9.

Conclusion

The present study deals with the collection of rhizosphere soil samples by medicinal plants for the isolation of Phosphate Solubilizing Fungus, *P. oxalicum* to highlight on major phosphate solubilizing fungi, which could be used for bio inoculums preparation in an eco-friendly and profitable manner. Due to all the results obtained by different tests in laboratory, *P. oxalicum* was recommended for Phosphate solubilizer in agricultural management. By observing all positive results of phosphate solubilization of *P. oxalicum* can be recommended as phosphate solubilizer.

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