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Isolation and Molecular Characterization of Plant Growth Promoting Fungi from Rhizospheric Soil

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Abstract

Aim: Phosphorus is abundant in soil in both organic and inorganic forms; nevertheless, it is unavailable to plants. Accordingly, soil becomes phosphorus deficient, making phosphate one of the most important nutrient elements limiting crop productivity. To prevent the phosphate deficiency, phosphate solubilizing microorganisms could play an important role in making phosphate available for plants by dissolving insoluble Phosphate. Materials and methods: The serial dilutions was done in rhizospheric soil and *humicola.sp*, fungi was isolated. The morphological and molecular characters were studied. Using pikovskaya media *humicola* fungi was identified to be phosphate solubilizing. This fungi helps to promote plant growth. Result: Phosphate in the soil is essentially unavailable to plants and use of plant associated organisms may help in solubilization of mineral phosphorous for easy uptake by the plants. Fungi have the ability to solubilize phosphorous by production of organic acids and are known to have a higher efficiency of solubilization than bacteria. Increase in cost of fertilizers and worldwide energy crises, low purchasing power of farmers, increase in cost of production restricted the use of chemical fertilizers alone as a source of plant nutrient. Under such condition it has become alternative to use all available resources of plant nutrients including microorganisms like Phosphate Solubilizing Microorganisms for sustainable soil fertility and productivity. A sterile rhizospheric fungus was isolated from rhizospheric soil of *spathoglottis plicata* and was found to be dominant with highest phosphate solubilization capacity. The fungus could identify by morphological features, molecular characterization was carried was found closest homolog to *humicola sp*, with maximum identity of 98%. Conclusion: The *humicola* is found to be in symbiotic association with the plant. It can be used as a substitute for chemical fertilizer. Therefore preserves the soil fertility.

Keywords
Phosphate solubilizing fungi, rhizospheric soil, *humicola sp*.

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1. INTRODUCTION

The abundant use of chemical fertilizers is hazardous to fauna and has led to the development of pest resistant plants. The soil present just above the roots influence the plant growth and it acts has a hub to several beneficial microorganisms therefore helps in maintaining ecology [1, 2].

Certain plants are capable of protecting themselves from infectious agents that are either inborn or inductive. Numerous PGPF has solubilized phosphates and various macro nutrients therefore they play a vital role in promoting plant growth [3, 4]. Phosphorous is one of the indispensable macronutrient that prudence the agricultural yield. It is essential for controlling the metabolic pathways. Few major functions of phosphorous is hardening of shoot and system, maturation of seedlings and crop growth [5].

Generally the total phosphorus content in every soil is 0.05% of which 0.1% is obtainable to plant [6] .Phosphorus is one of the most indispensable macronutrient that is required for biological growth and development [7]. But phosphorous in the soil is essentially unavailable to plants and use of plant associated organisms may help in solubilization of mineral phosphorous for easy uptake by the plants. Fungi have the ability to solubilize phosphorous by production of **organic acids and are known to have a higher efficiency of solubilization** than bacteria.

There are a number of Plant Growth Promoting Fungi(PGPFs) in rhizosphere soil, which have the ability to stimulate the plant immune response upon enemy attack and are considered as one of the safest mode for ISR(induced systemic resistance) and promotion of crop plants[8]. Increase in cost of fertilizers and worldwide energy crises, low purchasing power of farmers, increase in cost of production restricted the use of chemical fertilizers alone as a source of plant nutrient. Under such condition it has become alternative to use all available resources of plant nutrients including microorganisms like Phosphate Solubilizing Microorganisms for sustainable soil fertility and productivity. These Phosphate Solubilizing Microorganisms have ability to increase stress tolerant capacity in plant and induce disease resistance against soil borne pathogens. 'Phosphate' solubilizing capacity of Phosphate Solubilizing Microorganisms (PSM) varies with soil condition. Therefore, use of efficient isolates is necessary. Hence, the attempts were made to isolates efficient 'phosphate' solubilizing fungi from rhizosphere.

Therefore, the present study is investigated according to the following objectives:

- Isolation of fungal species from soil sample collected from Pearl Orchid Center, Palakad (Dt), Kerala.
- Sub culturing of fungal species for pure strains.
- Identification of fungi by morphological and molecular characters.
- Analysis of the phosphate solubilizing fungi.

2. MATERIALS AND METHODS

Soil collection:

The rhizospheric soils were collected from the orchid root at Pearl Orchid Centre, Palakad (Dt), Kerala.

Serial dilution technique:

A series of sterilized test tubes capped with cotton plugs were taken and marked as 10^{-1},10^{-2},10^{-3},10^{-4},10^{-5},10^{-6},10^{-7} .1g of enriched soil sample was inoculated into 10ml of the molecular grade water containing test tube marked 10^{-1} so that the diluted is brought about by 1:10 ratio. 1ml of 10 fold diluted sample was taken into a test tube containing 9ml of molecular grade water so that 10^{-2} dilution is prepared. The dilutions were carried up to 10^{-7}. For each transfer the dilution was increased by 10 fold, that is 1:10, 1:100, 1:1000, 1:10000, 1:100000 and so on.

Media preparation:

PDA Medium

Potato- 200g
Dextrose- 20g
Agar- 20g

200g of unpeeled potatoes was sliced and boiled in 1000 ml of distilled water for 30 min. The mixture was filtered using a filter paper. To the filtered potato infusion dextrose, agar and water was boiled to dissolve. The pH was noted to be 5.6. Autoclave was done at 121^{0}C for 15 min. 20-25ml portions were transferred to sterilized petriplates.

Pikovskaya's Medium

Calcium phosphate	- 5 g
Ammonium phosphate	- 0.5 g
Potassium chloride	- 0.2g
Magnesium sulphate	- 0.1 g
Dextrose	- 10.00g
Yeast extract	- 0.5 g
Agar	- 15 g
Manganese sulphate	- 0.0001 g
Ferrous sulphate	- 0.0001 g
Distilled water	- 1000 ml

31.30g of the above ingredients was suspended in 1000ml of distilled water. The mixture was boiled to dissolve the medium and sterilization was done by autoclaving at 121°C for 15 minutes. The pH was noted to be 7. It was mixed well and poured into sterile petri plates.

Isolation of fungi:
Suitable dilutions prepared were spread on plates containing Potato Dextrose Agar (PDA) medium. The plates were kept for incubation at 28±2°C for 5-7days. Fungal colonies were sub cultured several times on PDA plates till the appearance of pure cultures. The isolates were stored in refrigerator on PDA slants for further studies. (Fig 1)

Screening the isolates of phosphate soloubilizing fungi:
The isolates were screened by inoculating on plates containing Pikovskaya's Agar (PKA) medium amended with 0.5% tri-calcium phosphate (TCP) as insoluble phosphate source and were incubated at 28±2^{0}c for 5 days. Fungal colonies with clear halo zone around them were screened as phosphate solubilizers. (Fig 3)

Identification of fungi:
The fungi were identified based on macroscopic and microscopic features. For macroscopic, mycelium and pigmentation of the colony was observed and documented. Slide culture was prepared in order to identify spores and mycelia of pure fungal isolates for microscopic characterization. Accordingly the morphology of spores and mycelia of fungal isolates was examined and identified by lacto phenol cotton blue staining using optika microscope. The spores, hyphae and conidiophores arrangement were observed and recorded (Table 6 &Fig 2).

Genomic DNA isolation:
DNA was extracted from 1g of fungal hyphae using a CTAB method. The modifications were as follows: 650µl of CTAB (Table 1) and 300µl of Mercaptoethanol were used for grounding of, 0.8g of hyphae tissue in a prechilled mortar, the mixture was kept at water bath of 60˚C for 45min, after that 24:1 volume of chloroform and isoamyl alcohol were mixed separately (Table 2), and added double the volume to the extract. Then the extract was shaken vigorously and centrifuged at 15000rpm for 15min and the supernatant was collected, and washed thoroughly in 24:1 volume of chloroform and isoamyl alcohol until clear aqueous layer is formed, then the aqueous layer is taken into fresh centrifuge tube and 1ml of ice cold isopropanol is added, and kept in deep freezer for 1 hour to precipitate DNA then it is centrifuged at 1000rpm for 10minutes and pellet was washed thoroughly in 70% ethanol and dried completely in incubator for 30min then it is dissolved in TE buffer (Table 3) and used for further analyses.

Purification:
The crude DNA was purified using liquid-liquid extraction of phenol-chloroform method. The aqueous phase containing purified DNA is then dissolved and stored in 15Mm TE buffer.

PCR (Polymerase Chain Reaction:
SELECTION OF PRIMERS: The following are the primers used for amplification of 10-mer oligonucleotides with artibitary sequences namely,
Primer-1:**5'-TCCGTAGGTGAACCTGC**
(FORWARD PRIMER**)**
Primer-1:**5'-TCCTCCGCTTATTGATATGC**
(REVERSE PRIMER)

Reaction mixture:
The PCR reaction mixture consisted of 2µl of genomic DNA, PCR buffer 2µl, dNTP of 2µl, 0.5µl of primers and 0.5µl of taq polymerase enzyme in a 25 µl of volume. The denaturation step was during at 94˚C for 4 min and 40 seconds, annealing step at 54˚C for 40 seconds and extension step 72˚C for 40 seconds. The PCR reaction was carried out for 30 cycles, with a final extension at 72˚C for 9 min, and eventually stored at 4˚C (Table 4).

Agarose gel electrophoresis:
Electrophoresis was carried out in 1% agarose gel with 0.5×TBE buffer. After the complete running of the gel, with the banding patterns of DNA in gel were visualized with gel documented using gel documentation system (Biorad).

ITS gene sequencing:
The purified PCR products were directly sequenced with an ABI Prism 3110 Genetic Analyzer using Big Dye Terminator V 3.0 Cycle Sequencing chemistry.

Homology analysis:
In bioinformatics, BLAST for **B**asic **L**ocal **A**lignment **S**earch **T**ool is an algorithm for comparing primary biological sequence information, such as the amino acid sequences of proteins or the nucleotides of DNA and RNA sequences. A BLAST search enables a researcher to compare query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold.

3. RESULTS &DISCUSSION

Identification of Fungi:
There are around 5 colonies found and marked. Each fungal colony were examined and observed under Optika microscope. Out of 5 fungal isolated only one isolate was taken into the further studies. The dominant and whitish black color fungal isolate were sub cultured and the morphological characters were observed was shown in Table 5, Fig 1.

Phosphate solubilizing:
The morphologically identified fungus was taken into further analysis for its phosphate solubilizing ability. Therefore, the screening of phosphate solubilizing

fungi was done on pikovskaya media. After incubation, the observed colonies were morphologically distinct, both with and without halo zones. Those fungal colonies showing clear zone around the colonies were observed and recorded (Fig 3). The rhizospheric soil of orchid root was collected and the isolated microorganism fungi was helpful in phosphate solublilizer. Similarly [9] reported in the fungus of *A.niger* solubilized insoluble phosphate well in liquid medium supplemented with tri -calcium phosphate. The soil sample collected for screening of PSB and PSF from different rhizospheric and root regions are inoculated on Pikovaskya's agar plates have clear zone around it indicating phosphate solubilizing[10].

Reduction in pH:

Microorganisms which tend to decrease the pH of the medium during growth are efficient phosphate solubilizers. Contrary to the decreasing pH for individual cultures up to 48hrs and then acquiring constancy, the soluble phosphate concentrations continue to increase after 48hrs. This clearly suggests that pH drop is not the sole factor for phosphate solubilization. An initial increase in phosphate concentration followed by a gradual decrease in culture filtrate as observed in this study was also reported in earlier studies.

Increase titratable acidity (loss of protons) was observed to correlate with the reduction in pH of the medium as shown in the graph (Fig 4) Results indicate that fungi isolates that produced organic acid also solubilizes insoluble phosphate. (Fig 4).These pH reductions are due to secretion of organic acids excreted by phosphate solubilizing fungi. Decreasing the pH of the medium to an extent that causes solubilization of insoluble phosphate [11] a remarkable drop in pH of culture media was solubilized considerable amounts of phosphate [12].

Genomic DNA isolation:

The genomic DNA was isolated from *Humicola species* for further conformation at genetic level. The isolated fungal genomic DNA was further amplified with ITS1 primer and the results are recorded. (Fig 5)

PCR amplification of ITS 1 gene:

PCR reaction was performed in a thermal cycle. The universal primer

5'-TCCGTAGGTGAACCTGC (FORWARD PRIMER**)**

5'-TCCTCCGCTTATTGATATGC (REVERSE PRIMER**)** were used for the amplification of the ITS gene fragment. (Fig 6)

In the present study, the fungal DNA was subjected into PCR amplification using ITS primer shows 600 bp amplified product. The entire internal transcribed spacer (ITS) 1-5.8s-ITS2 ribosomal DNA region was amplified in different clinical fungal isolates [13].Internal transcribed spacer 1 (ITS1) and ITS2 were amplified by PCR to detect fungal pathogens in patients with ocular infections (endophthalmitis and keratitis). Fifty strains of 12 fungal species (yeasts and molds) were analyzed in this analysis [14].

Homology identification:

Alignment of the sequence from unknown fungal isolates against already submitted sequence in the Genbank database of National Centre for Biotechnological Information (NCBI). BLASTn resulted in the identification of fungi up to species level using this similarity search tool .The output of two sequences of BLAST shows the 100% similarity with the *Humicola*. Hence it is concluded that the identified organism through ITS are *Humicola species*.DNA sequences of *Aspergillus sp* identified by similarity searches in the GenBank (BLAST) gave *A. fumigatus* (57.6%), *A. niger* (28.6%), *A. tubingensis* (7.1%) and *A. flavus* (7.1%) from all sources investigated [15] .

Chemicals	Quantity	Concentration
NaCl	8.18 g	100mM
Tris HCL (Ph 8.0)	12.11g	100Mm
EDTA (Ph 8.0)	18.6 g	20Mm
CTAB	2 g	100Mm

Table .1: CTAB buffer preparation (100ml) 2% (W\V)

Solvents	Final concentration	For 25 ml of CIA
Chloroform	96%	24ml
Isoamyl alcohol	1%	1ml

Table .2: CIAA SOLUTION (Chloroform: Isoamyl alcohol) 24:1

Chemicals	Final concentration	For 250ml of TE
Tris Hcl	10Mm	1.0ml
EDTA	1Mm	0.2ml

Table .3: TE buffer preparation

S.no	Primer	Denaturation		Annealing		Extension	
1	ITS1-Forward primer	94˚C	4 min	54˚C	40 sec	72	40 sec
2	ITS1-Reverse primer	94˚C	40 sec	54˚C	40 sec	72	9 min

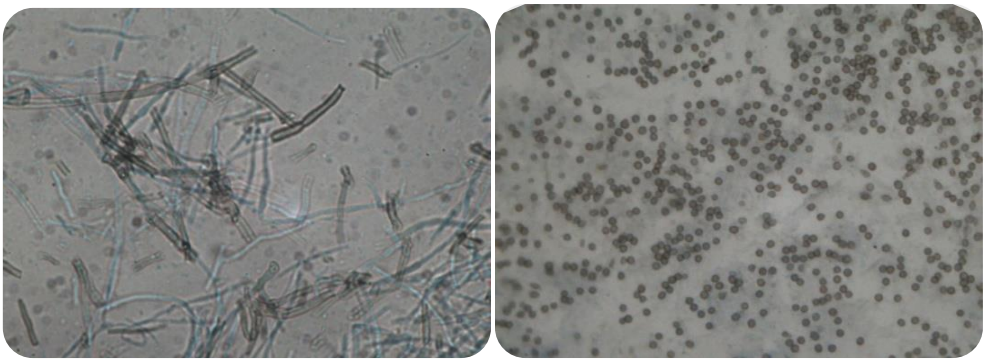
Table .4: PCR temperature

TEST	OBSERVATION
Cell shape	Spherical
Cell size	≥3.4 cm
Colony character	Whitish black
Spore shape	Globular
Hyphae	Branching filamentous

Table .5: Morphological and microscopical characteristics are isolated from pure culture

Fig .1: Isolation of pure culture by Potato Dextrose Media

DAY: 1 **DAY: 2**



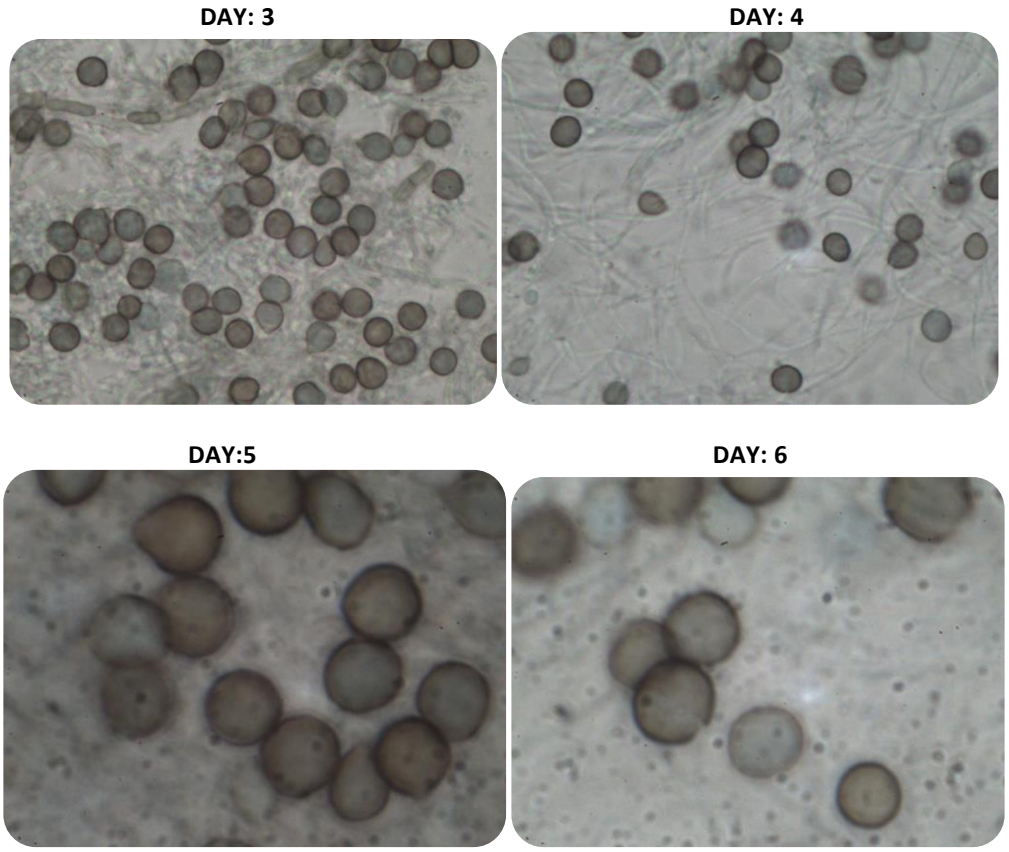


Fig 2: Image of phosphate solubilizing fungi (Optika microscope) showed that these were phosphate solubilizing fungi the *Humicola species*

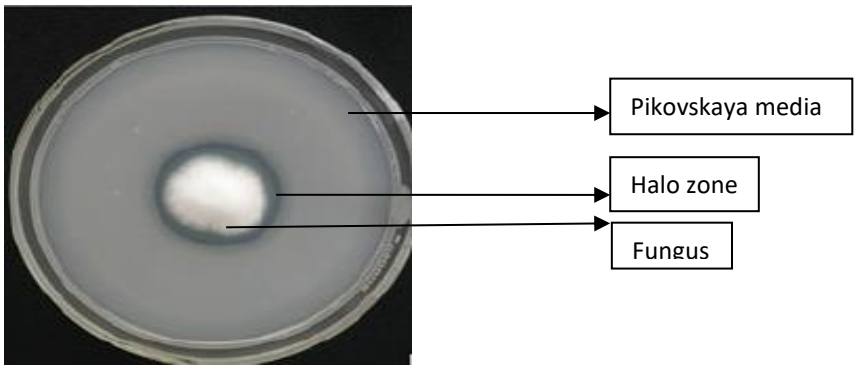


Fig. 3: Phosphate solubilizing fungi forming clear zone: *Humicola species*

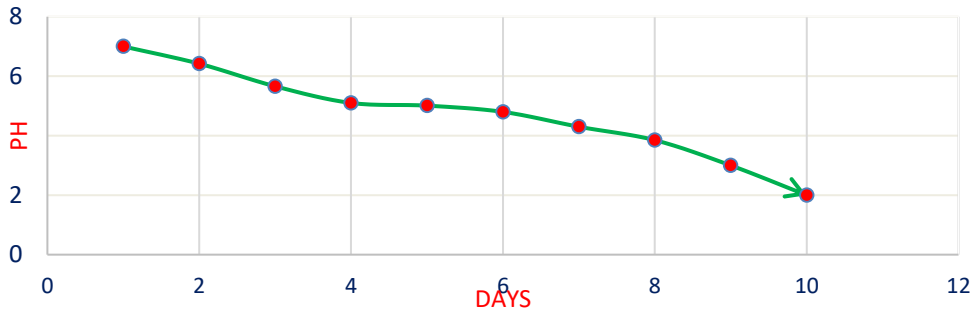


Fig 4: Reduction in pH, as a result of total organic acid produced by phosphate solubilizing fungi.

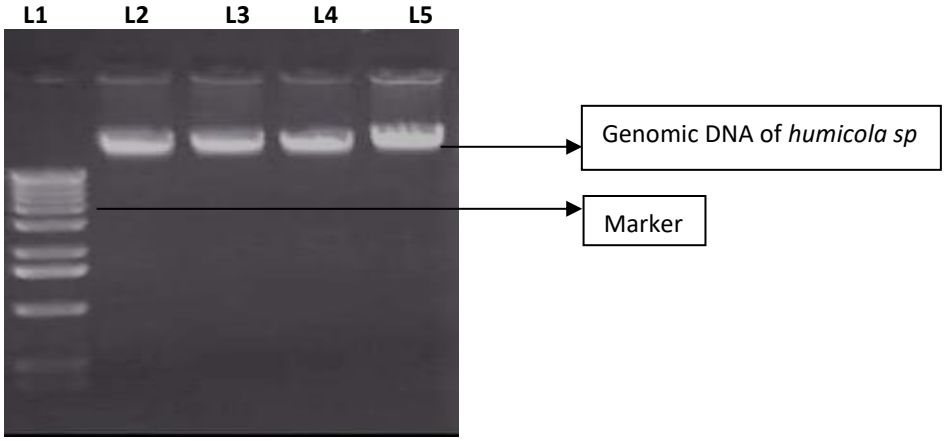


Fig .5: Genomic DNA sample in 1% agarose gel
Lane description:
1. L1- 100bp Marker
2. L 2, 3, 4, 5-*Humicola sp*

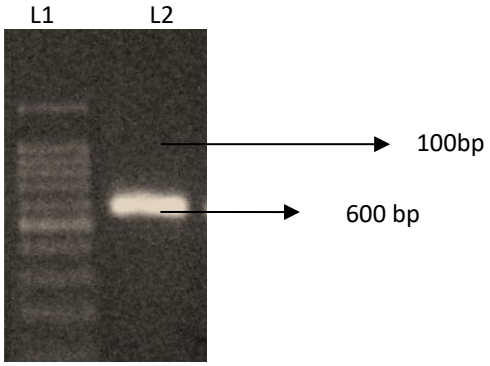


Fig .6: PCR amplification of ITS1 fragments from samples.
Lane description:
L1 – 1000 bp ladder
L2 – *Humicola sp*

4. CONCLUSION

Phosphates are essential nutrients required for the protectivity and productivity of the plants .Phosphate in the soil to support crop growth but the crop exhibit deficiency due to the presence of unavailable fractions. Exogenous application of these nutrients in the form of fertilizer also becomes unconventional because of the transformation into unavailable fractions soon after application and becomes accumulated in the soil. This crisis can be prevented by the identification of rhizospheric fungi which has the potential to transform various unavailable form of metal to available from which will be an alternative tool to alleviate phosphate deficiency in plants. Therefore in the present investigation, phosphate solubilizing fungi was isolated from the rhizospheric soil sample.

The ITS primer sequencing provides the better identification of unknown fungal isolates as compared to old phenotypic techniques and identification kits. The use of double set of primer instead of single set has the potential to make the technique more precise and accurate. More experimentation is required however for validity of this improved method in the identification of unknown fungi from other fields of studies. Thus the present study recommends the field application of phosphate solubilizing fungi i.e., *Humicola species* which can solubilize phosphate. This method will form an alternative eco-friendly approach to ease the use of such minerals in their inorganic form and provides to upheld agriculture.

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