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IDENTIFICATION AND CHARACTERISATION OF A PHOSPHATE SOLUBILISING ACTIVITY IN MORINGA LEAVES

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Abstract
Phosphate solubilization involves acid production, enzymatic activity, and microbial interactions. This study investigates the phosphate solubilizing activity in Moringa oleifera leaves, which are known to contain organic acids and phosphatases. Release of phosphate from insoluble calcium phosphate was found to be mediated by an oxalic acid producing fungal endophyte. Field studies using fenugreek confirmed the potential of Moringa leaf extracts as an inexpensive means of converting land containing insoluble phosphate to arable soil. Our findings suggest Moringa leaves can be used as a natural biofertilizer, contributing to reduced use of chemical fertilizers and promoting sustainable farming practices.

Keywords: Moringa oleifera, phosphate solubilization, organic acids, fungal endophytes, sustainable agriculture, biofertilizers

1. INTRODUCTION

As a critical nutrient for plant growth, the presence of inorganic and organic forms of phosphorus are important in soil (Mamathashree *et al*., 2018). Phosphate mobilization is mediated by soil microbes though organic acids, enzymes, and microbial activity (Barea and Richardson, 2015). Solubilization, on the other hand, can take place by acidification, chelation and proton exchange reactions (Ilmer and Schinner, 1995).
Moringa oleifera (drumstick tree), a drought-resistant plant with nutritional and medicinal properties, has recently been shown to enhance soil fertility in phosphorus-deficient soils (Paliwal *et al*., 2011).
As a natural fertilizer, fermented leaf juice of *Moringa* was first developed and applied to tomato and papaya crops resulting in better yield (Phiri, 2010). Diluted *Moringa* leaf extract significantly increased seed and seedling vigour in wheat (Afzal *et al*., 2008), maize (Yasmeen *et al*., 2012) and many grass species including *Cenchrus ciliaris*, *Panicum antidotala* and *Echinochola crusgalli* (Nouman *et al*., 2012).

Moringa leaves are themselves rich in phosphate, indicating that some phosphate solubilising activity is present in them. Although *M. oleifera* plant extract is known to possess diverse medicinal and biological activity on human and animals, little is known about its mechanism as bioorganic fertilizer (Abdalla, 2013).

2. MATERIALS AND METHODS

2.1 Plant Material

Fresh moringa *(Moringa oleifera*) leaves were collected from local organic farms. Leaves were carefully washed with distilled water to remove any surface contaminants and air-dried at room temperature.

2.2 Preparation of Leaf Extract

Freshly washed and dried moringa leaves were made into a slurry using a mixer. An aqueous extract was prepared by mixing distilled water, centrifuged to remove solid particles and stored until further use.

2.3 Detection of change of pH of Moringa leaves

The pH of the aqueous extract of the Moringa leaves was tested periodically from day 1 to day 7 using a digital pH meter. The aqueous extract was also titrated against 0.1N NaOH using phenolphthalein as an indicator (Nenwani *et al*., 2010). The volume of NaOH solution required to neutralize the acid in 5.0 ml of the extract from day 1 to day 7 was recorded.

2.4 Phosphate solubilizing activity of fermented *Moringa* leaf extract

The phosphate solubilizing activity of the leaf extract was tested on insoluble calcium phosphate. 5 ml of 0.5 % calcium phosphate was incubated with 1ml of stored filtrates for 6 days at room temperature. The amount of soluble phosphate was estimated according to Fiske-Subbarow method (Fiske and Subbarow, 1925). Ammonium molybdate was used as the acid reagent and 1, 2, 6-aminonaphtholsulfonic acid (ANSA) as reducing reagent. Aliquots (0.5ml) from the incubated samples were taken in the test tubes and made up to 1ml with distilled water. 1ml of acid and reducing reagents were added to all the test tubes and incubated for 30 minutes. 7 mL of distilled water was added to test tubes and the absorbance was read at 640 nm on a colorimeter. Potassium dihydrogen phosphate was used as a standard and 5 ml of 0.5 % insoluble calcium phosphate with 1 ml distilled water was used as the control.

2.5 Detection of phosphate solubilizing epiphytes and endophytes

Two types of media were used for the isolation of phosphate solubilising microbes. Pikovskaya's agar (Pikovskaya, 1948) and NBRIP medium (Nautiyal, 1999) were prepared according to the composition given in Table 1.0.

Table 1.0 Composition of Pikovskaya's (left) and NBRIP (right) agar in 100ml distilled water.

Yeast extract	0.05g
Dextrose	1g
$Ca_3(PO_4)_2$	0.5g
$(NH_4)_2SO_4$	0.05g
KCl	0.02g
$MgSO_4$	0.01g
$MnSO_4$	0.1g
$FeSO_4$	0.0001g
Agar	1.5g

Glucose	1g
$Ca_3(PO_4)_2$	0.5g
$MgCl_2$	0.5g
$MgSO_4$	0.025g
KCl	0.02g
$(NH_4)_2SO_4$	0.01g
Agar	1.5g

The medium was autoclaved at 15psi for 20 mins. Calcium phosphate and the inorganic salts were autoclaved separately and mixed just before the medium was poured into sterile Petri dishes.

Moringa leaves were surface sterilized with 70% ethanol and 1% mercuric chloride. The leaves were placed directly (for epiphytes) or crushed aseptically (for endophytes) and the exudate was plated on Pikovskaya's agar, NBRIP medium, and Potato Dextrose Agar (PDA) containing 1% streptomycin. All the Petri dishes were incubated at 28-30°C for 24-48 h.

2.6 Identification of fungal endophyte:

The fungal colony on PDA agar was stained with lactophenol blue and the morphology was observed under 40X magnification. The isolate was sent to Sakhala Enterprises, Bangalore, for identification by 18S rRNA sequencing.

2.7 Detection of phosphate solubility by TLC:

Thin-layer chromatography (TLC) of organic acids was carried out on Silica gel 60F254 sheets (Merck) with the following solvent systems:

a) Dichloromethane: methanol (1:1)
b) Ethyl acetate: acetic acid: water (3:1:1)

The samples analysed were the aqueous extract of Moringa leaves and the culture filtrate of phosphate solubilising endophyte. Oxalic acid (1%), citric acid (1%) and gluconic acid (1%) were used as standards. After the run, plates were air-dried and developed in potassium permanganate stain [$KMnO_4$ (75%), K_2CO_3 (5%) and NaOH (10%)]. The

stained plate was dried using a hair-dryer set on high heat till the organic acid spots appeared as yellow- coloured against a pink background.

3. FIELD STUDIES

4 pots with approximately 100g of autoclaved soil were taken. 5g of calcium phosphate was mixed with the soil in two of the pots. Moringa leaf extract fermented for 4 days was added to one pot with and one pot without the calcium phosphate. A fixed number of sprouted fenugreek seeds was added to all four pots and allowed to grow for two weeks. The number of seedlings in the pots were counted and related to the soil conditions.

4. RESULTS AND DISCUSSION

In organic farming, Moringa leaves have gained attention as an effective natural fertilizer. Rajamani *et al*. (2015) have shown that Moringa leaves not only enrich the soil with essential nutrients but also facilitate the solubilization of inorganic phosphates, thereby improving overall soil health and agricultural productivity.

An earlier study to determine the phosphate solubilising activity of Moringa leaves was undertaken by Deepthi N. (2019). Heat treatment did not destroy the activity and a decrease in pH was seen to mobilise phosphate. This was attributed to the presence of organic acids, but the acid was not clearly identified.

With this background, the study was repeated in order to identify the organic acid as well as the possible endophyte responsible for its production.

pH Measurement of Moringa Leaves Extract

The pH of *Moringa oleifera* leaves extract was measured from Day 1 to Day 6 to evaluate the changes in acidity over time. Fresh Moringa leaves were extracted in distilled water and allowed to settle. The pH was measured using a calibrated pH meter at the same time each day to ensure consistency. The pH of the Moringa leaves extract showed a decreasing trend from Day 1 to Day 6, dropping from 6.00 to 5.06 (Fig 1).

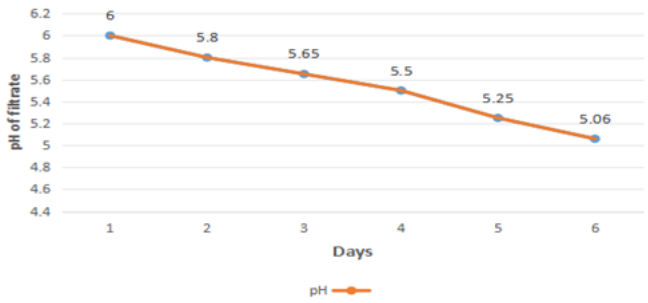

Fig 1. Decrease in pH of Moringa leaf extract from day 1 to day 6.

The decrease in pH indicates an increase in acidity in the Moringa leaves extract over the six-day period. This change is likely associated with the production of organic acids.

Assessment of Organic Acid Production Using Titration Method

The production of organic acids from *Moringa oleifera* leaf extracts was evaluated using a 0.1N sodium hydroxide (NaOH) as a titrant, and phenolphthalein as an indicator in the conical flask containing the Moringa leaves extract. There was a consistent requirement of NaOH to neutralize the organic acids in the Moringa leaves extract from day 1 to day 4 after which the acid concentration was stabilised. This suggests a buffering effect, possibly due to the presence of a weak organic acid.

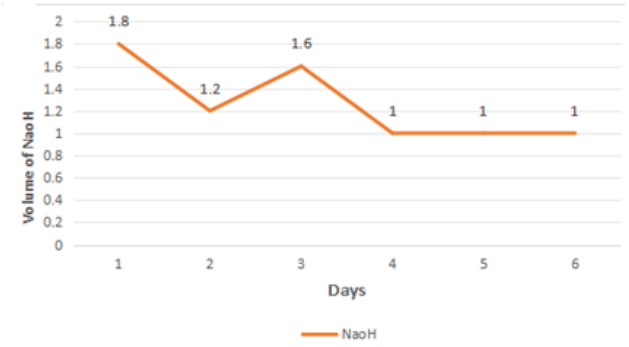

Fig 2. Titration of organic acid(s) in Moringa leaves against 0.1N NaOH.

The requirement of NaOH confirmed the presence of organic acids in the Moringa leaf extract over the six-day period. The stabilisation of the acid production after day 4 was seen in contrast to the steady decrease in pH seen in Fig 1. The volume of NaOH required to neutralise the acid component indicated the presence of a dicarboxylic acid.

Phosphate Solubilizing Activity of Moringa Leaves Extract

The phosphate solubilizing activity of *Moringa oleifera* leaves extract was evaluated over a period of six days. The leaf extract was incubated with insoluble calcium phosphate slurry and the phosphate released was measured using the Fiske-Subbarow method.

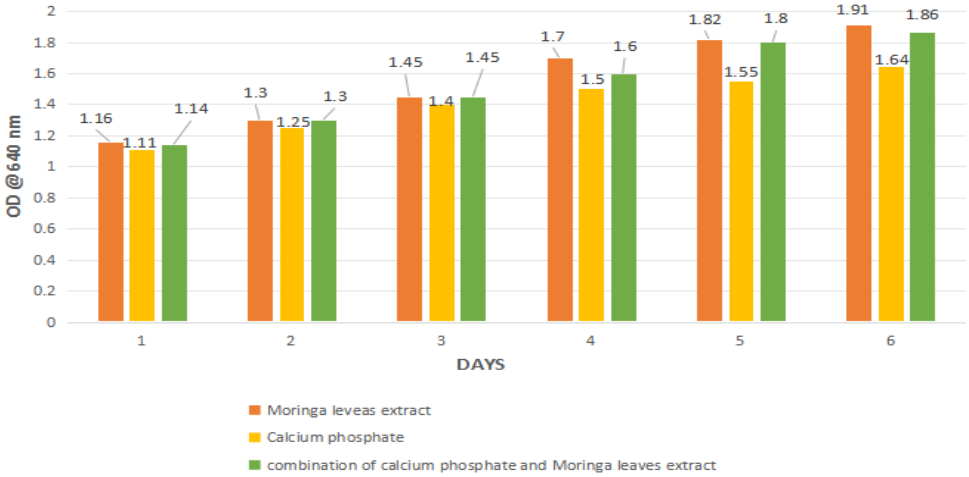

Fig 3. Phosphate solubilization activity of moringa leaves over time.

As seen in Fig 3, Moringa leaf extract enhances phosphate solubilization both on its own and in combination with calcium phosphate from day 1 to day 6. This is not unexpected since the leaves, by themselves are phosphorus rich. In addition to the existing phosphate, the phosphate from the insoluble calcium phosphate was released. The highest phosphate release activity was observed with the day 5 Moringa leaves extract, suggesting its efficacy in increasing soluble phosphorus levels in the medium.

Endophyte Isolation from *Moringa oleifera* Leaves

While plants are known to produce organic acids, the secretion of such molecules is mainly associated with the presence of endophytes (Adeleke and Babalola, 2021). Towards this, endophyte isolation was performed to identify the microbial community associated with *Moringa oleifera* leaves, specifically on two different media: Potato Dextrose Agar (PDA) and NBRIP (National Botanical Research Institute's Phosphate) medium.

Epiphytes were excluded by thoroughly washing the leaves with sterile distilled water, surface-sterilized using 70% ethanol followed by 10% bleach, and rinsed with sterile water. Leaf sections inoculated on PDA (plus 1% streptomycin) showed the presence of a single fungal colony on while no consistent results were obtained with NBRIP media (Fig 4) or Pikovskaya medium (data not shown).

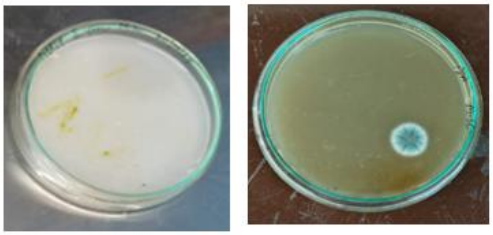

Fig 4. Isolation of endophytes from Moringa leaves. NBRIP agar (left) and PDA (right).

The fungal isolate was identified as *Aspergillus spp.,* characterized by its conidia, septate hyphae and typical microscopic appearance (Fig 5).

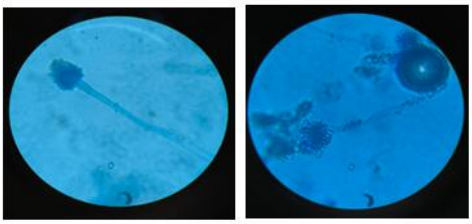

Fig 5. Identification of the Moringa leaf fungal endophyte as *Aspergillus spp*.

Identification of Fungal Endophyte

The fungal endophyte isolated from *M. oleifera* leaves was identified by 18S rDNA sequencing. A phylogenetic tree of the endophyte and closely related species was constructed based on the ITS (Internal Transcribed Spacer) sequences. Sequence analysis revealed a high similarity (99%) to *Aspergillus fumigatus* (Fig 6a and b). Hence, the isolate was identified as *Aspergillus fumigatus* (database accession number 0824_708_001_PCR_PDB_NS4_E10.AB1).

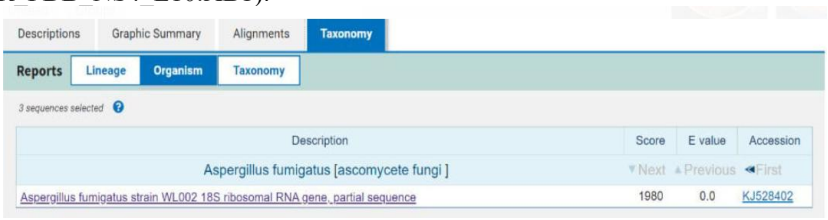

Fig 6a. Identification of the fungal isolate from *M. oleifera* leaves.

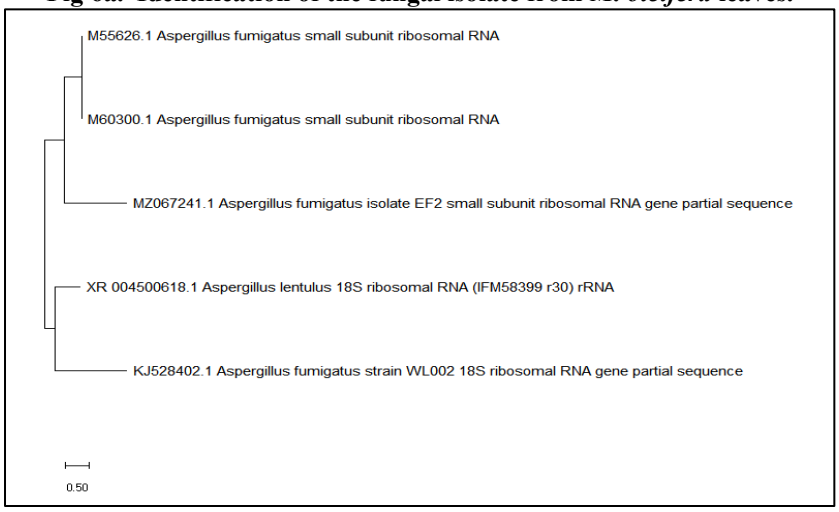

Fig 6b. Phylogenetic tree showing the relationship of the isolated strain to related species.

Secretion of Organic Acids by the fungal endophyte

Thin Layer Chromatography (TLC) was employed to analyze the presence of organic acids in the fungal culture broth and Moringa leaves extract. Citric acid, oxalic acid and gluconic acid were used as standards, with a solvent system of ethyl acetate: acetic acid: water (3:1:1). Upon staining with $KMnO_4$ stain, the fungal culture showed the presence

of oxalic acid while the Moringa leaf extract showed the presence of a number of organic acids (Fig 7).

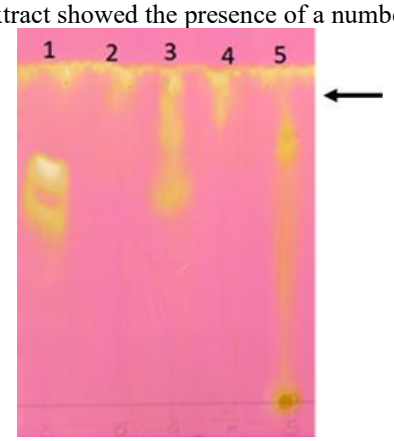

Fig 7. TLC of organic acids from the fungal endophyte (lane 4) and Moringa leaves (lane 5). Lanes 1, 2 and 3 are the citric acid, oxalic acid and gluconic acid standards.

The fungal culture broth exhibited a prominent spot corresponding to oxalic acid. The Moringa leaves extract revealed spots for both oxalic and gluconic acids, indicating the production of multiple organic acids.

Field Study on Phosphate Solubilization

After having established the phosphate solubilisation activity of Moringa leaves, a field study was undertaken to evaluate the effects of the leaves on the growth of Fenugreek (*Trigonella foenum-graecum*). A set of four pots were set up with the following combinations:

Pot 1: Soil only
Pot 2: Soil + Calcium phosphate
Pot 3: Soil + Calcium phosphate + Moringa leaves extract
Pot 4: Soil + Moringa leaves extract

Each set had three replicates, with uniform number (35) of sprouted fenugreek seeds planted in all pots. Seedling growth was monitored over a period of three weeks and the number of surviving seedlings recorded showed significant variations among the treatments.

Table 2: Effect of Moringa leaf extract on the growth of Fenugreek in soil with and without a) calcium phosphate and b) Moringa leaf extract.

Pot no	Treatment	No. of seedlings
1	Soil Only	13
2	Soil + Calcium Phosphate	15
3	Soil + Calcium Phosphate + Moringa Extract	22
4	Soil + Moringa Extract	30

Fig 8. In vivo effect of Moringa leaf extract on the growth of fenugreek in A: soil B: soil + calcium phosphate C: soil + calcium phosphate + Moringa extract and D: Soil + Moringa extract.

As seen in Table 2 and Fig 8, the addition of Moringa leaf extract enhanced the number of seedlings that survived even in the presence of insoluble phosphate. This suggests that Moringa leaf extract can be used as an eco-friendly and economically viable means of converting un-arable land containing insoluble phosphates into cultivable land.

Presence of Mucilage in *Moringa oleifera* Leaf Extract:
When the project was initiated in the month of May - during the summer season – it was observed that the leaf extract turned slimy (Fig 9). Within a couple of days, the extract congealed into a thick slurry, making it difficult to obtain a watery supernatant to determine phosphate solubilising activity.

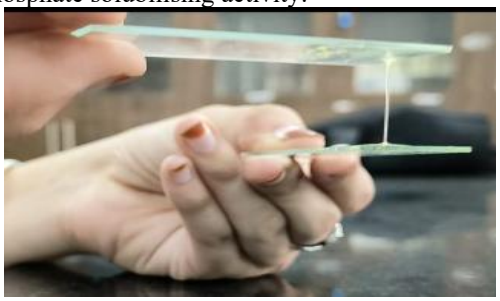

Fig 9. Demonstration of the presence of mucilage in Moringa leaf extract.

Mucilage is a gelatinous substance that can retain water and improve soil moisture retention. This characteristic can further enhance the extract's role in promoting plant growth, especially in arid conditions. However, the discovery of mucilage in Moringa leaves indicates that the preparations made in the summer months may not be effective in phosphate solubilisation.

To summarise our findings, *Moringa oleifera* leaf extract has significant potential in agriculture by enhancing soil and plant health through various mechanisms such as:
1. Organic acid production and pH regulation: The extract promotes organic acid production, lowering soil pH and improving nutrient uptake in alkaline soils.
2. Phosphate solubilization: Moringa extract enhances the solubilization of phosphorus, making it more accessible for plant absorption, which boosts root development and plant vigor.
3. Promotion of plant growth: Improved nutrient availability and soil conditions lead to increased biomass, healthier root structures, and higher crop yields.
4. Beneficial endophytes: The extract supports beneficial endophytes that enhance nutrient cycling and plant resilience while promoting microbial diversity in the soil.
5. Sustainability in agriculture: Using Moringa extract aligns with sustainable farming practices, reducing reliance on chemical fertilizers and fostering eco-friendly techniques.
For the first time, we have shown the presence of a fungal endophyte which produces oxalic acid capable of solubilizing phosphate.
In conclusion, *Moringa oleifera* leaf extract is a valuable natural resource for enhancing soil health and supporting sustainable agriculture. However, further research is needed to address the impact of mucilage and high summer temperatures on the efficacy of phosphate release from insoluble soil phosphates like aluminum or ferric phosphate.

5. ACKNOWLEDGEMENTS

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6. AUTHOR CONTRIBUTIONS AND STATEMENTS

DN and VB conceptualized the project; DN initiated the project and standardized the assays to measure the phosphate solubilizing activity; SS confirmed DN's results and isolated the fungal endophyte; DN, SS, NZS and VB were involved in manuscript preparation.
AI Statement: The authors declare that they have **not used** generative artificial intelligence, specifically ChatGPT, in the writing of this manuscript and/or in the creation of images, graphics, tables, or their corresponding captions.
Conflict of Interest: The authors state that they do not have any conflict of interest.
Informed Consent / Ethical Compliance: Authors state that there is no informed consent/human or animal involvement in the article.
Data Availability: All data included in this research article will be provided on request.

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