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Novel biofertilizers from nutmeg rhizosphere of Wayanad, Kerala

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Nutmeg, *Myristica fragrans* Houtt., an important spice crop from India, is mostly grown in parts of Kerala state. Here, we investigated the rhizosphere region of the nutrient exhaustive tree spice nutmeg for nitrogen fixers, phosphorus solubilizers and potassium solubilizers. In this study, we sought to find new biofertilizer strains from the nutmeg rhizosphere. Ten rhizosphere soil samples of nutmeg were collected from Wayanad district in Kerala. A total of 55 predominant isolates were obtained. The isolates produced indole acetic acid (IAA) in amounts between 0.4 and 11.81 µg mL^{-1}, fixed nitrogen in amounts between 2.1 and 3.5 mg g^{-1} of carbon source, solubilized phosphorus in amounts between 57.91 and 71.647 µg mL^{-1}, and solubilized potassium in amounts between 6.66 and 8.30 mg mL^{-1}. Based on the specific plant growth promoting traits and IAA production, 15 isolates were characterized and selected for abiotic stress tolerance (moisture and temperature) studies. With the molecular characterization of 16S rRNA, the six promising isolates were identified as *Staphylococcus equorum* (nitrogen fixer), *Staphylococcus pasteuri* and *Pseudomonas aeruginosa* (phosphate solubilizers), *Pseudomonas veronii* and *Sphingobacterium anhuiense* (potassium solubilizers). Possibly, it is the first report of novel biofertilizer strains from nutmeg growing areas of Wayanad district in Kerala.

Keywords: Abiotic stress tolerance, Indole acetic acid (IAA), *Myristica fragrans*, Plant growth-promoting rhizobacteria (PGPR), Spice

The living and efficient strains of microorganisms that aid in boosting crop output are termed as biofertilizers. Through nitrogen fixation, phosphorus solubilization, potassium mobilization or solubilization, zinc solubilization, siderophore and indole acetic acid (IAA) production, microorganisms can increase the nutrient uptake of plants. More to the primary mechanisms, microorganisms in the rhizosphere region also produce hormones that enhance plant growth, and compounds that prevent diseases. Due to the plant chemical signals, the rhizosphere region contains the most advantageous microbes as opposed to free soil. The native soil microflora contributes more to the plants because of their adaptability and competence to nutrients and space rather than introduced microorganisms. When these microorganisms are introduced as biofertilizers, the dependency on chemical fertilizers can be reduced, and lead to sustainable agriculture. Solid based or liquid types comprising of plant associated microorganisms have emerged as a cost effective and ecofriendly alternative to chemical fertilizers and pesticides[1]. Microorganisms also produce volatile organic compounds like acetone, 3-butane diol, 2-butanone, etc., promoting plant growth by enhancing physiological processes. Amendment of the microorganisms with locally available organic materials and nanoparticles are currently used to formulate biofertilizers[2]. Recently, to improve the beneficial characteristics of plants and microorganisms, genetic engineering technology is utilized to improve nutrient availability, soil fertility and crop yield[3].

Nutmeg is a spice crop cultivated for twin spices, the aril and kernel. With its pharmacological properties like anticarcinogenic, hepato-protective and anti-inflammatory activity, is mostly used in food flavoring, essential oil extraction and in traditional medicines. But nutmeg being a nutrient exhaustive crop, microorganisms can be quite important for sustaining soil health[4]. Recently, due to the change in climate, there has been a change in nutmeg productivity[5]. Root diameter, length, and density are important for improving the plant growth under drought conditions[6]. Plant rhizosphere favours the nutrient acquisition, especially nitrogen fixation, as a conducive environment with high carbon substrates

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and low oxygen is present[7]. Stress tolerant rhizosphere microorganisms mitigates abiotic stresses by enhancing antioxidant potential, improving nutrient acquisition, regulating the production of plant hormones, ACC deaminase, siderophore, exopolysaccharides and osmo-protectants resulting in increased crop yield[8].

Wayanad district in Kerala is a place well known for organic production of spices. The present nutmeg productivity[9] in Wayanad district is 370 kg ha^{-1}. Kerala, being the land of rainfall with acidic laterite soil, the hurdle of absorption of nutrients and growth of microorganisms exists. Hence, here, we have made an attempt was made to isolate, characterize, and identify the nitrogen fixers, phosphate solubilizers and potassium solubilizers from the rhizosphere soils of nutmeg from different locations of Wayanad district.

Materials and Methods

Nitrogen fixers, phosphate solubilizers and potash solubilizers were isolated using differential media in the serial dilution and plating technique. Nitrogen free Jensen's agar and Ashby's agar culture media were employed for isolation of nitrogen fixers, Pikovskaya's agar culture media for isolation of phosphorus solubilizers and Alexsandrov's agar culture media for isolation of potassium solubilizers. The screening of the predominant isolates were carried out for its plant growth promotion ability under *invitro* namely nitrogen fixation, phosphorus solubilization, potassium solubilization, and indole acetic acid (IAA) production. The quantitative estimations were carried out for nitrogen fixation[10,11], phosphorus solubilization[12], potassium solubilization[13] and IAA production[14].

Based on the *in vitro* screening, fifteen promising isolates of nitrogen fixers, phosphorus solubilizers and potassium solubilizers were subjected to morphological, cultural and biochemical characterization[15] and further screened for moisture stress tolerance[16] and for high temperature tolerance[16] under *in vitro*. Different moisture stress was induced with the addition of 10, 20, 30 and 40% polyethylene glycol (PEG 6000) and heated to tryptone soy broth (TSB) and made up to required quantity with unamended TSB. Fresh cultures of isolates were inoculated and incubated at room temperature (30°C) for 48 h. OD values were recorded at 24 and 48 h. Similarly, for temperature tolerance experiment, incubator was maintained at 30, 35, 40 and 45°C for 48 h. Five mL of TSB was taken in 50 mL of screwcap tubes, autoclaved, inoculated with fresh cultures and incubated at respective required temperatures. OD values were recorded at 24 and 48 h of incubation.

After the screening, compatibility of fifteen isolates were evaluated using cross streak and dual culture method[17]. The dual culture method was carried out in a Petri dish, by growing one bacterial isolate with spread plate technique, while the other in the centrally placed well. Based on the plant growth encouraging traits, abiotic stress tolerance and compatibility studies, the most compatible nitrogen fixers, phosphorus solubilizers and potassium solubilizers were subjected to molecular characterization by 16S rRNA sequencing. Forward and reverse sequences were aligned with DNA baser tool and identified with available sequences using BLAST. The Mega software 11 CLUSTAL W programme was used to align the sequences. The same programme was employed to create phylogenetic trees using the neighbour joining tree method[18].

Results and Discussion

The abundance of the beneficial microorganisms in the rhizosphere of nutmeg ranged from 4.67×10^3 to 140.67×10^4 CFU g^{-1} of soil (Table 1). The highest population was recorded in the case of phosphate solubilizers (81×10^4 to 140.67×10^4 CFU g^{-1} of soil). Maximum nitrogen fixers were found in Kolagappara region. Potassium solubilizers were found maximum (113.0×10^3 CFU g^{-1}) in Sulthanbathery region of Wayanad district. The variation in the population in different locations might be due to geochemical and physical conditions (pH, temperature, conductivity and rainfall)[19]. In the present study, changes in microbial population with environmental conditions were found, which is in agreement with the previous study.

A total of 55 predominant isolates of nitrogen fixers, phosphorus solubilizers and potassium solubilizers were obtained and examined for characters that foster plant growth through nitrogen fixation, phosphorus solubilization and potassium solubilization. Nitrogen fixation of 21 predominant isolates varied from 0.7 to 4.9 mg g^{-1} of carbon source utilized. Phosphorus solubilized by the 18 predominant isolates ranged from 12.98 to 71.64 µg mL^{-1} and potassium solubilization by the 16 predominant isolates ranged from 6.66 to 8.3 mg mL^{-1}. Quantity of IAA produced by the 55 isolates varied from 0.263 to

Table 1 — Population of nitrogen fixers, phosphorus solubilizers and potassium solubilizers in the rhizosphere soil of nutmeg

Location	Jensen's agar*	Ashby's agar*	Pikovskaya's agar**	Alexsandrow's agar*
Mankunnu High range (MH)	15.33[a]	9.33[c]	86.0[ef]	4.67[d]
Mankunnu Karappuzha river basin (MK)	6.33[bc]	4.33[d]	81.67[f]	4.67[d]
Kolagappara (KP)	15.67[a]	155.0[a]	94.33[d]	5.33[cd]
Vazhavatta (VV)	7.0[b]	nil	140.67[a]	nil
Pulpally (PP)	nil	nil	91.67[de]	nil
Nenmeni (NM)	6.0[cd]	nil	121.67[b]	9.33[b]
Manjappara (MP)	nil	13.33[b]	81.0[f]	4.67[d]
Karinkutty (KK)	5.67[cd]	15.0[b]	97.33[d]	6.33[c]
Sulthanbathery (SB)	5.33[d]	nil	107.33[c]	113.0[a]
Vythiri (VT)	nil	6.33[d]	120.00[b]	9.33[b]

[*×10^3 cfu/g); and **×10^4 cfu/g). Each value represents a mean of 3 replications. Mean values in a column sharing similar alphabets do not differ significantly in ranking as determined by the DMRT test ($P \leq 0.05$)]

Table 2 — Morphological and cultural characters of beneficial microbial isolates

Isolates*	Colony characters						Microscopic characters	
	Size**	Texture	Shape	Colour	Elevation	Margin	Gram staining	Shape of cell
VTN 2	Small	Dry	Circular	Off white	Raised	Entire	Positive	Cocci
KPN 3	Medium	Dry	Circular	Off white	Flat	Entire	Positive	Cocci
MKN	Small	Dry	Circular	Off white	Flat	Entire	Negative	Rod
KPN 1	Medium	Mucoid	Circular	Off white	Raised	Entire	Negative	Rod
VVN 2	Medium	Mucoid	Circular	Off white	Raised	Entire	Positive	Rod
PPP	Small	Dry	Circular	Off white	Flat	Entire	Positive	Cocci
KKP 1	Small	Dry	Circular	Off white	Raised	Entire	Positive	Rod
MKP 2	Small	Dry	Circular	Brownish	Flat	Entire	Negative	Rod
KPP 2	Small	Dry	Circular	Off white	Raised	Entire	Positive	Rod
KPP 1	Small	Dry	Circular	Off white	Flat	Entire	Positive	Rod
KKK 2	Small	Dry	Circular	Pink	Flat	Entire	Positive	Rod
MPK	Small	Dry	Circular	Yellowish	Raised	Entire	Negative	Rod
NMK 2	Small	Dry	Circular	Off white	Flat	Entire	Positive	Cocci
KPK 1	Small	Dry	Circular	Off white	Flat	Entire	Positive	Cocci
MHK	Small	Dry	Circular	Off white	Flat	Entire	Negative	Rod

[*First two alphabets: Location code; N: Nitrogen fixer; P: Phosphorus solubilizer; K: Potassium solubilizer. **<1 mm: Small and 1 mm: Medium]

11.810 µg mL^{-1}. Earlier reports reveal that some of the highest recorded *in vitro* values for potassium solubilization was 1.75 mg/L[20], nitrogen fixation of 13.38 mg/mL[21], phosphorus solubilization of 250.77 mg/L[22] and IAA of 91.7 µg/mL[23]. The results are in agreement with the present study where the values were in the same range.

Based on these experiments, a total of 15 most promising isolates of nitrogen fixers, phosphorus solubilizers and potassium solubilizers were selected. Morphological and cultural characterization were recorded (Table 2). Most of the isolates were offwhite and circular with dry texture. Ten isolates were Gram positive bacteria, and five isolates were Gram negative bacteria. Five of the isolates were cocci shaped and ten of the isolates were rod shaped. Based on the morphological and cultural characters and its comparison, with the standard keys, the isolates (MKN, KPN 1, MKP 2, MPK and MHK) were tentatively assigned to *Pseudomonas* sp. (Peix *et al.*)[24] and *Rhizobium* sp. (Martinus-Romero)[25], the isolates (KPP 2, KPP 1, KKK 2, VVN 2 and KKP1) were tentatively identified as *Bacillus* sp. (Logan and Vos)[26]. The isolates (VTN 2, KPN 3, PPP, NMK 2 and KPK 1) were assigned to *Micrococcus* sp. (Weiser *et al.*)[27].

In order to screen for moisture stress tolerance, the 15 predominant isolates were selected. The growth of isolates varied from 0.06 to 1.53 OD values. Highest growth of bacteria was observed at 10% moisture stress as compared to higher moisture stress. The 15 isolates were also screened for temperature tolerance at 30, 35, 40 and 45°C in TSB for 48 h. The growth of bacteria varied from 0.11 to 1.95 OD values. The growth of bacteria was maximum at 30°C as compared to other temperatures. In addition to the tolerance for abiotic stress, the fifteen isolates were evaluated for their compatibility among themselves by cross streak and dual culture method. The isolate (potassium solubilizer - KKK2) was found to be incompatible with other (nitrogen fixers and

Table 3 — Plant growth enhancing traits and abiotic stress tolerance level of beneficial isolates from rhizosphere soils of nutmeg

Isolate Code	IAA (µg/mL)	N fixed (mg/g C)	P solubilized (µg/mL)	K solubilized (mg/mL)	OD values (45°c 48 h)	OD values (40% PEG)	Compatibility among themselves	Identity of organism
VTN 2	11.81	2.1	-	-	0.113	0.443	+	*Staphylococcus equorum*
KPN 3	7.26	2.45	-	-	0.536	0.477	+	*Staphylococcus equorum*
PPP	1.98	-	71.647	-	1.047	0.209	+	*Staphylococcus pasteuri*
MKP 2	1.0	-	57.91	-	0.243	0.546	+	*Pseudomonas aeruginosa*
MPK	3.56	-	-	8.3	0.591	0.534	+	*Sphingobacterium anhuiense*
MHK	9.78	-	-	6.7	0.156	0.062	+	*Pseudomonas veronii*

[-: not assessed, Each value represents a mean of three replication]

Fig. 1 — 16S r RNA sequence of isolates (A) VTN 2 isolate obtained from Vythiri; (B) MHK isolate obtained from Mankunnu high range; (C) KPN 3 isolate obtained from Kolagappara; (D) MPK isolate obtained from Manjappara; and (E) PPP isolate obtained from Pulpally

phosphorus solubilizers) isolates tested and the other fourteen (nitrogen fixer, phosphorus solubilizer and potassium solubilizer) were found to be compatible. Compatible isolates are generally considered for consortia formation. Based on the earlier report[28], efficient mineral solubilizing microbes and nitrogen fixers were found compatible among themselves to increase the yield of the crop Amaranth.

The PGPR isolates were statistically ranked based on the features that promote plant growth, tolerance to abiotic stress and compatibility studies. The 6 promising isolates (VTN 2, KPN 3, PPP, MKP 2, MPK and MHK) were further confirmed by molecular characterization of 16S rRNA gene sequencing. The details are provided in the Table 3 and Fig. 1 except *Pseudomonas aeruginosa* (Migula)[29]. The phylogenetic analysis of the isolates with the existing relevant bacteria are compared (Fig. 2 A-F).

Based on the maximum homology with query sequence, the nitrogen fixers *viz.*, VTN 2 and

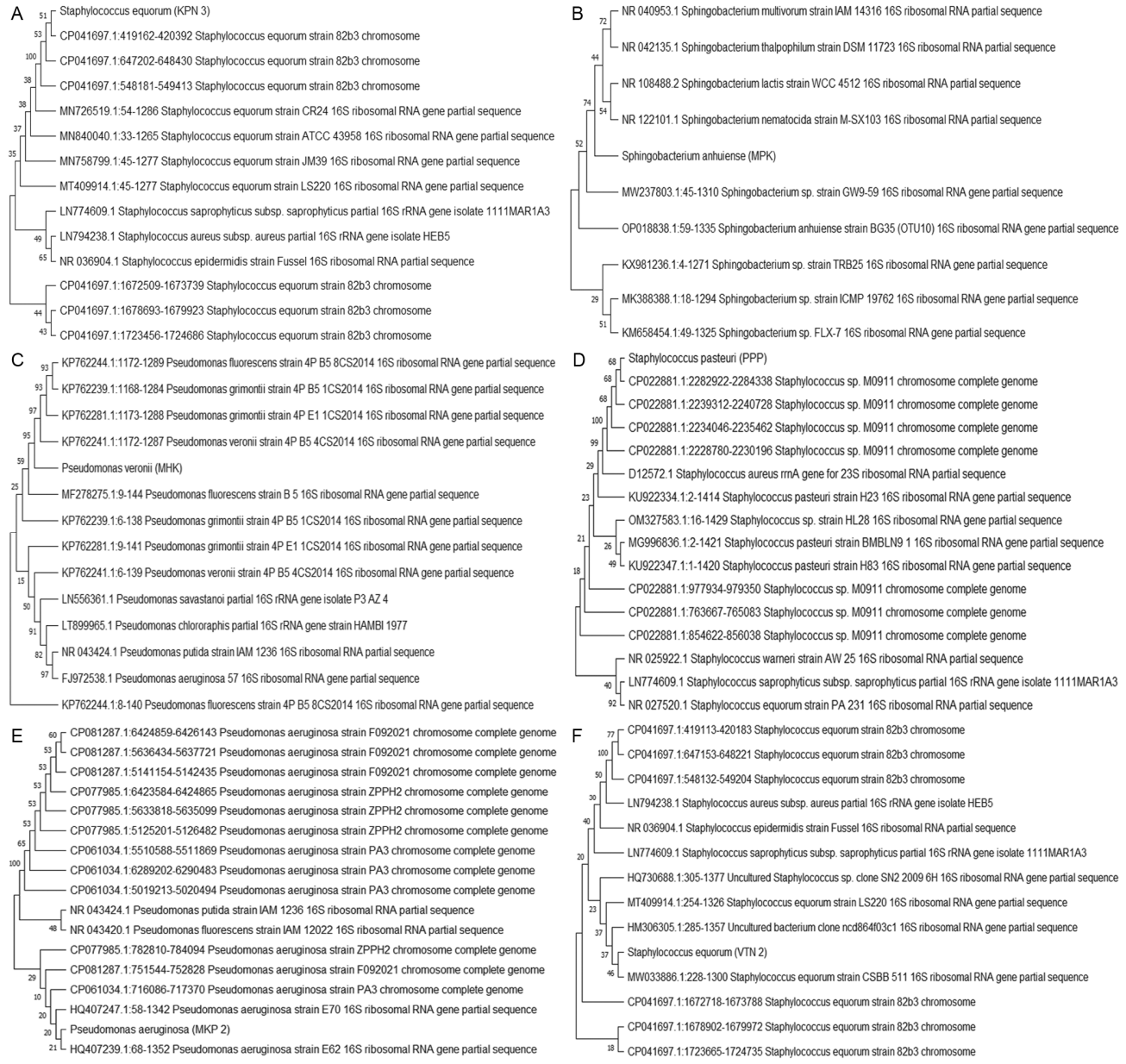

Fig. 2 — Phylogenetic analysis of (A) KPN 3; (B) MHK; (C) MKP 2; (D) MPK; (E) PPP; and (F) VTN 2 with existing strains of relevant bacterial species

KPN 3 had maximum homology with *Staphylococcus equorum* (Schleifer *et al*.)[30], and similarity percent of 83.26 and 99.35 (Accession number OQ789535), were obtained respectively. The results indicated urease activity and ammonia production by *Staphylococcus equorum* metabolism, a soil bacterium[31]. *Staphylococcus equorum* is reported to be a halotolerant and seed endophyte which enhanced shoot and root length along with the root weight of different plant species[32,33]. The reports of chickpea seed endophyte revealed IAA production of 52.33 µg per 100 mL and ammonia production of 5.31 $\mu g mL^{-1}$ along with the other plant growth promoting attributes which enhanced the root fresh and dry weight and shoot length. Non halophilic endophyte *Staphylococcus equorum* also contributed to the increased root weight and length in *Arabidopsis thaliana*. The isolates used in the current investigation, VTN 2 and KPN 3 had an IAA activity of 11.81 and 7.26 $\mu g\ mL^{-1}$, and nitrogen fixing capability of 2.1 and 2.45 mg/g of carbon source utilized, which confirms that the isolates can contribute to plant growth, and is also in agreement

with earlier studies. The phosphorus solubilizing isolates (PPP and MKP2) had maximum homology with *Staphylococcus pasteuri* (Accession number OQ789534) and *Pseudomonas aeruginosa* (Accession number OQ789533)[34]. Earlier studies revealed that *Staphylococcus pasteuri* can solubilize phosphorus[35] and potassium[36]. *Pseudomonas aeruginosa*, a soil bacterium has phosphate solubilization activity along with other plant growth enhancing attributes such as IAA, siderophore and HCN production[37], and these results are in agreement with the present study. In this study, *S. pasteuri* showed phosphate solubilization of 71.647 µg mL^{-1} and produced 1.98 µg mL^{-1} IAA and *P. aeruginosa* solubilized phosphorus of 57.910 µg mL^{-1} and produced 1.0 µg mL^{-1} IAA.

The potassium solubilizing isolates, *viz.*, MHK and MPK had maximum homology with *Pseudomonas veronii*[38] and *Sphingo bacterium anhuiense*[39] (Accession number OQ789532). *Pseudomonas veronii* is a rhizobacteria having IAA production[18], an endophytic nitrogen fixer[40] with biodegradable activity and mitigates water logging stress[41]. *Sphingobacterium* sp. is reported to be a plant growth enhancing bacteria which also produces secondary metabolites and enzymes[42] in the Himalayan region. In another study, IAA production of *P. veronii* was found positive and *Sphingobacterium prati* sp. *nov*. isolated from France had lignocellulosic biomass fractionation ability[43]. Endophyte (*Sphingobacterium changzhouense*)[44] from *Aloe vera* roots when inoculated to maize was effective for drought tolerance with enhanced root and shoot elongation[45]. In the present study, *P. veronii* had IAA production of 9.680 µg mL^{-1} and potassium solubilization of 6.7 mg mL^{-1} whereas, *S. anhuiense* had IAA production of 3.560 µg mL^{-1} and potassium solubilization of 8.3 mg mL^{-1}. The present *in vitro* experiments revealed that the isolates (MHK and MPK) with IAA production can contribute to the drought tolerance ability of the plants.

The present study revealed that *Staphylococcus equorum* (nitrogen fixer), *S. pasteuri* and *Pseudomonas aeruginosa* (phosphorus solubilizers), *P. veronii* and *Sphingobacterium anhuiense* (potassium solubilizers) could be possibly the first report of beneficial bacteria from the nutmeg rhizosphere in Wayanad district of Kerala, India.

Conclusion

The community structure of rhizophere is determined by the dynamic interactions between microbes and plants, microbes and microbes, and plants and microbes. The combination of the inoculants can provide the success of establishment of a plant and synergistic effects of consortium over conventional single inoculant. The acidic soil isolated free living rhizospheric bacteria can be exploited as biofertilizer for the nutrient exhaustive crop, and in this context, nutmeg and can lead to sustainable agriculture, especially in the hilly regions with heavy rainfall such as Wayanad. The six isolates VTN 2, KPN 3 (*Staphylococcus equorum* as nitrogen fixers), PPP and MKP 2 (*S. pasteuri* and *Pseudomonas aeruginosa* as phosphorus solubilizers) along with MHK and MPK (*P. veronii* and *Sphingobacterium anhuiense* as potassium solubilizers), could be developed as inoculant. However, further studies on multilocational trials are needed for its consideration as a commercial biofertilizer.

Conflict of Interests

Authors declare no competing interests.

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