Research Paper

Characterization of Native Phosphorus Solubilizing Fungal Species and Development of a Phosphorous Biofertilizer Formulation for Rice Cultivation

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Abstract

Inoculation of plants with phosphate solubilizing microorganisms (PSM) is a promising strategy to improve plant absorption of phosphorus (P) and thereby to reduce the usage of chemical P fertilizer. However, in Sri Lankan context, only a limited number of P biofertilizers has been identified. The objective of the study was to develop a low-cost P biofertilizer for rice cultivation in the low country wet zone. A phosphate solubilizing fungal isolate (Bentota isolate) isolated from rice rhizosphere was used to develop the P biofertilizer and the fungus was identified as *Penicillium oxalicum* through DNA sequencing. P solubilization ability of the fungus was tested in Pikovskaya's Broth Medium. Phosphate content released to the culture medium by *P. oxalicum* after seven days of incubation was 402.43 µg mL^{-1}. A low-cost growth medium with rice husks and rice seeds as the main ingredients was developed for mass multiplication of *P. oxalicum* was able to produce a sufficient colony count and survived up to 4 months in the growth medium maintaining a final population size of 22.7 x 10^8 CFU g^{-1} at room temperature and 33.5 × 10^8 CFU g^{-1} at 8 °C without losing the P solubilizing ability. The efficacy of the P biofertilizer was evaluated under field condition. Results revealed that the bioferlizer can reduce chemical P fertilizer usage by 50% without a yield reduction and can be used as alternative P fertilizer for rice cultivation in the Low Country Wet Zone in Sri Lanka.

Keywords: Biofertilizer, *Penicillum oxalicum*, Phosphate solubilization, Rice

Introduction

The problem of P deficiency in paddy soil is generally alleviated through the application of chemical P fertilizers despite of the high cost. At present, rice cultivation in Sri Lanka largely relies on chemical fertilizer (Amarasinghe *et al*., 2014). Phosphate anion in chemical P fertilizer is extremely reactive and large amount of applied chemical P fertilizer is fixed to form insoluble phosphate salt complexes depending on the pH of the soil. Thus, the plant utilization efficiency of chemical P fertilizer is only 5-25% (Zheng *et al*., 2017). Further, indiscriminate use of chemical P fertilizer potentially causes surface and ground water pollution, waterway eutrophication and development of high P concentrations in agricultural soils (Zhu *et al*., 2012). Under this scenario, there is an urgent need to investigate management strategies that are capable of reducing chemical P fertilizer usage and improving P fertilizer use efficiency.

A large number of soil microorganisms are capable of solubilizing insoluble soil phosphate. They are referred as Phosphate Solubilizing Microorganisms (PSM) and they increase the bioavailability of insoluble soil P for plant use. Inoculation of crops with PSM is a promising strategy to improve plant absorption of P (Gong *et al*., 2014). Occurrence of PSM has been reported from different environmental niches. In soil, phosphate solubilizing fungi (PSF) constitute from 0.05 to 0.1% of the total fungal population (De Bolle *et al*., 2012). High proportions of PSM are concentrated in the rhizosphere and rhizospheric fungi possessing P solubilizing activity are reported extensively (Rathore *et al*., 2014). Many studies have shown that growth and P uptake by plants can be enhanced by inoculation of PSF (De Bolle *et al*., 2012). Despite their great significance, Use of PSF is limited in commercial agriculture with conventional chemical P fertilizer. Lack of suitable low-cost media for mass multiplication of PSF and lack of evidence on field performance are considered as key bottlenecks for their wider use. Further, only a limited number of P biofertiizers have also been identified for rice cultivation in Sri Lanka. Therefore, the objectives of this study were to identify a phosphate solubilizing fungal isolate from rice rhizosphere, to identify low-cost medium for mass multiplication of the fungus and to study the performance of the developed P biofertilizer for rice under field condition in the Low Country Wet Zone (LCWZ) of Sri Lanka.

Materials and Methods

Molecular identification of P solubilizing fungal isolate used for the study

P solubilizing fungal isolate (designated as Bentota isolate) which was isolated from rice rhizosphere by Sandamali *et al* (2017) was selected for this study. Total DNA was extracted from a pure culture of Bentota isolate and subjected to the polymerase chain reaction (PCR) using universal primers, ITS 1 and ITS 4 and the purified PCR product was subjected to Sanger DNA sequencing (Macrogen, Korea). Sequence of the isolate was aligned with selected similar sequences obtained from National Center for Biotechnology

Information (NCBI) database using BioEdit software (7.2 version) (Kumar *et al*., 2018). A phylogenetic tree was constructed for the sequence of Bentota isolate and similar sequences available at NCBI using MEGA X software (Kumar *et al*., 2018) and the fungal species was identified. The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei,1987).

In vitro assessment of P solubilizing ability of the fungal isolate

P solubilization was quantified in Pikovskaya's Broth Medium amended with tri calcium phosphate at 5 mg L^{-1} (pH 7.2). Sterilized medium was inoculated with 5.00 mm size mycelial disk of 7 days old culture of Bentota isolate. Uninoculated medium was used as the negative control. Medium inoculated with 5.00 mm mycelial disk of 7 days old culture of *Aspergillus niger* was used as the positive control. The experiment was arranged in completely randomized design with four replicates. After inoculation, growth medium was incubated on a rotary shaker (160 rpm) at 30 °C for 7 days. Culture filtrate (5 mL) was withdrawn aseptically at 3, 5 and 7 days post incubation and soluble P content in the culture filtrate was measured by Molebdenum Blue Method at 420 nm (Singh and Reddy, 2011).

Studying the inoculation ability and P solubilizing ability of Bentota isolate in preserved cultures

Pure cultures of Bentota isolate were maintained in Potato Dextrose Agar (PDA) slants at 4 °C and used to test the P solubilizing and inoculation ability of Bentota isolate in preserved cultures with time up to three years. After 6 months, germinated rice seeds were inoculated with preserved culture of Bentota isolate (seeds were mixed for 24 hours with 1×10^8 CFU mL^{-1} spore suspension of Bentota isolate) and planted in pots with sterilized soil. Rhizospheric fungi were isolated using soil serial dilution plate technique on PDA medium one month after planting (Sandamali *et al*., 2017). Colony count of Bentota isolate was obtained using colony morphological characters after incubation at Room Temperature (RT) (30 ± 2 °C) for 7 days. Three replicates were performed. Plants, which were not inoculated with the fungus were used as the control.

P solubilizing ability of six-month-old preserved cultures of Bentota isolate was tested by inoculating the fungus on fresh Pikovskaya's Agar Medium and incubated at RT for 7 days (Pikoviskia , 1948). Pikovskaya's Agar Medium which were not inoculated with the microorganisms were used as the control. Three biological replicates were performed. P solubilizing ability was determined by formation of clear zone around the fungal colonies. The two experiments were conducted at 6 month intervals up to 3 years using the preserved cultures of Bentota isolate.

Multiplication, survival and P solubilizing ability of Bentota isolate in the growth medium with time under two storage temperature conditions

A low-cost growth medium was prepared using paddy husk and paddy seeds as the main ingredients. Packets containing the growth medium were prepared and sterilized. Sterilized, packets were allowed

to come to RT and inoculated with 7 days old pure culture of Bentota isolate grown in PDA medium (ten mycelial disks for each packet - 5.00 mm diameter). These packets were incubated under RT for 7 days. After the incubation period, inoculated packets were kept at two storage temperature conditions (at RT & at 8 °C under refrigerated condition). Six packets were kept at each temperature. Viability and P solubilizing ability of Bentota isolate on the growth medium was determined at 14-day intervals up to 5 months under the two storage temperature conditions. The viable population of the fungal isolate was determined by serial dilution plate technique on PDA medium and P solubilizing ability on Pikovskaya's Agar Medium respectively. At each sampling, 1.0 g from the medium was taken at 2 week intervals and a dilution series was prepared. 100 µL from each dilution was inoculated on to the PDA medium and fresh Pikovskaya's Agar Medium. Colony count on PDA plates and colonies with clear zones on Pikovskaya's Agar plates were recorded after 7 days of incubation at RT (Rajput *et al*., 2014). Colony count data were analyzed using SAS 9.1.3 statistical software package.

Evaluation of the P biofertilizer under field condition

An experiment was designed with five P fertilizer treatments to study the performance of P biofertilizer under field condition. The experiments were conducted using Bw 367 rice variety in two consecutive seasons (2017 *Yala* and 2017/2018 *Maha*) in a paddy field at Palathota, Kalutara of LCWZ of Sri Lanka which had an initial soil available P content of 6.25 mg kg^{-1}. The experiment was carried out in a randomized complete block design with three replicates and consisted with five treatments ie: T_1- Department of Agriculture (DOA) recommended rate of TSP (55 kg ha^{-1}) (positive control), T_2 - No P fertilizer (negative control), T_3 - TSP 50% of DOA recommended rate + P biofertilizer, T_4 - P biofertilizer + Compost, T_5 - P biofertilizer only). Whenever bio fertilizer is applied, germinated rice seeds were treated with the P biofertilizer for 24 hours before sowing (2 kg of P biofertilizer for 20.5 kg of paddy seeds). Further, field application was done at 3 weeks after sawing (2 kg of P biofertilizer for 1 ac). Nitrogen and potassium fertilizer were applied for all treatments according to the DOA recommendation (Urea - 100 kg ha^{-1}; Muriate of Potash - 110 kg ha^{-1}). Plot yield was obtained at the end of the harvesting. Data were statistically analyzed using SAS 9.1.3 statistical software package.

Results and Discussion

Identification of Bentota isolate by DNA sequencing

Sequence similarity analysis done using BioEdit (version 7.2.5) for the ITS region of the fungus revealed that the Bentota isolate shows more than 99% similarities with genetic sequence data of *Penicillium oxalicum* isolates available at the NCBI (Table 1). The evolutionary relationship built with Bentota isolate and the similar sequences available at the NCBI showed that Bentota isolate clusters with already reported *P. oxalicum* isolates. These molecular analyses shows that Bentota isolate belongs to *P. oxalicum* (Figure 1).

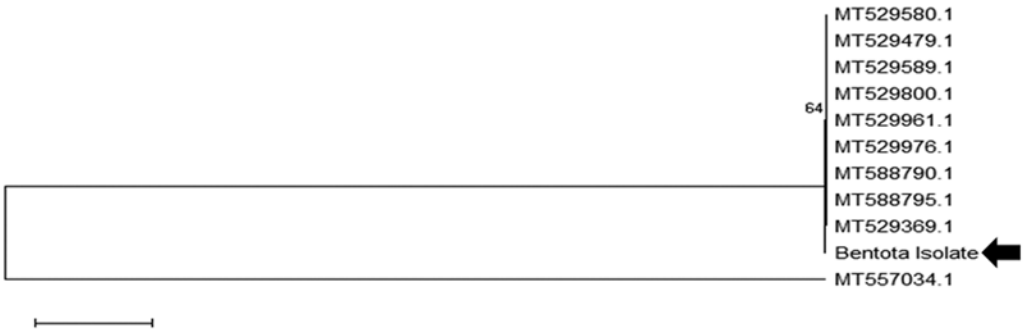

Figure 1. Evolutionary relationship of Bentota isolate with similar sequences in the NCBI

Table 1. Similarity between the IST region of Bentota isolate and similar isolates available at the NCBI

	Similarity with IST region of Bentota isolate %
P. oxalicum; MT588795	99.6
Penicillium sp.; MT588790	99.6
Fungal sp. isolate; MT 57034	35
P. oxalicum; MT529976	99.6
P. oxalicum; MT529961	99.6
P. oxalicum; MT529800	99.6
P. oxalicum; MT529589	99.6
P. oxalicum; MT529580	99.6
P. oxalicum; MT529479	99.6
P. oxalicum; MT529369	99.6

Quantitative P solubilizing ability of *P. oxalicum*

Soluble P content in the culture filtrates gradually increased with time up to 7 days. However, P solubilizing ability of A. niger was higher than *P. oxalicum* (Table 2).

Inoculation ability and P solubilizing ability of preserved cultures of P.oxalicum on Pikovskaya's agar medium

Sufficient population of *P. oxalicum* survived in the rice rhizosphere when plants were inoculated with preserved cultures of the fungus up to 36 months. Further, preserved cultures of *P. oxalicum* showed P solubilizing ability on Pikovskaya's Agar Medium up to 36 months (Table 3) showing the stability and the suitability of this fungal isolate for P biofertilizer development.

Table 2. Quantitative phosphate solubilizing ability of *P. oxalicum* and A. niger with time

Fungal isolate	Soluble Phosphate content in the filtrate (µg mL^{-1})		
	3 DAI	5 DAI	7 DAI
P. oxalicum	125.67	214.60	402.43
A. niger	332.42	439.03	555.01
Uninoculated control	7.00	7.16	7.08

(Values represents means of four replicates) (DAI – Days After Inoculation)

Survival and P solubilizing ability of *P. oxalicum* in the growth medium

Population of *P. oxalicum* in the growth medium under RT was comparable to initial up to 3 months and then reduced drastically after 4 months. A similar pattern of change in the population was observed when the growth medium was kept under 8 °C. However, the population decline of *P. oxalicum* was lower in the growth medium under 8 °C compared to RT storage condition.

Table 3. Population in rhizospheric soil and P solubilizing ability of *P. oxalicum* preserved cultures on Pikovskaya's Agar Medium

	Age of preserved culture of *P. oxalicum* (Months)					
	06	**12**	**18**	**24**	**30**	**36**
Population of *P. oxalicum* in rice rhizosphere (CFUg^{-1})	42 x10^5	58 x10^5	62 x10^5	44 x10^5	55 x10^5	46 x10^5
Phosphate solubilizing ability	+	+	+	+	+	+

(Represent means of three replicates)

P solubilizing ability of *P. oxalicum* existed in low-cost growth medium until the end of survival of *P. oxalicum* on that medium (Table 4). The results of this study provide evidences for existence of a sufficient population of the fungus in the growth medium up to 4 months and suitability of identified growth medium for a commercial preparation of *P. oxalicum*. Results of the statistical analysis is shown in Table 5.

Evaluation of developed P biofertilizer under field condition

Highest grain yield was recorded in TSP added plot and a similar yield was obtained in P biofertilizer + 50% TSP plot. It shows that the possibility of cutting down the recommended dose of TSP fertilizer by 50% under LCWZ condition. Yield was increased significantly by the application of P biofertilizer compared to No P fertilizer plot. There was no significant difference in yield between developed P biofertilizer + Compost added plot and only P biofertilizer added plot (Table 6).

There are several reports on plant growth promoting and biocontrol activity of *P. oxalicum* and it is widely used as a P solubilizing fungus and biocontrol agents in various crops. Biocontrol activity of *P. oxalicum* against Fusarium wilt of tomato was recorded by Larena *et al*. (2002).

Table 4. Survival and P solubilizing ability of *P. oxalicum* in the developed growth medium with time under two storage temperature

Storage Temperature		**WAI**									
		2	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**
At RT	Population of *P. oxalicum* CFUg^{-1}(x 10^8)	51.6	59.2	44.2	44.8	42.7	40.8	34.5	22.7	0.02	0
	P solubilizing ability	+	+	+	+	+	+	+	+	+	-
At 8 °C	Population of *P. oxalicum* CFUg^{-1}(x 10^8)	47.2	49.8	48.3	49.2	49.5	46.8	42.2	33.5	10.5	1.08
	P solubilizing ability	+	+	+	+	+	+	+	+	+	+

(Values represents means of six replicates) (WAI – Weeks After Inoculation)
(+) - Shows P Solubilizing Ability (-) - No P Solubilizing Ability

Table 5. Analysis of variance (ANOVA) of survival of *P. oxalicum* in the growth medium with time under two storage temperature

Source of variation	DF	p value	CD 5%
Treatments	19	0.000	
Factor A (time)	09	0.000	2.64×108
Factor B (Storage T)	01	0.000	1.18×108
A×B	09	0.000	3.74×108
Error	95		
Total	119		

Table 6. Effectiveness of the P biofertilizer under field condition in LCWZ

Treatment	Yield (tha^{-1})	
	2017 *Yala*	**2017/2018 *Maha***
T_1 Positive control (TSP)	1.893[a]	2.019[a]
T_3 P biofertilizer + 50% TSP	1.790[a]	1.943[a]
T_4 P biofertilizer + Compost	1.568[b]	1.446[b]
T_5 P biofertilizer	1.524[b]	1.455[b]
T_2 No P fertilizer	1.198[c]	1.092[c]
CV%	12.57	7.747

Means in each column followed by the same letters are not significantly different (p=0.05)

Increasing growth and nutrient uptake of wheat and maize plants by inoculation with *P. oxalicum* in alkaline soils was reported by Singh and Reddy (2011). Yin *et al*. (2015) reported P solubilization and growth promotion of maize by *P. oxalicum* in calcareous soil. These evidences show the potentiality of the developed P biofertilizer for other crops. The most important requirement of a growth medium for mass multiplication of beneficial microorganisms is the ability to produce sufficient population of the microorganisms and maintaining the population for a considerable time period. Rajput *et al*. (2014) have reported the amendment of carbon and nitrogen sources to organic substrate resulted in increased conidia production of *Trichoderma pseudokongii* on wheat straw, rice husk and millet grains. In the present study, rice seeds were added to enhance the growth and conidia production of *P. oxalicum*. Filamentous fungi need high oxygen content for growth, especially for sporulation. Thus, a solid medium with good aeration was selected for mass multiplication of *P. oxalicum*. Easy sterilization, easy manufacturing, low-cost and availability in adequate quantities, chemical and physical stability, assurance of sufficient shelf-life of the product, easy handling in the field and ease of releasing functional microorganism in to soil or seeds are the most important criteria that have to be considered when selecting a suitable carrier medium for multiplication of microrganisms (Ichriani *et al*., 2018). The growth medium was developed in the study considering aforementioned criteria. Cost of production is very important in developing biofertilizer and price of fertilizer shall not exceed conventional fertilizer to assure market sustainability. Approximate production cost of 1 kg of the P biofertilizer is Rs. 50.00. Amount of the biofertilizer required for one-acre paddy field for a season is 6 kg and approximate cost for P biofertilizer for one acre is approximately Rs. 300.00.

Conclusions

P solubilizing fungal isolate (Bentota isolate) used for the study was identified as an isolate belonging to *P. oxalicum*. The

P biofertilizer developed by multiplying *P. oxalicum* in low-cost growth medium could be stored at RT for 4 months with a final population size of 22.7 x 108 $CFUg^{-1}$ of the fungus. The P biofertilizer can be used as alternative P fertilizer for rice cultivation in the LCWZ. Further investigations are needed to study the potential of using the developed P biofertilizer for other crops.

References

Amarasinghe UGS, Ranawake AL, Senanayake SGJN 2014. Fertilizer response of some Sri Lankan traditional rice cultivars during vegetative growth phase. International Journal of Scientific and Research Publications 4(7):1-7.

De Bolle S, Gebremikael MT, Maervoet V, De Neve S 2012. Performance of phosphate-solubilizing bacteria in soil under high phosphorus conditions. Biology and Fertility of Soils DOI 10.1007/s00374-012-0759-1.

Gong M, Du P, Liu X, Zhu C 2014. Transformation of organic P fractions of soil and plant growth promotion by phosphate solubilizing ability of Penicilllium oxalicum I1. Journal of Microbiology 52(12):1012-1019.

Ichriani, GI, Syehfani NY, Handayanto E 2018. Formulation of Biochar-Compost and Phosphate Solubilizing Fungi from Oil Palm Empty Fruit Bunch to Improve Growth of Maize in an Ultisol of Central Kalimantan. Journal of Ecological Engineering 19(6): 45-55.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35:1547-1549.

Larena I, Melgarego P, De Cal A 2002. Production, survival and evaluation of solid-substrate inocula of Penicillium oxalicum, a biocontrol agent against Fusarium wilt of Tomato. Biological control 92(8): 863-869.

Pikoviskia RI 1948. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Microbiologia 17: 362-370.

Rajput AQ, Khansada MA, Sahsad S 2014. Effect of different substrate and carbon and nitrogen sources on growth and shelf life of Trichoderma pseudokoningii. International journal of agriculture and biology 16: 893-898.

Rathore P, Phanse N, Patel B 2014. Screening for Microorganisms Possessing Phosphate Solubilizing Potential. Indian Journal of Research 3(1): 172- 173.

Sandamali TGI, Mithrasena YJPK, Silva JN 2017. Antifungal activity and phosphate solubilization ability of fungi isolated from rice rhizosphere in low country wet zone of Sri Lanka. Proceedings of International symposium, University of Ruhuna Sri Lanka 2017: 23-25.

Saitou N, Nei M 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Singh H, Reddy S 2011. Effect of inoculation with phosphate solubilizing fungus on growth and nutrient uptake of wheat and

maize plants fertilized with rock phosphate in alkaline soils. European journal of soil biology 47: 30-34.

Yin Z, Shi F, Jiang H, Roberts DP, Chen S, Fan B 2015. Phosphate solubilization and promotion of maize growth by *Penicillium oxalicum* P4 and *Aspergillus niger* P85 in a calcareous soil. Canadian Journal of Microbiology 61: 913-923.

Zheng B, Hao X, Ding K, Zhou G, Chen Q, Zhang J, Zhu Y 2017. Long-term nitrogen fertilization decreased the abundance of inorganic phosphate solubilizing bacteria in an alkaline soil. Scientific Reports: DOI: 10.1038/srep42284.

Zhu HJ, Sun LF, Zhang YF, Zhang X, Qiao JJ 2012. Conversion of spent mushroom substrate to biofertilizer using a stress-tolerant phosphate-solubilizing Pichia farinose FL7. Bioresource Technology 11:410-416.