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Full Paper

Rhizobacteria in *Cyperus iria* L.: Elucidating its plant growth-promoting potentials

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Abstract

Excessive use of synthetic agents in agricultural production entails negative impacts in the environment. Hence, the search for effective and environment-friendly methods is imperative. This study explored the rhizosphere of *Cyperus iria* L., a common rice weed, for potential plant growth-promoting rhizobacteria. Fifteen rhizobacterial isolates were tested *in vitro* for plant growth-promoting characteristics such as phosphate solubilization, ammonia production, catalase production, and antifungal activity. Phosphate solubilization was tested by plating the isolates on Pikovskaya agar while ammonia production was determined via Nessler's reagent. Catalase production was determined using 3% hydrogen peroxide, while antifungal activity was tested against a plant pathogen, *Rhizoctonia solani*. Results showed that among the fifteen rhizobacterial isolates, five were phosphate solubilizers while eight showed antifungal activity against *R. solani*. All isolates tested positive for catalase test and negative for ammonia production. Based on the *in vitro* screening, the highest phosphate solubilization was observed in *Curtobacterium* sp. while significant antifungal activity against *R. solani* was demonstrated by *Bacillus* sp. It can be concluded that the rhizosphere of *C. iria* is associated with bacteria that can be further studied to elucidate its plant growth-promoting potential.

Keywords - ammonia production, antifungal activity, catalase activity, phosphate solubilization, plant growth-promoting rhizobacteria

Introduction

Intensification of agricultural production has resulted in the increased use of chemical substances to improve crop production. However, the excessive use of synthetic agents entails negative environmental impacts (Aktar et al., 2009). Several sectors have recognized the detrimental environmental effects of these chemicals, which resulted to a stronger call to practice organic agriculture (Organic Agriculture Act of 2010). Soil microorganisms are receiving attention as they show promising potentials in promoting plant growth (Kloepper et al., 2004). Plant growth-promoting rhizobacteria (PGPR) stimulate growth through the production of phytohormones (Maheshwari et al., 2015) and help increase plant nutrient uptake efficiency through siderophore production (Sayyed et al., 2013), nitrogen fixation (Figueiredo et al., 2008), and mineral solubilization (Yazdani et al., 2009). Also, PGPR enhance plant protection through antibiosis such as antifungal activity by inducing systemic responses or by producing substances such as hydrogen cyanide (HCN) to kill plant pathogens (Castro et al., 2009; Tariq et al., 2017).

Weeds have been a persistent problem in crop production (Peterson & Peterson, 1999). Weeds are considered unwanted because they can reduce crop yield as a result of weed-crop competition (Fickett et al., 2013; Javaid et al., 2007; Siddiqui et al., 2010). Also, weeds could have an allelopathic effect (Javaid et al., 2007) and serve as alternative hosts for pests and diseases (Lopes et al., 2003). Despite the negative impact of weeds in agriculture, they can be a source of biopesticides (Krishna et al., 2012) and novel microorganisms with plant growth-promoting activities (Sarathambal et al., 2013).

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Cyperus iria L., commonly known as "payong-payong" in the Philippines, is one of the common weeds in the irrigated and rain-fed lowland rice fields. It belongs to the genus *Cyperus* which had been considered as the "world's worst weed" (Holm et al., 1977) and continues to cause major losses in crop production across the world (Peerzada, 2017). It is known to co-exist in fields planted with rice, corn, sugarcane, cotton, and various vegetables (Mishra et al., 2016). The adverse impact of *C. iria* on rice as a competitor and an alternative host to pests and diseases had been established. However, little is known about its associated rhizobacteria. Hence, this study aimed to explore the rhizosphere of *C. iria;* specifically, to isolate and identify its associated rhizobacteria and evaluate its plant growth-promoting attributes including its antifungal effect against *Rhizoctonia solani*, a plant pathogenic fungus which infects a wide host range.

Materials and Methods

Collection of Rhizosphere Samples

Rhizosphere of *C. iria* was collected from a rice field in Midsayap, Cotabato, Philippines. The root system along with the bulk soil was removed from 0-20 cm depth at five randomly selected locations in the sampling site. After the plant was uprooted, the soil attached to its roots was collected by shaking and subsequent brushing of the remaining soil (cf., Yanai et al., 2003). The collected soil samples were carefully collected and transferred to sterile sampling bags and were labeled properly.

Isolation of Rhizobacteria

Ten grams of the composite soil samples were diluted in 90 mL of sterile distilled water in a 250-mL flask. The soil suspension was diluted up to 10^{-7}. Dilution factors 10^{-4} to 10^{-7} were spread on nutrient agar in triplicates and incubated for 24 hrs at 37 ± 2°C. Fifteen well-isolated colonies were randomly picked and re-streaked on nutrient agar plates to obtain pure cultures. The pure cultures were stored in a refrigerator and were sub-cultured every two weeks to maintain their viability.

In vitro Screening for Growth-Promoting Activities

Phosphate solubilization

The phosphate solubilizing activity of the rhizobacteria was tested in Pikovskaya agar. Each isolate was inoculated at the center of the agar plate in triplicates and incubated for seven days at 36 ± 2°C (cf., Sharma et al., 2011). A clearance or halo zone around the bacterial colony indicates positive result for phosphate solubilization (Figure 1a), while the absence of a halo zone indicates otherwise (Figure 1b).

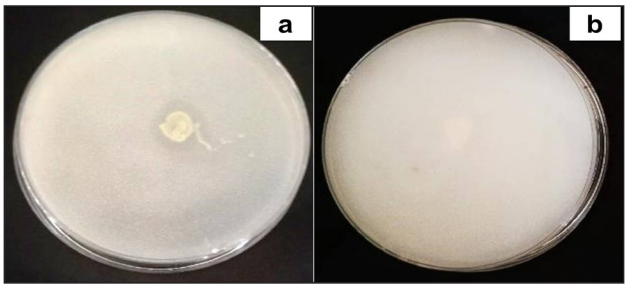

Figure 1. Phosphate solubilization test of PGPR isolates. a) PGP4 showing positive result for phosphate solubilization and b) PGP2 showing absence of halo zone.

The Phosphate Solubilization Efficiency (PSE) of the isolates was determined using the formula of Edi-Premono and Vleck (1996). The colony and the halo diameter were measured using a vernier caliper.

$$\text{PSE (mm)} = \frac{\text{Colony Diameter + Halo Zone Diameter}}{\text{Colony Diameter}}$$

Antifungal activity

The antifungal activity of the isolates was determined against the common plant pathogen *Rhizoctonia solani* Kühn obtained from the Plant Pathology Department, College of Agriculture, University of Southern Mindanao, Kabacan, Cotabato. A 6 mm mycelial plug obtained by puncturing the agar plate grown with *R. solani* (Figure 2a) using a sterile cork borer was inoculated at the center of a Potato Dextrose Agar (PDA) plate streaked with the rhizobacterial isolate in a square pattern (Figure 2b).

The distance of the mycelial plug from the edges of the square streak was approximately 2 cm. The inoculated PDA plates were incubated at 28 ± 2°C and the radial growth of the fungal colonies after five days was measured (Elkahoui et al., 2012). The percent inhibition (PI) of the fungal radial growth was calculated using the formula of Tiwari et al. (2016):

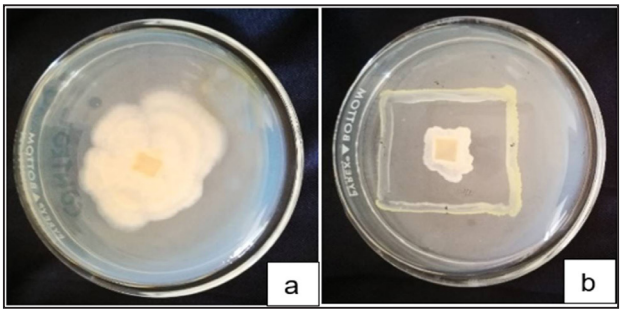

Figure 2. Antifungal Activity Test. a) Control plate containing pure culture of *R. solani* and b) dual culture assay of *R. solani* and PGP1 showing its zone of inhibition.

$$PI = \frac{(R - r)}{R} \times 100$$

where the *PI* corresponds to percent inhibition; *R* is the radial growth of the pathogen in the control plate and *r* is the radial growth of the fungal colony interacting with the antagonistic bacteria.

Catalase activity

The catalase test was performed by dropping 3% hydrogen peroxide to a 48-hour old rhizobacteria placed in a clean glass slide. Formation of effervescence indicates positive result for catalase activity (Rorth & Jensen, 1967).

Ammonia production

The production of ammonia was tested in 48-hour old isolates inoculated in 10 mL peptone water incubated at 30°C. Nessler's reagent (0.5 mL) was added after the incubation. The appearance of brown to yellow coloration indicates positive result for ammonia production (Capuccino & Shermann, 1992).

Characterization of the Rhizobacterial Isolates

Bacterial isolates were culturally and morphologically characterized. Cultural characteristics such as shape, elevation, margin, color (pigmentation), and opacity on nutrient agar plates were described and recorded. Morphological characteristics such as cell shape and gram reaction of each isolate were determined and the motility was observed through hanging drop method (Sharma et al., 2012).

All rhizobacterial isolates were sent to Philippine Genome Center (PGC) to confirm their identity. The DNA was extracted following the protocol of TRITON X-100 AMPure kit. The 16S rDNA gene of the extracted DNA was amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') with the thermal cycling parameters at 95°C for 5 minutes; 30 cycles at 95°C for 1 minute, 60°C for 45 seconds, 70°C for 1 minute; then 72°C for 10 minutes; and hold at 4°C, resulting in a specific DNA fragment of approximately 1400 bp. The PCR products were subjected to capillary sequencing. Both forward and reverse sequences were viewed in BioEdit, and sequence comparison was done in BLAST in GenBank of the National Center for Biotechnology Information (NCBI) and cross checked at http://www.ezbiocloud.net/eztaxon. The matched sequence with the highest percent homology was considered as the identity of the isolated rhizobacteria. Sequences with ≥ 97% homology with the sequences from the database were identified at a species level (Stackebrandt & Goebel, 1994) while sequences with percent homology below 97 were identified at the genus level.

Statistical Analysis

The Phosphate Solubilization Efficiency (PSE) and Percent Inhibition (PI) were statistically analyzed using one-way Analysis of Variance (ANOVA) and separated with Tukey's HSD Test ($p < 0.05$) in SPSS.

Results

Out of fifteen bacteria isolated from the rhizosphere of *C. iria*, nine showed positive result in at least one of the tested parameters for plant growth-promotion. Five isolates tested positive for phosphate solubilization. Among the phosphate solubilizers, isolate PGP4 exhibited the highest activity (3.49 PSE). There is significant difference ($p < 0.01$) among the PSE of the isolates. The result implies that there is variability on the phosphate solubilization efficiency of the rhizobacteria isolated from the common weed, *C. iria* (Table 1).

Eight isolates showed antifungal activity against *R. solani*. Isolate PGP1 (63.46%) showed the highest activity against the fungal pathogen. All isolates were capable of producing catalase while none were observed to produce ammonia (Table 1).

Characterization of the Rhizobacterial Isolates

The color of the rhizobacterial colonies varied on nutrient agar. The colony of all isolates were round

and raised with an entire margin. In terms of opacity, the isolates varied from opaque to translucent. The microscopic characterization revealed that seven out of nine possible PGPR isolates were gram positive. All isolates were rod in shape while only four are motile. The molecular identification based on percent homology revealed that the isolates belonged to five genera specifically *Bacillus* (PGP1, PGP2, PGP5, PGP6), *Rhizobium* (PGP3), *Curtobacterium* (PGP4), *Chromobacterium* (PGP7), and *Fictibacillus* (PGP8, PGP9) (Table 2).

Discussion

Phosphorus is considered as the second most required nutrient by plants (Pradhan & Sukla, 2006). However, a large concentration of phosphorus in the environment is in insoluble form (Liu et al., 2008). Phosphate solubilizing bacteria play an important role in the transformation of insoluble phosphates into utilizable forms. In the current study, the PSE of all rhizobacteria isolated from *C. iria* ranged from 1.37-3.49. These values are within the range of the reported PSE of known phosphate solubilizers such as *Acinetobacter* sp. and *Aeromonas* spp. associated in the rhizosphere of *Coffea arabica* L. (Muleta et al., 2013). Notably, Muleta et al. (2013) also reported *Pseudomonas* spp. exhibiting a PSE as high as 33.92. The PSE of rhizobacteria could fluctuate due to some factors such as the nutritional richness of the soil, physiology and growth status of microorganisms, the extent of vegetation, ecological conditions, soil types, agronomic practices, land use systems, plant types, soil physico-chemical properties, and its interactions with other soil microorganisms (Seschachala & Tallapragada, 2012). With the exessive and irresponsible application of synthetic chemicals by conventional farming, physico-chemical characteristics of the soil were greatly atlered which could negatively affect beneficial microorganisms reducing it's plant growth-promoting potential (Mabe, et al., 2017).

Catalase functions as an antioxidant enzyme which primarily protects aerobic bacteria and the plant from the damaging effects of hydrogen peroxide (H_2O_2) and the pathogens (Quan et al., 2008). In the study of Senthilraja et al. (2013), catalase positive rhizobacteria provide protection in tomato against blight disease. In addition, an increased level of catalase in tomato and myrtle can help improve the photorespiratory activity of the plants (Mittova et al., 2004).

In contrast to other rhizobacteria, all isolates in this study were negative for ammonia production. It implies that the isolates do not participate in nitrogen fixation, a process known to benefit the plant. In this study, *Bacillus* (Allagheny et al., 1996) and *Rhizobium* (Wagner, 2011) are bacterial genera known to include species that are nitrogen fixers. However, the amount of nitrogen produced during the test might not be at the detectable level that can be observed qualitatively (Zeng et al., 2014).

The antagonistic activity of the isolated PGPR against *R. solani* is an indication of the presence of natural biocontrol agents in the environment. *R. solani* is a fungal pathogen of many economically important crops such as rice. At present, the main control measure of this pathogen is the use of chemical fungicides (Bartholomäus et al., 2017). However, excessive and irrational use of fungicides

Table 1. Plant growth-promoting potential and antifungal activity of the isolated rhizobacteria.

Isolate	Phosphate solubilization	*PSE*[1]	Antifungal activity	Percent Inhibition (%)	Catalase activity	Ammonia production
PGP1	+	2.66^{b}	+	63.46^{a}	+	-
PGP2	-	-	+	49.95^{e}	+	-
PGP3	+	2.19^{d}	+	44.52^{g}	+	-
PGP4	+	3.49^{a}	+	50.71^{de}	+	-
PGP5	+	1.37^{e}	-	-	+	-
PGP6	-	-	+	42.84^{h}	+	-
PGP7	-	-	+	49.08^{f}	+	-
PGP8	+	2.43^{c}	+	54.64^{b}	+	-
PGP9	-	-	+	52.76^{c}	+	-

[1]/PSE and percent inhibition with the same letter superscript are not significantly different at 5% level (Tukey's Test)

Table 2. Cultural, Morphological and Molecular Characterization of potential PGPR.

Isolate	Cultural Characteristics					Morphological			Molecular Identity		
	Color	Shape	Margin	Elevation	Opacity	Shape	Gram Stain Reaction	Motility	Closely related taxa	Accession number	Percent Homology
PGP1	W	Round	E	Raised	O	Rod	G+	N	*Bacillus* sp. strain FA2-30	KY476206.1	90.12%
PGP2	W	Round	E	Raised	O	Rod	G+	N	*Bacillus* sp. strain JSB10	MG742210.1	95.16%
PGP3	M	Round	E	Raised	T	Rod	G-	M	*Rhizobium pusense* strain KSB	MF135560.1	83.60%
PGP4	Y	Round	E	Raised	O	Rod	G+	N	*Curtobacterium luteum* strain S2-185	JQ660078.1	90.02%
PGP5	M	Round	E	Raised	O	Rod	G+	N	*Bacillus* sp. strain PL18-3	EU912460.1	88.43%
PGP6	YO	Round	E	Raised	O	Rod	G+	M	*Bacillus* sp. strain CMS1	MH633708.1	85.13%
PGP7	M	Round	E	Raised	T	Rod	G-	M	*Chromobacterium haemolyicum* strain EAPL14	JX500185.1	81.25%
PGP8	M	Round	E	Raised	O	Rod	G+	M	*Fictibacillus phosphorivorans* strain LMS42	MK050852.1	87.47%
PGP9	YO	Round	E	Raised	T	Rod	G+	N	*Fictibacillus nanhaiensis* strain FJAT-46876	MG651494.1	88.20%

Legend: W= White; M= Milky; Y= Yellow; YO= Yellow orange; E= Entire; O= Opaque; T= Translucent; G+= Gram positive; G- =Gram negative; M= motile; N= non-motile

is rampant, which not only hastens environmental deterioration but also entails human health hazards. Also, it could promote the development of resistance to currently applied fungicides. As a result, more concentrated and potent agents are formulated to control the resistant pathogens which only increase the loading of toxic residues in the environment (Thind & Gupta, 2014).

Conclusion

This study revealed a potential association of PGPR with the common weed *C. iria*. Specifically, the rhizobacterial isolates showed various characteristics that may contribute to plant growth promotion such as phosphate solubilization and antifungal activity. These isolates can be further investigated and manipulated through biotechnology to develop biological active formulations such as biofertilizers and biocontrol agents for plant growth improvement.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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