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Identification and characterization of plant growth-promoting properties of bacterial endophytes from selected Zingiberaceae plants

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ABSTRACT

Aims: This study aims to isolate, characterize and screen the plant growth-promoting bacteria from Zingiberaceae plants. Plant promoting activities such as indole-3-acetic acid (IAA), phosphate solubilization, zinc solubilization and nitrogen-fixing capabilities are determined, and the IAA production of selected isolates are optimized.
Methodology and results: Endophytic bacteria were isolated from the plant samples by surface sterilization on nutrient agar (NA) plates and incubated at 30 °C for 2-3 days. The bacteria were identified based on their phenotypic characteristics and 16S rRNA gene sequence analyses. All isolates were identified as genera *Bacillus*, *Lysinibacillus*, *Kerstersia*, *Klebsiella* and *Brucella*. The isolates exhibited phosphate solubilization (1.5 ± 0.75-37.5 ± 8.75 Solubilization Index, SI), zinc solubilization (2.5 ± 0-60 ± 1.5 SI) and IAA production (0.1 ± 0.2-115.7 ± 1.6 µg/mL), while 3 isolates possessed nitrogen-fixing capabilities. Five isolates (PHAS-2, PWS-2, PWR-2, PHBS-2 and SCG-2) were selected for IAA optimization. Isolate PWR-2 produced the maximum IAA at 447.7 ± 0 µg/mL when tryptophan concentration was maintained at 1.0%.
Conclusion, significance and impact of study: Genera of bacteria included *Bacillus*, *Lysinibacillus*, *Kerstersia*, *Klebsiella* and *Brucella* were successfully isolated from Zingiberaceae plants. All the isolates showed the capability to produce IAA, while some isolates exhibited phosphate solubilization and zinc solubilization, and a few possessed nitrogen-fixing capabilities. The potential IAA production isolates could be applied for the enhancement of agricultural production that will be becoming a more widely accepted practice.

Keywords: Endophytic bacteria, indole-3-acetic acid, plant growth-promoting activity, phosphate solubilization, zinc solubilization

INTRODUCTION

Endophytic bacteria are the beneficial bacteria that colonize in plant tissues and interact among themselves and with invaders to influence the growth of plants (Chen *et al.*, 2014). They play an important role in host plants by promoting nutrient uptake and plant growth by phytohormone production (Spaepen *et al.*, 2007). Indole-3-acetic acid (IAA) is an auxin responsible for cell elongation, cell division and differentiation in plants (Taghavi *et al.*, 2009). The other phytohormones such as cytokinins are involved in the cell cycle progression; gibberellins are regulated the reproductive organ formation and ripening of fruit and viable seeds; ethylene is involved in a functioning of the shoot, root and root hair growth; and abscisic acid (ABA) is synthesized in response to abiotic stresses and activates the genes responsible for stress resistance (Tsukanova *et al.*, 2017). Siderophores are produced by microorganisms and plants growing under low iron conditions (Ahmed and Holmström, 2014). Endophytic bacteria also exhibit phosphate (P) solubilization capacity, including the release of complexing or mineral-dissolving compounds and P during substrate degradation (biological P-mineralization) (Sharma *et al.*, 2013; Walia *et al.*, 2017; Di Benedetto *et al.*, 2019). These bacteria also enhance nitrogen (N) availability in the rhizosphere (N-fixing bacteria and nitrifying bacteria) and zinc solubilization potential (Saravanan *et al.*, 2007).

Plant growth-promoting bacteria include *Bacillus*,

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Rhizobium, *Acinetobacter*, *Alcaligenes*, *Azotobacter*, *Arthrobacter*, *Enterobacter*, *Pseudomonas*, *Serratia* and *Burkholderia* (Kloepper *et al.*, 1989) are distributed in plant rhizospheres (Akinrinlola *et al*., 2018). It has been reported that *Lysinibacillus capsici* strain was found in the rhizosphere of a pepper plant (Burkett-Cadena *et al.*, 2019), while Proteobacteria, Actinobacteria and Bacteroidetes are mainly distributed in plants in extreme environments (Zhang *et al.*, 2019). Diverse bacteria from plant roots such as *Enterobacter*, *Pseudomonas* and *Azospirillium* have also been reported to produce IAA (El-Khawas and Adachi, 1999; Jasim *et al*., 2014). It has been described that *Pseudomonas*, *Bacillus*, *Agrobacterium* and *Enterobacter* strains from ginger plants exhibited antimicrobial and IAA activity (Chen *et al.*, 2014), while a *Pseudomonas* strain from ginger rhizome is able to produce IAA (Jasim *et al.*, 2014). Besides, *Bacillus cereus* RBacDOB-S24 and *Pseudomonas aeruginosa* BacDOB-E19 isolated from different varieties of turmeric (*Curcuma longa* L.) from South India exhibited biocontrol properties against *Pythium aphanidermatum* and *Rhizoctonia solani* pathogens of turmeric (Vinayarani and Prakash, 2018), while *Serratia* sp. ZoB14 isolated from ginger rhizome had inhibitory activity toward *Pythium myriotylum* (Sabu *et al.*, 2019). In addition, *Bacillus* strains stimulated for plant growth and produced biological control agent for displaying antagonistic activity towards *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium solani* were isolated from different root, leaf and stem of pearl millet (Kushwaha *et al.*, 2020).

The plant family Zingiberaceae are well-known for medicinal values and are distributed widely throughout the tropics, particularly in Southeast Asia (Kumar *et al.*, 2013). In Thailand, *Curcuma parviflora* Wall., *Globba winitii* C.H.Wright and *Boesenbergia rotunda* (L.) Mansf., within this family are medicinal plants. Currently, *B. rotunda* extract has been reported contains panduratin A that exhibits anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) property (Kanjanasirirat *et al*., 2020). Studies on isolation and characterization of endophytic bacteria as IAA producers from these plants are interesting in the improving of plant growth and their production. Here, the endophytic bacteria which isolated from Zingiberaceae ginger family in Thailand were characterized and screened for their plant growth-promoting activities, and the selected isolates were optimized for IAA production.

MATERIALS AND METHODS

Sample collection and isolation of endophytic bacteria

Three plant species *Curcuma parviflora* Wall., *Globba winitii* C.H.Wright. and *Boesenbergia rotunda* (L.) Mansf. were collected from Mueang District, Udon Thani (17° 25' 21.468" N, 102° 48' 54.9" E), Mueang District, Nong Bua Lamphu (17° 15' 39.096" N, 102° 31' 38.741" E) and Amphoe Ban Phaeng, Nakhon Phanom Provinces (17° 28' 13.588" N, 104° 39' 28.207" E) and kept separately in sterile plastic bags at 4 °C. Rhizomes, stems and leaves of the plant samples were pretreated as described by Riker and Riker (1936). The plant samples were first washed with tap water to remove the attached soil. Then, each plant part was cut with a sterile surgical knife and then immersed in 3% (v/v) NaOCl (Clorox) and 95% (v/v) ethanol for 1 min each before washing with sterile distilled water. Each sample was then cut into pieces 0.5 × 0.5 cm, placed on nutrient agar (NA) plates and incubated at 30 °C for 2-3 days. Different colonies were selected and purified on NA plates and the selected isolates were preserved in 20% (v/v) glycerol at –20 °C.

Characterization of the isolates

Phenotypic characterization

The bacterial cell morphology was recorded and physiological characteristics were also accessed which include growth in NaCl (3% and 5%) at pH (5 and 9) and temperature (40 °C and 48 °C). Biochemical characteristics of oxidase, catalase, Simmon's citrate, arginine, indole, Methyl Red (MP)-Voges-Proskauer (VP), nitrate and hydrolysis of aesculin, reduction, arginine, casein, gelatin, starch and Tween 80 were also performed as described by Barrow and Feltham (1993) and acid production from carbohydrates were followed as previously reported by Tanasupawat *et al*. (1998).

Genotypic characterization

The 16S rRNA gene was analysed by amplifying using the universal primers 20F (5'-GAGTTTGATCCTGGCTCAG-3') and 1530R (5'-GTTACCTTGTTACGACTT-3') (Weisburg *et al.*, 1991). The cycling program of PCR condition was initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation 94 °C for 1 min, annealing at 50 °C for 2 min, elongation at 72 °C for 2 min. The PCR was ended with a final extension at 72 °C for 3 min and amplified product was cool at 4 °C (Peker *et al*., 2019). The PCR products were sequenced using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991) (Macrogen, Seoul, Korea). Homology search was conducted on the EzBioCloud database using the BLAST algorithm (Yoon *et al.*, 2017). The 16S rRNA gene sequences were manually verified and multiple alignment was conducted using BioEdit software (Hall, 1999). A phylogenetic tree was constructed based on the neighbor-joining (NJ) method (Saitou and Nei, 1987) using MEGA 6.0 software (Tamura *et al.*, 2013). Evolutionary distances among the strains were analyzed based on Kimura's two-parameter method (Kimura, 1980). Confidence values of the nodes were evaluated using the bootstrap resampling method with 1,000 replications (Felsenstein, 1985).

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Determination of plant promoting activities

A single colony of bacteria was inoculated onto nutrient broth and incubated at 30 °C for 24 h. The suspension culture (10^6 CFU/mL) was subsequently used for plant growth-promoting assays.

Phosphate solubilizing activity

A suspension of isolates was swabbed on nitrogen-free (NF) agar (Tariq *et al*., 2015) and incubated at 30 °C for 2 days. The culture on NF agar plates was used as an inoculum by a cork-borer technique and inoculated on PVK agar plates containing (g/L) 10 glucose, 0.5 yeast extract, 0.5 $(NH_4)_2SO_4$, 0.1 $MgSO_4 \cdot 7H_2O$, $5Ca_3(PO_4)_2$, 0.2 KCl, 0.002 $MnSO_4 \cdot 2H_2O$, 0.002 $FeSO_4 \cdot 7H_2O$ and 15 agar (Pikovskaya, 1948). Phosphate solubilization ability was observed by the formation of halo zones around the colonies on Pikovskaya agar plate supplemented with tricalcium phosphate [TCP, $CaF_3(PO_4)_2$] as a source of phosphorus. After incubation, clearing zones formed by the isolates were determined/quantified according to Nautiyal (1999) using the solubilization index as follows:

Solubilization index (SI) = [Colony diameter + clear zone diameter (mm)]/Colony diameter (mm)

Zinc solubilizing activity

A suspension of isolates was swabbed on nitrogen-free (NF) agar (Tariq *et al*., 2015) and incubated at 30 °C for 2 days. The culture on NF agar plates was used as an inoculum by a cork-borer technique, inoculated on mannitol salt agar (MSA) and incubated at 30 °C for 3-5 days. The isolates that produced halo zones around the colony indicating a positive zinc solubilization ability. The clearing zone formed by the isolates were determined using the equation above.

Nitrogen-fixing characterization

The nitrogen-fixing activity was determined using Nessler's reagent (Vogel and Svehla, 1979). The isolates were cultivated in nitrogen-free (NF) broth and incubated in a shaker (180 rpm) at 30 °C for 48 h. Then, the broth was centrifuged at 3,000 rpm for 10 min. The supernatant was tested with Nessler's reagent in the dark. The colorimetric analysis was conducted on a microplate reader (CLARIOstar Plus, BMG Labtech) at 560 nm after 20 min incubation.

IAA production

The isolates were cultivated in NF broth with 1% (w/v) tryptophan on an incubator shaker (180 rpm) at 30 °C for 48 h. IAA production was evaluated using Salkowski's reagent (Ehmann, 1977). The broth was centrifuged at 4,000 rpm for 10 min and the supernatant was collected. The optical density (OD) of the samples was measured at 530 nm after 20 min by a microplate reader (Vaghasiat *et al*., 2011). The IAA production was determined and expressed as µg/mL based on the standard curve of IAA (Rahman *et al*., 2017).

Optimization for IAA production

Five isolates (PHAS-2, SCG-2, PWS-2, PHBS-2 and PWR-2) were selected for optimization of IAA production. The optimization of IAA production according to Mohite (2013) using yeast extract-malt extract-dextrose (YMD) broth supplemented with tryptophan as the basal medium. Different carbon sources (1%, w/v) (glucose, mannitol, sucrose, glucose with mannitol and glucose plus sucrose), 1% (w/v) of nitrogen sources [sodium nitrate, ($NaNO_3$), potassium nitrate (KNO_3), peptone, potassium nitrate coupled with peptone and sodium nitrate coupled with peptone], and tryptophan concentrations [0.1%, 0.5%, 1% and 1.5% (w/v)] were optimized. Effects of pH (5 to 9) and temperature (30 to 37 °C) on IAA production were also tested. Sodium phosphate buffer (100 mM) for pH 5–8 and Glycine-NaOH buffer (100 mM) for pH 9 were used to maintain the pH value of the medium. All experiments were optimized for 48 h using the one-factor-at-a-time (OFAT) method.

Statistical analysis

Data were statistically analyzed using the Statistical Package for the Social Sciences (SPSS, Statistics version 24.0.0.0) with one-way analysis of variance (ANOVA), while grouping was assessed by Duncan's multiple range tests at a *p*-value of 0.05 (Duncan, 1955). The data were expressed as mean values of triplicates ± standard deviation.

RESULTS AND DISCUSSION

Isolation and identification of isolates

In this study, 13 isolates were isolated from Zingiberaceae. Among 13 isolates, SCS-2, PHAS-2 and PHCG-3 were isolated from *Curcuma parviflora* Wall.; PWR-3, PWS-2, PWR-2, PZR-1, PHCL-1, SCS-3, SCG-2 and SCS-4 were isolated from *Globba winitii* C.H.Wright.; and PHBS-2 and SKR-3 were isolated from *Boesenbergia rotunda* (L.) Mansf. All isolates were formed milky-white, circular and convex colonies. Their 16S rRNA gene sequence accession number, similarity (%) and nearest type strains were described in Table 1. Seven isolates were Gram-positive rods including SCS-2, PHAS-2, PWR-3, PHCG-3, PWS-2, PWR-2 and PHBS-2 while 6 isolates were Gram-negative rods including PZR-1, PHCL-1, SCS-3, SCG-2, SCS-4 and SKR-3. All the isolates were grown in 3% and 5% NaCl (except isolate PHCG-3), at 40 °C, pH 5 and pH 9 but were negative from VP test. The differential phenotypic characteristics of the strains were shown in Table 2.

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Table 1: Identity of 13 bacterial isolates from Zingiberaceae in this study.

Plant sample	Province	Isolate no.	Accession number	Similarity (%)	Nearest type strain
Curcuma parviflora Wall.	Udon Thani	SCS-2	LC556996	99.71	*Bacillus siamensis* KCTC 13613^T
Curcuma parviflora Wall.	Nong Bua Lamphu	PHAS-2	LC556998	99.86	*Bacillus siamensis* KCTC 13613^T
Globba winitii C.H.Wright.	Nakhon Phanom	PWR-3	LC557000	99.78	*Bacillus siamensis* KCTC 13613^T
Curcuma parviflora Wall.	Nong Bua Lamphu	PHCG-3	LC556997	100.0	*Bacillus cereus* DSMZ 31^T
Globba winitii C.H.Wright.	Nakhon Phanom	PWS-2	LC557001	100.0	*Bacillus cereus* DSMZ 31^T
Globba winitii C.H.Wright.	Nakhon Phanom	PWR-2	LC556999	99.93	*Bacillus mobilis* MCCC 1A05942^T
Boesenbergia rotunda (L.) Mansf.	Nong Bua Lamphu	PHBS-2	LC557007	100.0	*Lysinibacillus capsici* PB300^T
Globba winitii C.H.Wright.	Nakhon Phanom	PZR-1	LC557002	99.93	*Kerstersia gyiorum* LMG 5906^T
Globba winitii C.H.Wright.	Nakhon Phanom	PHCL-1	LC557003	99.64	*Klebsiella pneumoniae* subsp. *pneumoniae* JCM 1662^T
Globba winitii C.H.Wright.	Nakhon Phanom	SCS-3	LC557004	99.79	*Klebsiella pneumoniae* subsp. *ozaenae* ATCC 11296^T
Globba winitii C.H.Wright.	Nakhon Phanom	SCG-2	LC557005	99.92	*Brucella oryzae* NBRC 102588^T
Globba winitii C.H.Wright.	Nakhon Phanom	SCS-4	LC557006	100.0	*Brucella cytisi* ESC1^T
Boesenbergia rotunda (L.) Mansf.	Nong Bua Lamphu	SKR-3	LC557008	99.92	*Brucella intermedia* NBRC 15820^T

Six Gram-positive rod-shaped bacteria containing SCS-2, PHAS-2, PWR-3, PHCG-3, PWS-2 and PWR-2 belonged to the genus *Bacillus*, while PHBS-2 belonged to the genus *Lysinibacillus* based on 16S rRNA gene sequence and phylogenetic tree analysis (Figure 1). The isolates SCS-2, PHAS-2 and PWR-3 were closely related to *Bacillus siamensis* KCTC 13613^T with 99.71-99.86% similarity. However, they were clustered in one branch of *Bacillus siamensis* KCTC 13613^T, *Bacillus velezensis* CR-502^T and *Bacillus subtilis* JCM 1465^T, therefore they were identified as *Bacillus* sp. They showed positive for catalase, oxidase, MR test, hydrolysis of aesculin and casein and growth at 48 °C but were negative for VP test and starch hydrolysis. Their variable phenotypic characteristics are presented in Table 2.

Isolates PHCG-3 and PWS-2 were closely related to *Bacillus cereus* DSMZ 31^T with 100% similarity, but they were shared branch of *Bacillus cereus* DSMZ 31^T, *Bacillus mobilis* MCCC 1A05942^T and *Bacillus wiedmannii* FSL W8-0169^T with 100% bootstrap. Similarly, isolate PWR-2 was closely related to *Bacillus mobilis* MCCC 1A05942^T with 99.93% similarity (Table 1), but it was clustered in same branch with PHCG-3 and PWS-2 (Figure 1). Therefore, PHCG-3, PWS-2 and PWR-2 were identified as *Bacillus* sp. They showed positive for citrate utilization, acid production from L-arabinose, D-fructose, D-maltose and D-trehalose but were negative for VP test, hydrolysis of casein, gelatin, starch and Tween 80. Their variable phenotypic characteristics are presented in Table 2. Another isolate PHBS-2 was closely related to *Lysinibacillus capsica* PB300^T with 100% similarity (Table 1), but *Lysinibacillus capsica* PB300^T was shared branch with *Lysinibacillus macrolides* DSM 54^T (Figure 1), therefore it was identified as *Lysinibacillus* sp.

On the other hand, six Gram-negative rod-shaped bacterial isolates PZR-1, PHCL-1, SCS-3, SCG-2, SCS-4 and SKR-3 belonged to the genera *Kerstersia*, *Klebsiella* and *Brucella*. They grew maximally in 5% NaCl at 48 °C and showed positive for citrate utilization, nitrate reduction and aesculin hydrolysis. Acid was produced from D-cellobiose, D-fructose and D-maltose. Their variable phenotypic characteristics are shown in Table 2. Due to isolate PZR-1 was closely related to *Kerstersia gyiorum* LMG 5906^T with 99.93% and *Kerstersia similis* LMG 5890^T with 99.42% similarity (Table 1), in addition they were clustered in the one branch, then it was identified as *Kerstersia* sp. In the meanwhile, isolate PHCL-1 closely related to *Klebsiella pneumoniae* subsp. *pneumoniae* JCM 1662^T and *Klebsiella variicola* subsp.

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Table 2: Differential phenotypic characteristics of the isolates.

Characteristic	Isolate no.												
	SCS-2	PHAS-2	PWR-3	PHCG-3	PWS-2	PWR-2	PHBS-2	PZR-1	PHCL-1	SCS-3	SCG-2	SCS-4	SKR-3
Gram staining	+	+	+	+	+	+	+	-	-	-	-	-	-
Growth in 5% NaCl	+	+	+	-	+	+	+	+	+	+	+	+	+
Growth at 48 °C	+	+	+	-	-	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	-	+	+	-	-	+	+	+	-
Oxidase	+	+	+	+	+	-	-	+	-	-	+	+	+
Citrate utilization	-	+	-	+	+	+	+	+	+	+	+	+	+
Indole	-	-	+	-	-	+	-	+	+	+	-	+	-
Methyl red (MR)	+	+	+	+	-	-	-	+	-	+	-	+	+
Nitrate reduction	+	-	+	+	+	-	+	+	+	+	+	+	+
Hydrolysis of:													
Aesculin	+	+	+	+	-	+	+	-	+	+	+	+	+
Arginine	+	-	+	+	-	-	+	+	+	-	+	+	-
Casein	+	+	+	-	-	-	-	-	-	-	-	+	-
Gelatin	-	+	-	-	-	-	-	-	-	-	-	-	-
Starch	-	-	-	-	-	-	-	+	+	-	+	+	-
Tween 80	-	+	-	-	-	-	-	-	-	-	+	-	-
Acid from:													
L-Arabinose	+	+	-	+	+	+	+	+	-	+	+	+	+
D-Cellobiose	+	+	-	+	+	-	-	+	+	+	+	-	+
D-Fructose	+	+	-	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	-	+	+	-	-	+	+	+	+	+	+
D-Lactose	-	+	-	-	+	-	-	-	-	+	-	-	+
D-Mannitol	-	+	+	-	+	-	-	+	-	+	-	-	+
D-Maltose	+	+	-	+	+	+	+	+	+	+	+	+	+
D-Raffinose	-	+	+	-	+	-	-	+	-	+	-	-	+
D-Ribose	+	+	-	-	+	+	+	-	-	+	-	+	+
D-Sorbitol	+	+	-	+	+	-	+	-	+	+	+	+	+
D-Trehalose	+	+	-	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	+	-	-	+	-	-	-	-	+	-	-	+

+ positive reaction; - negative reaction.

tropica SB5531^T with 99.64% and 99.56% similarity, respectively, but they were also clustered with one branch. Isolate PHCL-1 was therefore identified as *Klebsiella* sp. In case of SCS-3, it had 99.79% similarity to *Klebsiella pneumoniae* subsp. *pneumonia* JCM 1662^T and 99.64% similarity to *Klebsiella pneumoniae* subsp. *ozaenae* ATCC 11296^T and it is located at the same node with *Klebsiella pneumoniae* subsp. *ozaenae* ATCC 11296^T, therefore it was identified as *Klebsiella* sp. (Figure 1).

Isolates SCG-2 and SCS-4 were closely related to *Brucella oryzae* NBRC 102588^T (99.92%) and *Brucella cytisi* ESC1^T (100%) (Table 1), respectively and shared the node as shown in Figure 1. Isolate SKR-3 was closely related to *Brucella intermedia* NBRC 15820^T with 99.92% similarity and *Brucella pseudintermedia* ADV31^T with 97.73% similarity, but it was clustered in the one branch with *Brucella intermedia* NBRC 15820^T and *Brucella pseudintermedia* ADV31^T. Therefore, SCG-2, SCS-4 and SKR-3 were identified as *Brucella* sp. Their phenotypic characteristics are presented in Table 2.

Screening of plant promoting activities

Among the 13 isolates, 9 isolates exhibited the ability to dissolve phosphate, while 10 isolates had zinc solubilizing property and 3 possessed nitrogen fixation ability. Their phosphate solubilizing ability in Pikovskaya medium varied from 1.5 ± 0.75 to 37.5 ± 8.75 (Table 3). Isolate PWR-2 exhibited the highest level of solubilized phosphorus (37.5 ± 8.75). The zinc solubilizing ability of the 13 isolates varied from 2.1 ± 0.25 to 60 ± 1.5 using ZnO^{3+} as a source of insoluble zinc and isolate SKR-3 exhibited the highest solubilized zinc (60 ± 1.5) (Table 3). The isolates produced IAA (0.1 ± 0.2 to 115.7 ± 1.6 µg/mL) in NF medium when 1% L-tryptophan was used as a substrate. Isolates SCG-2 and PWR-2 produced the highest levels of IAA at 115.7 ± 1.6 and 111.3 ± 1.6 µg/mL, respectively (Table 3).

SCS-2, PHAS-2, PWR-3, PHCG-3, PWS-2 and PWR-2 identified as *Bacillus* species could produce IAA (6.0 ± 1.5 to 111.3 ± 1.6 µg/mL). However, *Bacillus* strains are distributed in soils, sediments, food (Liu *et al*., 2017) and

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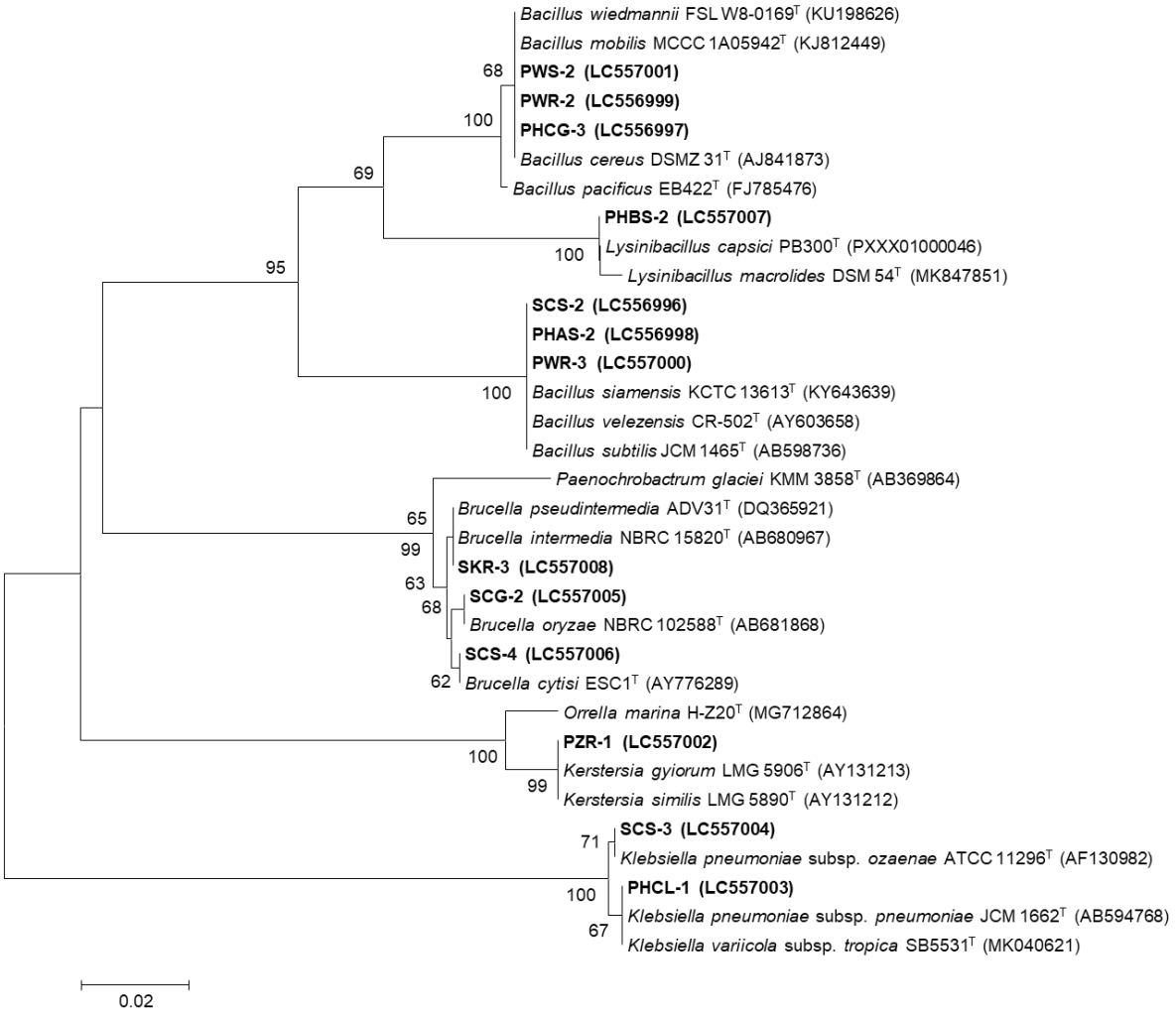

Figure 1: Neighbor-joining tree based on the 16S rRNA gene sequences showing relationships of the isolates in genera *Bacillus*, *Lysinibacillus*, *Kerstersia*, *Klebsiella*, *Brucella* and related type strains. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates and only values >50% are indicated. Scale bar 0.02 represents substitutions per nucleotide position.

plant rhizospheres (Kumar *et al*., 2011; Akinrinlola *et al*., 2018). *Bacillus amyloliquefaciens*, *B. subtilis*, *B. tequilensis* and *Bacillus* strains have reported to produce IAA and exhibited antagonistic activity to plant pathogenic fungi (Kushwaha *et al.*, 2020; Shahid *et al*., 2021).

Besides, our isolates which identified as *Lysinibacillus* species (PHBS-2), *Kerstersia* species (PZR-1), *Klebsiella* species (PHCL-1 and SCS-3), *Brucella* species (SCS-4, SCG-2 and SKR-3) also exhibited IAA production (Table 3). *Lysinibacillus* strains were reported found in the roots of maize (Pereira *et al*., 2011), the rhizosphere of a pepper plant (Burkett-Cadena *et al*., 2019) and durum wheat rhizosphere (Di Benedetto *et al*., 2019). On the other hand, *Kerstersia* strains were found in clinical samples (Coenye *et al*., 2003) while *Klebsiella* and *Brucella* strains were endophytic bacterial species isolated from plants (Velasco *et al*., 1998; Tripathi *et al*., 2006; Hördt *et al*., 2020). It has been reported that different strains of endophytic bacteria have different effects on growth-promoting in various plants. For example, strains of *Bacillus*, *Pseudomonas*, *Stenotrophomonas* and *Staphylococcus* were considered to have IAA, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and siderophore effects in ginger (Jasim *et al*., 2014) while *Pseudomonas fluorescens*, *B. megaterium* and *Enterobacter ludwigii* strains from ginger plants produced high IAA (Chen *et al*., 2014). *Bacillus megaterium*, *B. safensis*, *B. simplex* and *Paenibacillus graminis* strains increased the growth of soybean and wheat (Akinrinlola *et al*., 2018). These endophytic bacteria are useful for seed germination and proliferation of root nodules (Wahyudi *et al*., 2011).

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Table 3: Phosphate solubilizing, zinc solubilizing and indole-3-acetic acid (IAA) production of the isolates.

Isolate no.	Phosphate solubilizing SI	Zinc solubilizing SI	IAA (μg/mL)	N_2 Fixing (NH_4^+)
SCS-2	4.0 ± 0.5	ND	97.1 ± 1.6^{B}	ND
PHAS-2	5.0 ± 11.8	22.5 ± 0.2	35.5 ± 1.2^{E}	ND
PWR-3	3.7 ± 1.9	ND	85.7 ±1.4^{C}	ND
PHCG-3	ND	34.0 ± 0.0	6.0 ± 1.5^{F}	ND
PWS-2	25.0 ± 5.3	2.5 ± 0.5	57.8 ± 2.6^{D}	ND
PWR-2	37.5 ± 8.75	44 ± 7.0	111.3 ± 1.6^{A}	0.091 ± 0.003
PHBS-2	ND	2.5 ± 0.0	37.3 ± 1.7^{E}	ND
PZR-1	1.5 ± 0.75	38.5 ± 0.3	80.0 ± 2.2^{C}	0.079 ± 0.004
PHCL-1	ND	37.5 ± 0.0	53.6 ± 2.8^{D}	0.065 ± 0.004
SCS-3	4.5 ± 1.5	52.5 ± 2.7	0.8 ± 0.3^{F}	ND
SCG-2	2.1 ± 1.0	2.1 ± 0.3	115.7 ± 1.6^{A}	ND
SCS-4	29.0 ± 8.0	ND	89.5 ± 4.0^{B,C}	ND
SKR-3	ND	60.0 ± 1.5	0.1 ± 0.2^{F}	ND

SI, solubilization index; ND, not detected. Values marked by different superscripts are significantly different (*p*-value less than 0.05).

Effect of different carbon and nitrogen sources on IAA production

Based on the result of IAA production, the capacity of phosphate, and zine solubilizing, the representative strains, PHAS-2, SCG-2, PHBS-2, PWS-2 and PWR-2 were selected for further IAA optimization. The effect of various carbon sources on the selected isolates for IAA production (Figure 2A), revealed that isolate PWS-2 produced the maximum IAA concentration at 11.0 ± 0.4 μg/mL when using sucrose as a carbon source, followed by isolate PWR-2 (10.9 ± 2.7 μg/mL) produced from glucose. Based on the statistical analysis, production of IAA from isolate PWS-2 was not significantly different at alpha 0.05 when using glucose, sucrose or glucose coupled with sucrose as a carbon source. This result indicated that sucrose and glucose were optimum for IAA production from most of the strains, whereas sucrose was an unsuitable carbon source for isolates PWR-2, SCG-2 and PHBS-2 since they produced low IAA concentrations of 5.9 ± 0.5, 5.9 ± 0.4 and 6.4 ± 0.6 μg/mL, respectively.

Various nitrogen sources were also investigated for IAA production when glucose was used as a carbon source. Among the five nitrogen sources, peptone was suitable for isolates to produce IAA, while isolate PHBS-2 produced the maximum IAA at 12.8 ± 2.9 μg/mL using KNO_3 as a nitrogen source. Concentration of IAA produced from peptone as a nitrogen source was 11.5 ± 0.3, 11.2 ± 1.1, 11.1 ± 0.6, 10.1 ± 0.7 and 9.4 ± 0.2 μg/mL from isolates PWR-2, PWS-2, PHAS-2, PHBS-2 and SCG-2, respectively (Figure 2B). The result was supported by statistical analysis that peptone was the most suitable nitrogen source because the IAA production was not significantly different from 4 isolates (PWR-2, PWS-2, PHAS-2 and PHBS-2) when using peptone as a nitrogen source. Mohite (2013) reported that the degree of IAA production depends on the isolates, carbon sources (glucose, mannitol and sucrose) and nitrogen sources (peptone, $NaNO_3$ and KNO_3).

Effect of L-tryptophan concentration on IAA production

The effect of L-tryptophan (%) on IAA production of 5 isolates was examined when glucose and peptone were used as a carbon and nitrogen source, respectively. The result demonstrated tryptophan as a key factor to improve IAA production. As the concentration of L-tryptophan increased from 0.1 to 1.5%, IAA production from isolates SCG-2 and PHAS-2 increased steadily (Figure 2C). However, the concentration of tryptophan maintained at 1.0% was sufficient to produce maximum IAA at 447.7 ± 0 μg/mL from isolate PWR-2. This result was supported by Costacurta and Vanderleyden (1995) who reported that tryptophan was generally considered as an IAA precursor to enhance IAA biosynthesis from bacteria.

Effect of pH and temperature on IAA production

The effects of pH and temperature on IAA production were investigated when glucose and peptone were used as a carbon and nitrogen source, respectively. Results of the pH effect showed the maximum concentration of IAA obtained was 139.4 ± 23.7 μg/mL from isolate PWS-2 at pH 9. Similarly, IAA was also produced at a higher concentration at pH 9 by isolates PHAS-2, SCG-2, PWR-2 and PHBS-2 (72.4 ± 1.5, 69.8 ± 0.9, 66.2 ± 1.4 and 64.5 ± 0.7 μg/mL, respectively). Production of IAA reduced when pH value was lower than 8, while it was suppressed when pH value decreased to 5 (Figure 2D). Therefore, we concluded that pH value was a significant factor for IAA production. Weak alkaline (pH value 8-9) promoted the production of IAA from selected isolates. Statistical analysis results confirmed that low concentration of IAA was produced at pH values in the range 5-7 and did not significantly differ within the group. Our results agreed with other studies. IAA production by isolates was highest at pH 9 and decreased below pH 6 (Acuña *et al.*, 2011). Mohite (2013) also reported that pH 9 was the optimal

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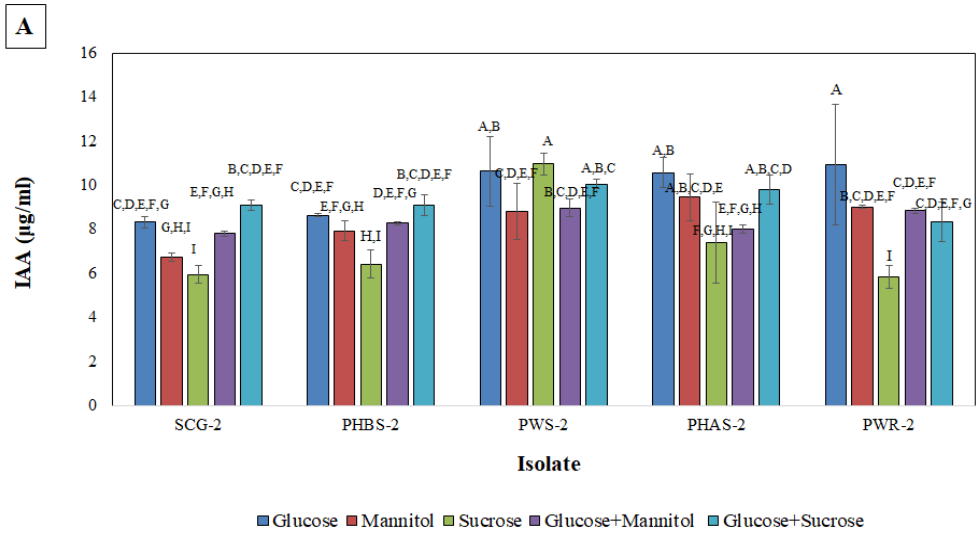

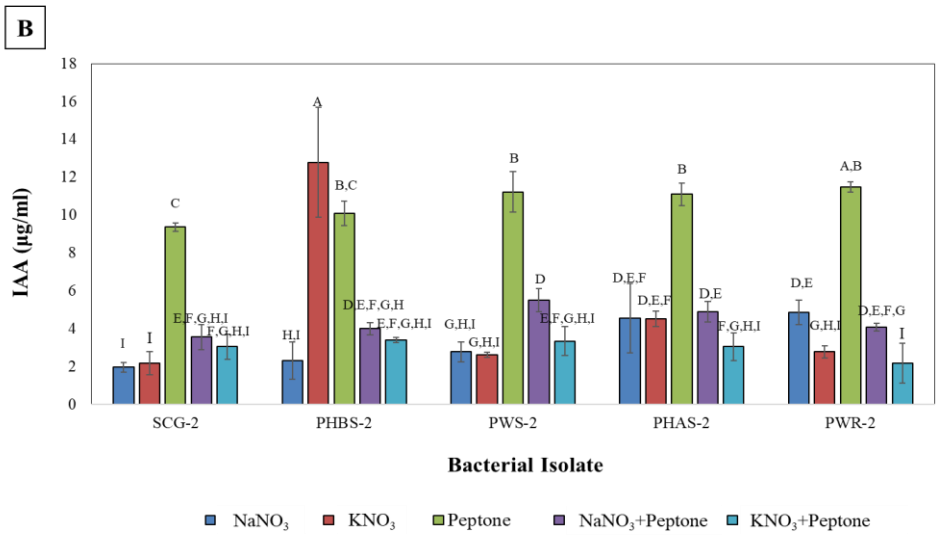

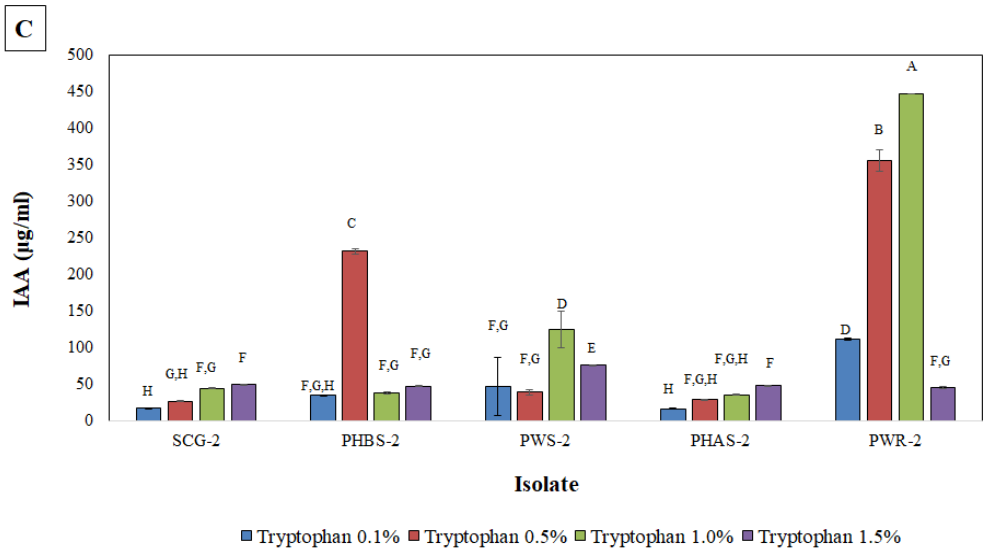

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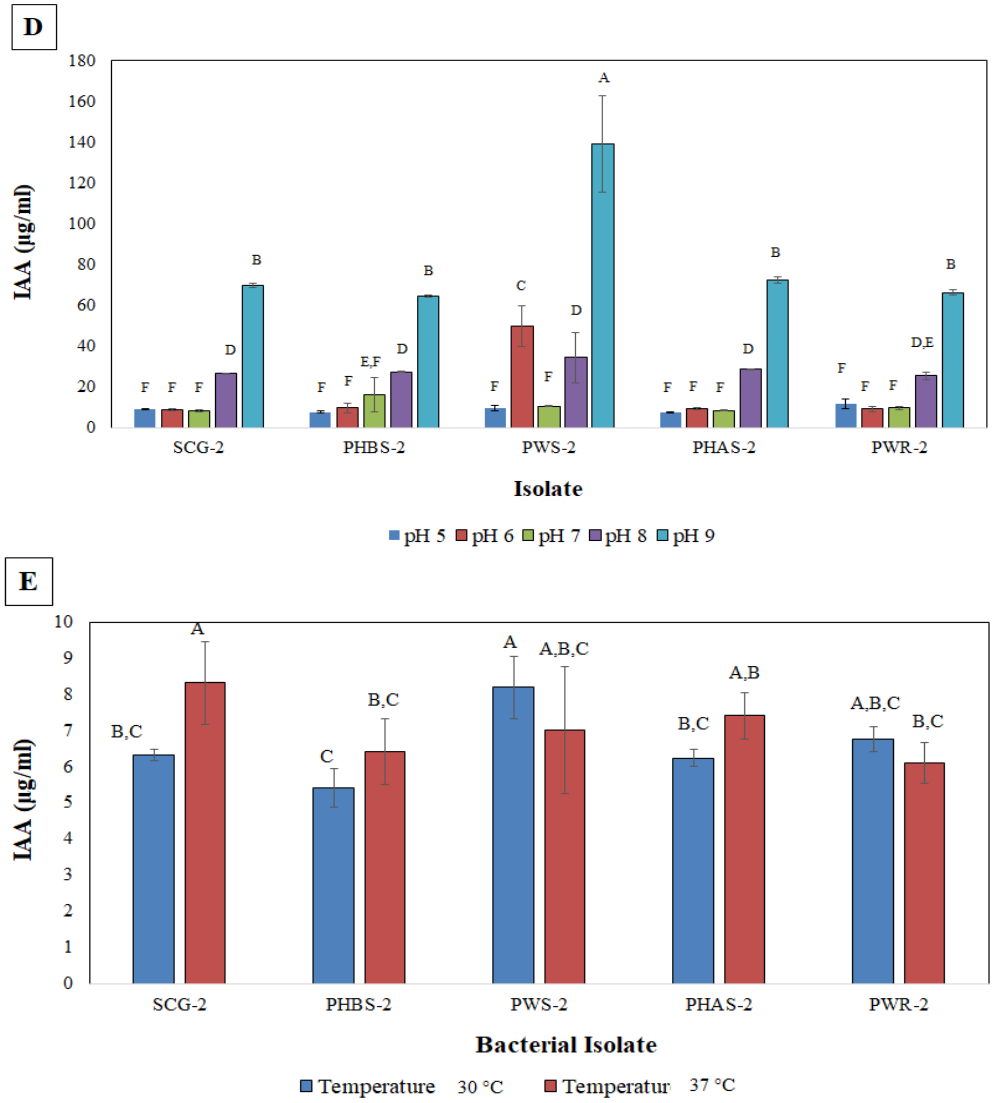

Figure 2: Optimization for IAA production. A) Effect of various carbon sources, B) Effect of various nitrogen sources, C) Effect of various tryptophan concentrations, D) Effect of various pH and E) Effect of various temperatures on IAA production by the selected isolates. Error bars represent standard deviation from triplicate experiments. Values marked by different alphabets show significantly different IAA (p<0.05).

condition for IAA production and under acidic pH (below 6) it was unfavorable.

Production of IAA at 30 and 37 °C was also compared**.** Results showed that isolates PWS-2 and PWR-2 were preferably cultivated at 30 °C for IAA production compared to 37 °C**.** However, isolates SCG-2, PHAS-2 and PHBS-2 were more effective for IAA production at 37 °C (Figure 2E). Maximum IAA production of 8.3 ± 1.1 µg**/**mL was obtained from SCG-2, followed by PHAS-2 (7.4 ± 0.6 µg/mL) and PWS-2 (7.0 ± 1.8 µg/mL) at 37 °C, while IAA production of isolates PWS-2 and PWR-2 at 8.2 ± 0.9 µg/mL and 6.8 ± 0.3 µg/mL were obtained at 30 °C**.** However, these results were not statistically different, and they were included in the same group**.** Accordingly, optimization of IAA production by bacteria from *Stevia rebaudiana* rhizosphere was obtained at 37 °C and pH 9 (Chandra *et al.*, 2018). The optimal condition for IAA production from *Rhizobium* sp. at 36 °C, pH 6.5 maximized IAA concentration at 166 µg/mL when a tryptophan concentration of 1 g/L was used (Lebrazi *et al.*, 2020). Therefore, the optimal condition also depended on the microbial strains.

From this study, the optimization condition for IAA production showed that glucose and peptone were suitable as carbon and nitrogen sources and supplemented with 1% tryptophan at pH 9 and temperature in the range 30-37 °C for 48 h. The optimum condition gave the maximum IAA to 447.7 µg/mL by

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isolate PWR-2, compared to 111.3 µg/mL before optimition using NF broth with 1% (w/v) tryptophan for at 30 °C for 48 h.

As previously reported, the isolates were obtained in this study belong to genera as mentioned above such as *Bacillus* species (SCS-2, PWR-3 and PWR-2), *Kerstersia* species (PZR-1) and *Brucella* species (SCG-2 and SCS-4) produced more than 80.0 µg/mL of IAA (Table 3). Besides IAA activity, 4 isolates (PHAS-2, SCG-2, PWS-2 and PWR-2) exhibited phosphate and zinc solubilizing, while PHBS-2 a representative strain of *Lysinibacillus* species produced only zinc solubilizing. Therefore, 5 isolates including PHAS-2, SCG-2, PWS-2, PWR-2 and PHBS-2 were selected to optimize the condition for IAA production. The maximum IAA production of 447.7 µg/mL was obtained from *Bacillus* sp. PWR-2 at optimized conditions. Our results on optimization IAA is considered as effective tool for screening beneficial bacteria suggesting that IAA producing bacteria have profound effect on plant growth (Mohite, 2013; Akinrinlola *et al.*, 2018). Inoculation with IAA producing bacteria will induce the proliferation of lateral roots and root hairs. Many strains of *Bacillus* species from this research produced high IAA and they will be useful for seedling treatment for plant production, however their antagonistic activity for the effective biocontrol of crop plants should be further study.

CONCLUSION

In this study, 13 endophytic bacteria associated with Zingiberaceae plants were obtained including *Bacillus* species (SCS-2, PHAS-2, PWR-3, PHCG-3, PWS-2 and PWR-2), *Lysinibacillus* species (PHBS-2), *Kerstersia* species (PZR-1), *Klebsiella* species (PHCL-1 and SCS-3) and *Brucella* species (SCS-4, SCG-2 and SKR-3). Among the 13 isolates, isolate PWR-2 (*Bacillus* sp.) showed high IAA production, phosphate and zinc solubilizing activity. It is suggested that this isolate will be useful as phytohormone producing bacteria for the seed germination and the growth improvement of plant.

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