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The biochemical characteristics of phosphate bacteria capable of increasing soil phosphorus bioavailability in Andisols

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Abstract

N-Acyl Homoserine Lactone (N-AHL) has been known as the quorum sensing (QS) signals that control phosphate bacteria (PB) activities in enhancing soil phosphorus (P) availability. This study was aimed at determining: 1) the bacteria biochemical characteristics capable of soil P solubilizing, 2) the N-AHL production types and 3) the best plant roots extract as a N-AHL source. The PB species were determined using 16S rRNA analysis. The determination of the organic acids, phosphatase, phytase, N-AHL and dissolved P was carried out using HPLC, para-Nitro Phenyl Phosphate (pNPP), Na-phytate and spectrophotometry methods, respectively. The PB isolates obtained were classified as *Pseudomonas trivialis*, *P. putida* and *P. fluorescens*. The PB secreted citric, lactic, malonic, oxalic and acetic acids amounting to 156.25 mg. kg^{-1}. The PB phosphatase and phytase activities ranged from 12 to 47 mg PO_4^{3-} dm^{-3} h^{-1}. The P solubilized in the PB Pikovskaya inoculated was greater with the amounts between 147.66 and 194.61 mg P kg^{-1} as control compared (31.06 mg P kg^{-1}). The PB inoculation also produced greater mineralized organic P (63.69 mg P kg^{-1}) than the control (23.7 mg P kg^{-1}). The PB secreted N-AHL greatest was butanoyl-AHL (C_4-AHL). The 30% of root extract corn seemed to be the best source of N-AHL as it could increase P dissolution even 300% in comparison with control variant.

1. Introduction

While phosphorus (P) soil content varies widely only a small proportion of P is available to plants (Banach et al., 2015). Water insoluble inorganic P forms are found as P bearing constituent soil minerals like apatite and strengite (Brogowski and Kwasowski, 2009; Belgaroui et al., 2016). Unavailable organic P is contained in various organic substances such as inositol phosphate, esters, phospholipids, nucleic acids and some derivatives of phosphonic acid (Abbasi et al., 2015; Banach et al., 2015; Delfim et al., 2018). In some soil types, significant P amounts remain unavailable due to the chemical fixation and organic colloids sorption. About 50% of adsorbed P is organic matter bound (Brogowski and Kwasowski, 2009; Banach et al., 2015; Belgaroui et al., 2016). Soil microbes has been widely known to be capable of P solubilizing, including the following bacterial genera *Pseudomonas*, *Bacillus* and *Escherichia*, the fungal genera *Aspergillus*, *Penicillium* and *Culvularia* and the Actinomicetes genus *Streptomyces* (Gao et al., 2016; Rathi and Gaur, 2016). Soil bacteria capable of enhancing P soil bioavailability adsorbed through production of organic acids (citric, malic, oxalic, gluconic and acetic acids); chelation of Al, Fe and Ca cations by organic acids containing carboxyl and hydroxyl anions; ligand exchange between organic anions and Al, Fe and Ca-bound phosphate; and colloid adsorbtion sites blocking by organic acids and charged cells surfaces; produce phosphatase and phytase improve P availability through organic P mineralization (Castagno et al., 2011; Azeem et al., 2014; Abbasi et al., 2015; Belgaroui et al., 2016; Stella and Sahren, 2016).

Microbe gene expression as a response to the surrounding environment is largely governed by their population density which can be detected by autoinducing substances referred to as quorum sensing (QS) signaling molecules (Palmer et al., 2014; Barriuso, 2015; Mahmoudi, 2015). Generally, gram negative bacteria produce autoinducers N-Acyl Homoserine Lactone (N-AHL) form, the derivatives of which include N-butanoyl (BHL; C_4), N-hexanoyl (HHL; C_6), N-octanoyl (OHL; C_8), N-decanoyl (DHL; C_{10}) and N-dodecanoyl (dDHL; C_{12}) Homoserine Lactones (Rani et al., 2011; Ransome et al., 2013; Zhang et al., 2016).

These QS signaling molecules are also produced by plant roots. In the rhizosphere, there are species or microbial populations as a form of mutual interactions between plants and microbes (Badura, 2004; Palmer et al., 2014; Rathi et al., 2016). In soils, microbes can transfer genetic materials not only to the other microbes, but also to the plant as a way of communication between the soil, plant and microbes (Kobus, 1999; Gao et al.,

2016; Liu et al., 2017). The rhizosphere and plant roots, therefore, are the potential source of QS molecules for microbes.

The objectives of this research were as follows: 1) to identify the PB capable of enhancing P availability biochemical characteristics, 2) N-AHL types determination produced by PB, and 3) the plant roots extract determination and examination N-AHL potential source. The formulated research hypothesis assumed that the plant roots extract could be used as N-AHL source for PB biochemical communication.

2. Materials and methods

2.1. PB determination

Three PB (*Pseudomonas trivialis*, *P. putida* and *P. fluorescens*) colonizing Andisols were analysed using 16S rRNA amplification with 1492R primers (5'-TAC CTT GTT ACG ggY ACTT-3') and 800R sequencing primer (5'-Cag TAC GGT ATC TAA TCC-3') determined at MACROGEN Inc. (908 World Meridian Venture Center, #60-24, Gasan-dong, Geumchun-gu, Seoul 153-781, South Korea). Briefly, Next Generation Sequencing (NGS) Pacific Biosciences' RS (PacBio) technique was applied. The Basic Local Alignment Search Tool (BLASTN) with MEGA 6 bioinformatics data analysis was employed to analyze the results and to compare the sequences with known bacteria samples in the GenBank (National Center for Biotechnology Information) (Janda and Abbott, 2007; Kumar et al., 2008).

2.2. Organic acids secretion

An organic acids secreted by PB were tested in a 100 cm^3 Pikovskaya medium (Pikovskaya, 1948) and in a 100 g sterile Andisol. The organic acid samples from the Pikovskaya medium were blended, centrifuged at 4,500 rpm for 15 min. and then filtered with a 0.2 µm nitrocellulose Millipore 246 filter. The secreted organic acids were measured using an HPLC (Hitachi, column OOF4250-CO/10 µm LaChrom Ultra C 18 (2 m) 100 A 150 × 4.60 mm 10 m KPOW 490065-1 Phenomenex). The filtrate was eluted with 0.02 N H_2SO_4 and distilled water (60:40 v/v) at a flow rate of 0.6 $cm^3\,min^{-1}$ for 15 min. at 25°C under a wavelength of 214 nm (Yuquan et al., 2018). The organic acids were identified by comparing their retention times and peak areas from organic acid standards (oxalic, citric, malic, lactic and acetic acids) by three repetitions at concentrations of 0, 12.5, 25, and 50 mg kg^{-1} (Weimin et al., 2016).

2.3. Phosphatase and phytase activities

PB were cultured in a 100 cm^3 Pikovskaya and in a 100 g sterile Andisol to assess phosphatase and phytase activities. After one week, 5.0 cm^3 of para-Nitro Phenyl Phosphate (pNPP) (Sigma, USA) or 5.0 g of Na-phytate (Sigma, USA) were added to the PB culture. After incubation (1 h), the PB cultures were stained with ammonium vanado molybdate and read at a 413 nm wavelength. The PB phosphatase and phytase activities were expressed in mg P cm^{-3} culture h^{-1} incubation (Azeem et al., 2014; Villamizar et al., 2019).

2.4. Inorganic P solubilization

Ten cm^3 of three PB culture (10^8 CFU cm^{-3}) were grown in a 100 cm^3 Pikovskaya medium (Pikovskaya, 1948) and in a 100 g steril Andisol treated with 1,000 ppm P (2.82 g phosphate rock (PR), 0.5 g CaP, 0.4 g AlP or 0.4 g FeP). The experiment was arranged in a completely randomized design (CRD) with one factor (PB isolates) by three replications. The factor consisted of 11 treatments comprising 1 control treatment (no inoculation) and treatments with 10 different PB isolates. The PB incubation period lasted one week at 25°C incubated temperature. The following variables soluble P, P solubilization efficiency, pH and PB population were measured.

2.5. Organic P mineralization

The mineralization ability was determined based on PB cultured in a 100 cm^3 Pikovskaya medium and in a 100 g steril Andisol. The experiment was arranged in a CRD with one factor (PB isolates) and three replications. The factor consisted of 11 treatments comprising 1 control treatment (no inoculation) and treatments with 10 different PB isolates. Ten cm^3 of three PB culture (10^8 CFU cm^{-3}) were grown in a 100 cm^3 Pikovskaya medium treated with organic P sources (up to 0.5 cm^{-3} pNPP or up to 0.5 g Na-phytate) (Azeem et al., 2014). The PB phosphatase and phytase activities were determined after one week incubation (25°C). The organic mineralized P, P mineralization efficiency, phosphatase and phytase activity and PB populations were measured.

2.6. N-AHL determination

Pseudomonas trivialis, *P. putida* and *P. fluorescens* were cultured and harvested during the stationary growth phase. PB pellets and root samples (rice, corn, bamboo, bananas and peanuts) were extracted with 4% chloroform and then dissolved in acetonitrile (Rani et al., 2011). The N-AHL from the PB and plant roots were analyzed by HPLC and compared against the standard N-AHL, $C_{4,6,8,10,12}$-AHL (Sigma-Aldrich, Germany), dissolved in acetonitrile (Merck, India) at a concentration of 50 mM.

2.7. Plant roots extract as PB N-AHL source

The experiment was arranged in a CRD with two factors. The first one was the type of plant roots extract comprising P1 = rice (RR), P2 = corn (CR), P3 = bamboo (BmR), P 4 = banana (BnR) and P 5 = peanut (PaR). The second factor was the rate of root extracts comprising D0, D1, D2, D3, D4, and D5 (0, 10, 20, 30, 40 and 50% roots extract, respectively). Each treatment was inoculated with PB 1 cm^3 (10^8 CFU cm^{-3}) and incubated for 5 days (until the end of the logarithmic growth phase) at 25°C. The PB growing medium was sterile liquid Pikovskaya medium (Pikovskaya, 1948).

2.8. P soluble determination

The PB was grown (25°C) in a Pikovskaya medium and treated with $Ca_3(PO_4)_2$ and $AlPO_4$ (Sigma, USA), $FePO_4$ (Aldrich, USA), pNPP or Na-phytate as sources of P. The amount of dissolved P

was determined calorimetrically by staining the samples with ammonium vanadomolybdate and read at a wavelength of 413 nm using a UV-VIS-1240 Shimadzu spectrophotometer.

2.9. Statistical analysis

The data of Pikoskaya P solubilization and P mineralization, Pikovskaya pH, PB population, solubilization index, soluble zone efficiencies, efficiencies dissolving P and PB phosphatase and phytase activity by three repetitions were analyzed using F-test with α 5%. Significantly different data were analyzed of mean using Duncant Multiple Range Test (CoStat-Statistics Software).

3. Results

3.1. PB species

The results of BLASTN analysis using 16S rRNA amplificons indicated that PB isolate 1 was classified as *Pseudomonas trivialis* (query coverage 95%; e-value 0.099% max; QV ≥ 16 at 740, QV ≥ 20 at 740; and GC 51.0%; BlastN J4PX2VSS013 ID). The isolate 5 was identified as *P. putida* (query coverage 82%; e-value 0.095% max; QV ≥ 16 at 569, QV ≥ 20 at 301; and GC 55.0%; BlastN J4R1ECDA012 ID) whereas the isolate 9 as *P. fluorescens* (query coverage 94%; e-value 0.099% max; QV ≥ 16 at 745, QV ≥ 20 at 743; and GC 52.0%; BlastN ID J4R7TRV6013).

3.2. Inorganic P solubilization and organic P mineralization

The organic acids secreted by PB were found as oxalic, citric, malic, lactic and acetic acids. The most secreted acid was citric acid meanwhile lactic acid was noted as the least. The pattern did not differ among PB isolates except for citric acid. PB inoculation increased the secretion of organic acid in the order of citric > malic > acetic > oxalic and lactic acids (Fig. 1 and 2).

The ability to dissolve inorganic P compounds and mineralize organic P compounds differed among the PB. Inorganic P was the most effectively solubilized by 1, 5 and 9 grown in a Pikovskaya medium than by other obtained isolates (Table 1). The P solubilization was the highest at CaP, followed by PR, AlP and FeP. Similarly, the isolates 1, 5 and 9 were capable of min-

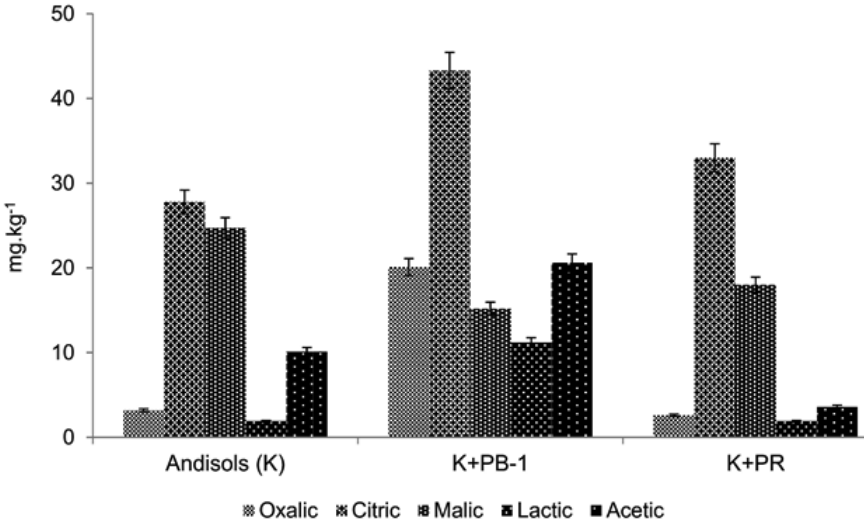

Fig. 1. Organic acids phosphate bacteria (PB) secretion in Andisols (for 4 weeks at 25°C incubated temperature). PB-1 = PB isolate 1; PR = 2.5 g phosphate rock 100 g^{-1} soil. The values of citric and malic acid for K + PB-1 (43.3 and 15.2) are 10 times of actual values

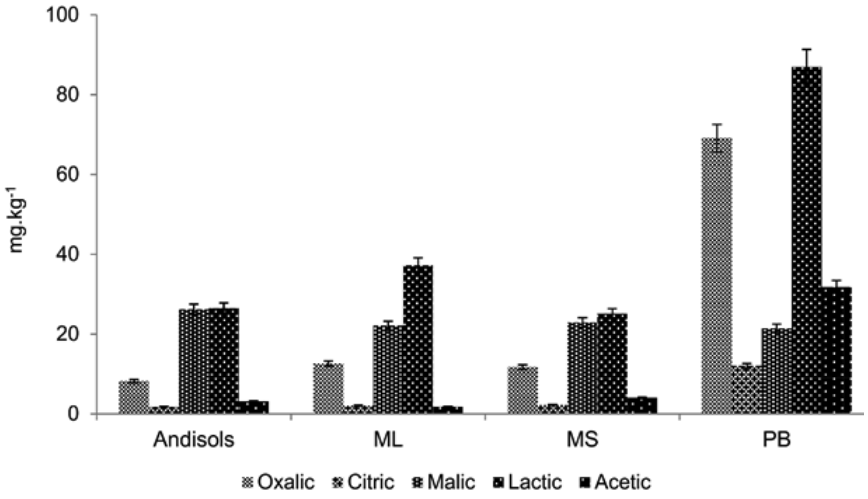

Fig. 2. Organic acids phosphate bacteria (PB) secretion in Andisols (for 4 weeks at 25°C incubated temperature). ML = liquid carrier medium; MS = solid carrier medium; PB = PB inoculant. The values of citric and malic acids in PB (87 and 21.4) are 10 times of actual values

Table 1
P solubilization and P mineralization on Pikovskaya by phosphate bacteria (PB) with various P sources (for 1 week at 25°C incubated temperature)

PB isolate	PR	CaP	AlP	FeP	pNPP	Na-phytate
mg P kg^{-1} (water soluble)						
Control	34.92 e	46.28 f	37.20 e	35.27 g	0.79 e	0.79 d
1 (Ja.b2)	198.17 b	238.61 d	101.63 d	127.20 c	134.24 b	181.76 a
2 (Jt. b2)	129.28 d	240.68 d	111.95 c	74.20 e	157.21 b	50.29 c
3 (Uja. b1)	151.94cd	197.36 e	94.62 d	156.88 b	74.45 d	60.19 c
4 (Ktt. b2)	156.09cd	290.18 a	84.97 d	75.61 e	155.23 b	182.95 a
5 (Bt. b2)	234.47 a	292.66 a	105.76 d	69.96 f	188.89 a	184.92 a
6 (Bt. b5)	165.17 c	264.39 b	125.56ab	74.20 e	102.56 c	74.84 c
7 (Pt. b2)	173.42 c	220.18 d	130.51 a	84.80 d	100.19 c	49.10 c
8 (Pa.b1)	170.53 c	225.62 d	122.26 b	77.03 e	176.2 ab	112.07 b
9 (Uka.b2)	170.12 c	204.70de	112.36 c	195.04 a	187.31 a	176.61 a
10 (Uka.b1)	221.27 a	236.55 c	120.20 b	69.25 f	85.14 d	101.38 b

Numbers in column followed by the same letter are not different according to DMRT α 5%. Isolate 1 = *Pseudomonas trivialis*; isolate 5 = *P. putida*; isolate 9 = *P. fluorescens*; Phosphate Rock (PR); CaP = $Ca_3(PO_4)_2$; AlP = $AlPO_4$; FeP = $FePO_4$; pNPP = para-Nitro Phenyl Phosphate; Na-phytate.

Table 2
Pikovskaya pH, PB population, solubilization index (SI), efficiencies of soluble zone (ESZ) and efficiencies dissolving P (EDP), PB phosphatase (Afo) and phytase (Afi) activity

PB isolate	pH	Log PB population (cfu dm^{-3})	SI	ESZ (%)	EDP (%)	Afo (mg PO_4^{3-} dm^{-3} h^{-1})	Afi (mg PO_4^{3-} dm^{-3} h^{-1})
1 (Ja.b2)	3.73 b	14.12 a	1.53 c	52.32 e	328.49 b	33.9 b	45.9 a
2 (Jt. b2)	3.88 b	13.90 a	1.51 c	50.94 e	250.87 d	39.7 b	12.7 c
3 (Uja. b1)	3.96 b	12.98 b	1.42 c	41.95 f	290.69 c	18.8 d	15.2 c
4 (Ktt. b2)	3.71 b	13.04 b	1.50 c	50.41 e	279.62 cd	39.2 b	46.2 a
5 (Bt. b2)	3.90 b	13.87 a	1.49 c	79.33 d	347.20 a	47.7 a	46.7 a
6 (Bt. b5)	4.05 a	13.49 ab	1.86 b	86.33 c	298.59 c	25.9 c	18.9 c
7 (Pt. b2)	3.81 b	12.95 b	1.91 b	91.31 c	291.49 c	25.3 c	12.4 c
8 (Pa.b1)	3.88 b	13.02 b	3.16 a	215.35 a	281.26 c	44.5 ab	28.3 b
9 (Uka.b2)	3.55 b	14.21 a	2.83 a	126.91 b	346.60 a	47.3 a	44.6 a
10 (Uka.b1)	3.62 b	13.54 ab	1.88 b	88.12 c	317.05 b	21.5 d	25.6 b

Numbers in the column followed by the same letter are not different according to DMRT α 5% (control: pH = 6.00 c, phosphatase activity = 0.2 e, and phytase activity = 0.2 d).

eralizing more organic P compounds as compared to the other isolates. The solubilization of inorganic P correlated with decreases in pH and increases in PB population, solubilization index and solubilization efficiency (Table 2).

3.3. PB Quorum Sensing Signal

The maximum expression of PB in dissolving P compounds was influenced by the optimum population through quorum sensing signal. The PB isolates produced five types of N-AHL quorum sensing signaling molecules comprising N-butanoyl (BHL), N-hexanoyl (HHL), N-octanoyl (OHL), N-decanoyl (DHL) and N-dodecanoyl (dDHL) Homoserine Lactones. BHL was produced in the highest concentrations at 10–15 times more than any others. The PB isolates produced N-AHL substances in the order of BHL > HHL > OHL > DHL > dDHL (Fig. 3). *Pseudomonas fluorescens* produced relatively more N-AHL than *P. putida* and *P. trivialis* (Fig. 4). Similarly, the P source affected the production of N-AHL by PB in the sequence of CaP > pNPP > FeP > AlP > Na-phytate (Fig. 5).

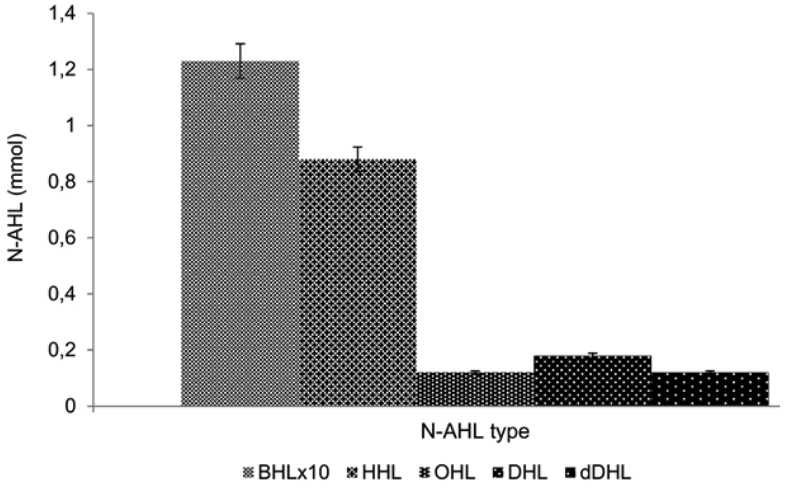

Fig. 3. The five N-AHL (mmol) was extracted from plant roots types and phosphate bacteria (N-butanoyl (BHL; C_4), N-hexanoyl (HHL; C_6), N-octanoyl (OHL; C_8), N-decanoyl (DHL; C_{10}) and N-dodecanoyl (dDHL; C_{12}) homoserine lactones)

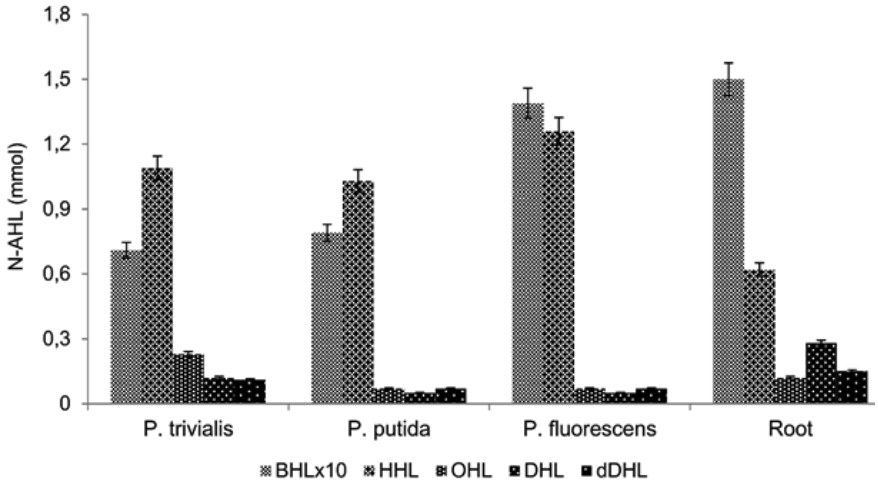

Fig. 4. The N-AHL (mmol) according to the PB types and plant roots extracted

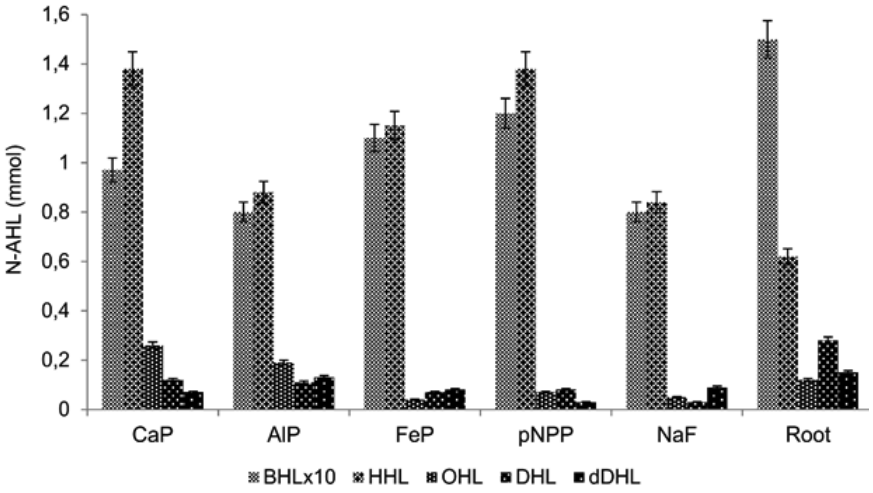

Fig. 5. The N-AHL (mmol) according to the P source and plant roots extracted

3.4. P Solubility by PB

The amount of soluble P in Pikovskaya was affected by the PB genera, so *P. fluorescens* produced twice as much as *P. putida* or *P. trivialis* (Fig. 6a). The PB were able to solubilize different P source types, in the order of CaP > AlP > FeP > pNPP > Na-phytate (Fig. 6b).

It was found that the roots extract of rice, corn, bananas, peanuts and bamboo produced similar types and amounts of N-AHL. The 30% of N-AHL corn root extract seemed to be the

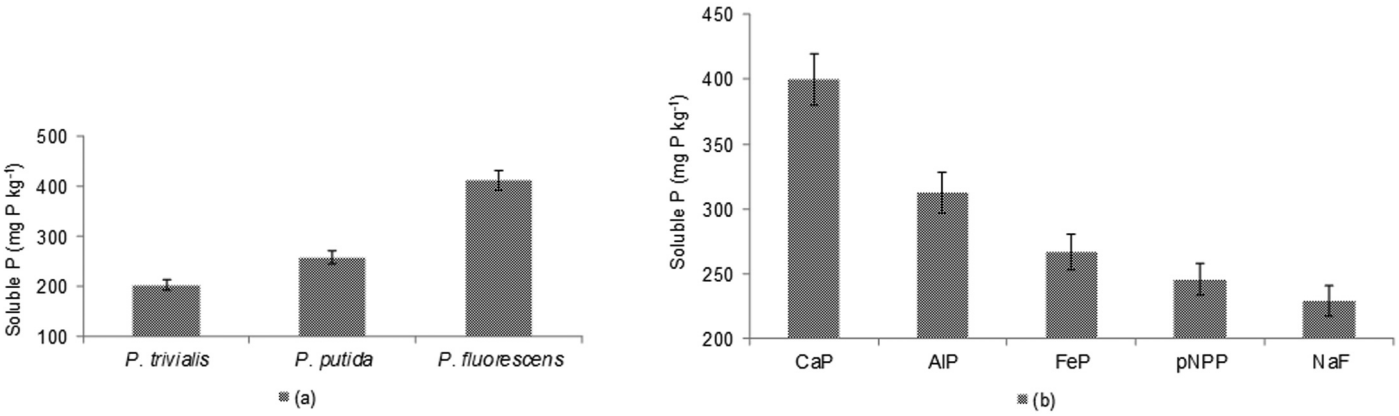

Fig. 6. Soluble phosphorus in Pikovskaya according to PB types (a) and P sources (b) for 5 days at 25°C incubated temperature

best of N-AHL source as it could increase P dissolution (even by 300%) in comparison to controls (Fig. 7). Consequently, the rhizosphere is the soil zone with the largest microbial population because plant roots secrete compounds like N-AHL (Fig. 8) required for microbial growth.

4. Discussion

The genetic analysis results indicated that three PB isolates were classified as *Pseudomonas trivialis*, *P. putida* and *P. fluorescens*. The evolutionary distance was 1.388 µm between *P. putida*

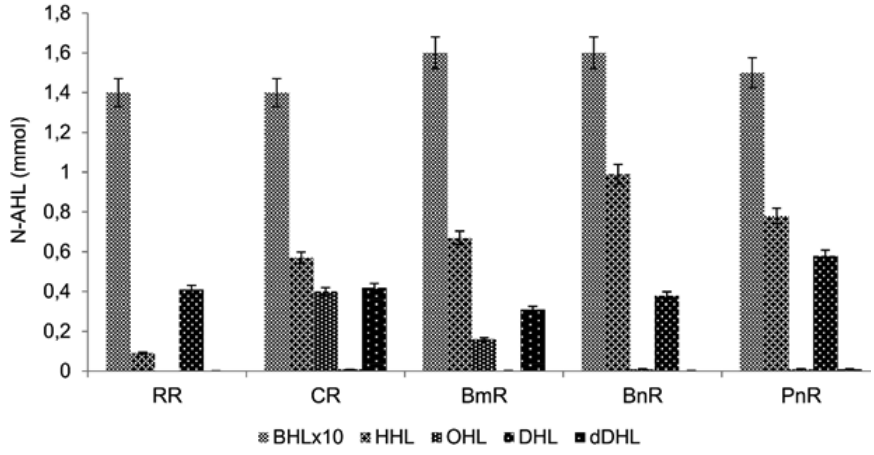

Fig. 7. The N-AHL (mmol) according plant roots extract (RR = rice, CR = corn, BmR = bamboo, BnR = banana and PaR = peanut plants root)

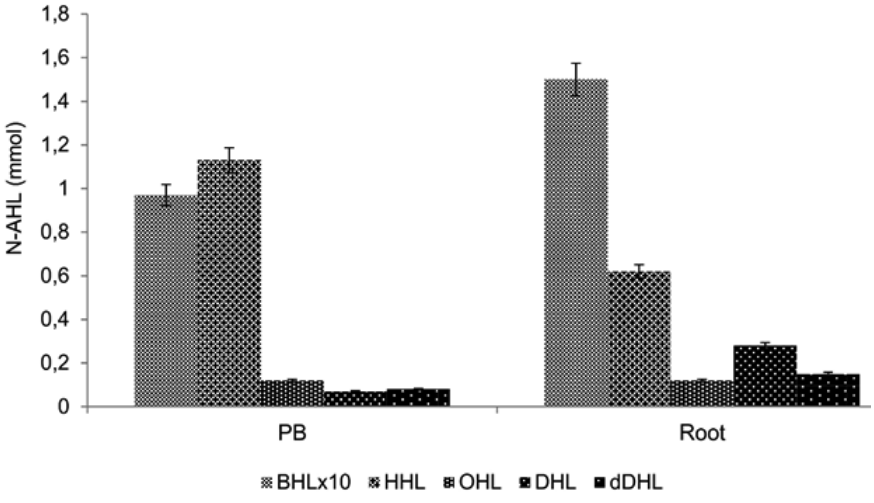

Fig. 8. The N-AHL (mmol) according to the N-AHL sources

and *P. trivialis*, 0.024 µm between *P. trivialis* and *P. fluorescens* and 1.409 µm between *P. fluorescens* and *P. putida* (Janda and Abbot, 2007; Kumar et al., 2008).

PB inoculation increased the secretion of organic acids in Andisols. Many studies have demonstrated that one of the mechanisms by which PB solubilize inorganic P is through the secretion of organic acids of low molecular weight (oxalic, citric, malic, lactic and acetic acids) through their primary metabolisms (Castagno et al., 2011; Stella and Syahren, 2016; Weimin et al., 2016; Yuquan et al., 2018). The secreted organic acids decrease soil pH, what affects on P solubility (Abbasi et al., 2015). The types and amounts of secreted organic acids are influenced by genetic and environmental factors (Gao et al., 2016; Saeid et al., 2018). The P solubility from AlP is greater than from PR and FeP (Rathi and Gaur, 2016; Stella and Syahren, 2016).

PB enhance organic P mineralization through the production of phosphatase and phytase for organic P degrading. Phosphatase is an enzyme that hydrolyzes organic P into inorganic P/ortho-phosphoric acid (–2 and –1) (Azeem et al., 2014; Abbasi et al., 2015; Belgaroui et al., 2016). Phytase enzyme catalyzes the hydrolysis of phytic acid, glucose 6-phosphate and glycerol 1-phosphate into inositol and orthophosphoric acid (Patki et al., 2015; Rathi and Gaur, 2016). A PB inoculant is viable if its population varied between 10^7 and 10^{10} cfu g^{-1} or cm^{-3} (Gao et al., 2016; Rathi and Gaur, 2016) and must be able to dissolve at least 30% of P (Rathi and Gaur, 2016; Saeid et al., 2018).

QS signaling molecules are complex protein sequences that represent overall gene topologies. The N-AHL was N-hexanoyl-(C6-HSL), N-octanoyl-(C8-HSL) and N-decanoyl Homoserine Lactone (C10-HSL) (Rani et al., 2011; Götz-Rösch et al., 2015). N-AHL activity reached a maximum level in their middle logarithmic phase and decreased in the stationary phase allowing bacteria to regulate their physiological activities by sensing these bacterial communities and population density (Ransome et al., 2013; Tan et al., 2014; Zhang et al., 2016; Liu et al., 2017).

The PB population and amount of soluble P were influenced by the PB isolate, P source type and the incubation length (Weiland-Bräuer et al., 2015; Saeid et al., 2018). The PB ability to release P was related to the PB population, while PB population was proportional to the concentration of N-AHL (Weiland-Bräuer et al., 2015; Zhang et al., 2016). QS allows bacteria to regulate their physiological activities by sensing the bacterial communities and the population density (Tan et al., 2014; Villamizar et al., 2019). QS regulate the bacterial population and the behavior of microbial cells in a particular environment (Wong et al., 2013; Palmer et al., 2014).

The area around the roots (rhizosphere) is known as a zone where the populations of microbes find an optimal condition for growth. This is due to plant roots secreting food source compounds and communication compounds for microbes with microbes and microbes with plants. Rice, corn, bamboo, banana and peanut roots extracts have equal potential to be used as a N-AHL source. Plant roots extract can be used as a N-AHL source because the metabolites that plant roots secrete include N-AHL (Tan et al., 2014; Dangjarean et al., 2015; Ma et al., 2016). The rhizobacteria produce plant growth-promoting factors and N-AHL which have potential as plant growth promoting agents (Palmer et al., 2014; Dangjarean et al., 2015; Liu et al., 2017). Plant roots produce and exude QS molecules as a means of mutual interactions between plants and microorganisms in the surrounding environment (Kobus, 1999; Badura, 2004; Götz-Rösch et al., 2015).

5. Conclusions

PB were identified as *Pseudomonas trivialis*, *P. putida* and *P. fluorescens*. The PB secreted citric, lactic, malonic, oxalic and acetic acids amounting to 156.25 mg kg^{-1}. The PB phosphatase and phytase activities produced by PB ranged from 12 to 47 mg PO_4^{3-} dm^{-3} h^{-1}. The P solubilized in Pikovskaya PB inoculated was much greater between 147.66 and 194.61 mg P kg^{-1} as control compared (31.06 mg P kg^{-1}). PB inoculation produced greater mineralized organic P (63.69 mg P kg^{-1}) than the control (23.7 mg P kg^{-1}). PB secreted N-AHL was greatest as butanoyl-AHL (C_4-AHL). The corn root extract 30% dose was the best PB N-AHL source as it could increase P dissolution by 300% compared to control.

Acknowledgements

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