Can Tho University Journal of Science
Special issue: Agriculture
website: sj.ctu.edu.vn

DOI:10.22144/ctu.jsi.2018.093

Phosphate solubilization, indole-3-acetic acid synthesis and nitrogen fixation ability of various indigenous microorganism communities from different agriecosystem habitats

Le Thi Xa[1], Ngo Thi Phuong Thao[1] and Nguyen Khoi Nghia[2*]

[1]*Biotechnology Research and Development Institute, Can Tho University, Vietnam*

[2]*Department of Soil Science, College of Agriculture and Applied Biology, Can Tho University, Vietnam*

[*] *Correspondence: Nguyen Khoi Nghia (email: nknghia@ctu.edu.vn)*

ARTICLE INFO

Received 23 May 2018
Revised 12 Jun 2018
Accepted 03 Aug 2018

KEYWORDS

Biological nitrogen fixation, indigenous microorganism communities, indole-3-acetic acid, nifH gene, phosphate solubilization

ABSTRACT

Bio-fertilizer formulation from indigenous microorganism communities (IMOCs) is great suitable methods applied widely in the eastern part of world for the extraction of minerals, enhancement of agriculture and waste management although its functionalities have been unknown. The aim of this study was to assess phosphate solubility, indole-3-acetic acid (IAA) biosynthesis and nitrogen fixation efficacy of various IMOCs from different farming systems within Soc Trang province of Vietnam. Phosphate solubilization and synthesis IAA abilities of collected IMOCs were investigated in National Botanical Research Institute's Phosphate (NBRIP) liquid media containing tricalcium phosphate (TCP) as the sole P source. This medium was supplemented with and without tryptophan (100 mg/L) for IAA synthesis capability evaluation while nitrogen fixation ability was tested in liquid N-free Burks media. Besides, nifH functional gene involving in nitrogen fixation was also detected by specific polF/polR primers. The results showed that all surveyed IMOCs were found to solubilize TCP with a various extent, and the maximum amount of P_2O_5 solubilized was over 2,000 mg/L. In regard to the IAA biosynthesis, all IMOCs were able to biosynthesize considerably IAA with the highest IAA amount of 56.6 mg/L. All surveyed IMOCs had potential in nitrogen fixation when the primer amplified nifH gene successfully from DNA of collected IMO, and eight out of 15 IMOCs proved their nitrogen fixation with quantity varied between 1.0 and 6.5 mg/L N. In conclusion, all collected IMOCs had beneficial functions for plants like phosphate solubility, IAA synthesis and biological nitrogen fixation which can be exploited for enhancing soil fertility and plant growth.

Cited as: Xa, L.T., Thao, N.T.P. and Nghia, N.K., 2018. Phosphate solubilization, indole-3-acetic acid synthesis and nitrogen fixation ability of various indigenous microorganism communities from different agriecosystem habitats. Can Tho University Journal of Science. 54(Special issue: Agriculture): 39-48.

1 INTRODUCTION

At the present, environmental protection has the foremost importance. Many technologies available for enhancement of agriculture, management of agricultural waste, etc. have been applied widely. The concept indigenous microorganism (IMO) is developed by Cho (1997) from the Janong Farming Institute, South Korea. IMO-based technology is a great technology applied in the eastern part of the world. IMO cultures contain consortia of beneficial microorganisms comprising of fungi and bacteria that are deliberately collected and cultured from soils to enhance organic matter degradation (Reddy, 2011). IMOC is a group of innate microbial consortium that inhabits the soil and the surfaces of all living things. It has the potential in biodegradation, bioleaching, bio-composting, nitrogen fixation, phosphate solubilization, soil fertility improvement and in the production of plant growth hormones as well (Kumar and Gopal, 2015). In facts, the positive effects of IMOCs on soil physical, chemical and biological properties and soil enzyme activities, soil healthy and crop yield were proven by many previous studies (Sumathi *el al*., 2012; Koon *el al*. 2013; Mbouobda *el al*., 2013). In addition, application of IMOCs resulted in increasing plant/leaf growth, earlier germination, increased seed yield and increased chlorophyll contents (Sekhar and Gopal, 2013). According to Chiemela *el al.* (2013b), many studies indicated that application of IMOCs in agriculture is environmentally friendly method and helps to enhance organic matter decomposition, plant nutrition, soil fertility, crop yields and resistance to plant diseases. Application of IMOCs was effective in compost production since it promotes the rapid degradation of agricultural and plant residues, producing large amount of micronutrients in the soluble form that are very easily to be taken up by plants (Chiemela *el al*., 2013a). Recently, in Hawaii, use of IMOCs to treat disease deadly caused by *Ceratocystis* sp. in Ohia trees has brought about a big surprised efficacy in rapid stopping this deadly plant disease, and scientists were so interested to know the mechanisms of biocontrol functions towards this deadly plant pathogen. In general, it is well known that IMOCs bring many benefits to plants and have been applied broadly in agriculture. However, deep and scientific knowledge of IMOCs is still lacking and should be scientifically elucidated. Therefore, the aim of this study was to assess the phosphate solubilization, biological nitrogen fixation and IAA synthesis abilities of several collected IMOCs from different agri-ecosystem habitats.

2 MATERIALS AND METHODS

2.1 Collection and cultivation of IMOCs

IMOCs were collected from different farming systems including grapefruit, rice, vegetable, sugarcane, maize, orange, banana, bamboo, shallot, grassland, etc. within the Soc Trang province, Vietnam by following the method described by Cho (1997). At each sampling site, three plastic baskets (25x15x8 cm) were used, corresponding as 3 replicates of each sampling site. Each basket was filled with 0.5 kg of steamed rice and covered on the top of the basket with cloth and waist belt. The baskets were buried under the soil at each sampling site and covered the top of baskets with leaf litters for four days. After four days of incubation when the microorganisms grew over the rice surface, before harvesting the fermented rice with indigenous microorganisms, the dark color mold infested rice parts were removed, the bright color mold infested rice parts were taken, put into a glass jar and carried to the laboratory. This source of microorganisms was called IMOC1. Collected IMOCs were mixed with brown sugar with a ratio of 1:1 (w/w) until the mixed material became gooey; the mixed material was stored in the ceramic pot in a cool area and away from direct sunlight for seven days for fermentation. After seven days of fermentation, this source of microorganisms was called IMOC2. The IMOC2 was kept in the refrigerator at 4^{o}C for further studies.

2.2 Determining the phosphate solubility and IAA synthesis abilities of collected IMOCs

2.2.1 Phosphate solubilizing ability of collected IMOCs

An aliquot of 10 grams of each IMOC was put into a 250 mL glass bottle containing 90 mL sterilized distilled water on an orbital shaker at a speed of 90 rpm for an hour, then 1 mL of the microbial solution was added into a 100 mL Erlenmeyer flask containing 49 mL NBRIP liquid medium. The composition of NBRIP liquid medium (g/L) was, D-glucose (10 g), $Ca_3(PO4)_2$ (5.0 g) $MgCl_2.6\ H_2O$ (5.0 g), $MgSO_4.7H_2O$ (0.25 g), KCl (0.2 g), $(NH4)_2SO_4$ (0.1 g), pH=7,0±0.2 (Mehta and Nautiyal, 2001). Each IMOC contained 3 replicates. Samples were put on the orbital shaker and shaken with a speed of 90 rpm in the dark and under laboratory conditions for 20 days. The concentration of phosphorus dissolved in the liquid medium by microbes was determined after 2, 5, 9, 15 and 20 days of incubation. An aliquot of 1 mL IMOC cultures was centrifuged at 12,000 rpm for 5 minutes. The centrifuge sample was diluted 100 times with MQ water, then 1 mL of solution was added to the new Eppendorf 2 mL, then

added 200 µL of ascorbic acid ammonium molybdate reagent solution to the sample with a ratio of 5:1(v/v) and mixed well for 1 min. The samples were let to stand for 20 minutes at room temperature. Optical density was taken at 880 nm with the help of spectrophotometer (Spectrometer Thermo Scientific, Multiskan Spectrum). Concentration of P_2O_5 produced by cultures was measured with the help of standard graph of P_2O_5 obtained in the range of 0-1 mg/mL.

2.2.2 IAA synthesis ability of collected IMOCs

An aliquot of 10 grams of each IMOC was put into a 250 mL glass bottle containing 90 mL sterilized distilled water on an orbital shaker at a speed of 90 rpm for an hour, then 1 mL of the microbial solution was added into a 100 mL Erlenmeyer flask containing 49 mL NBRIP liquid medium with tryptophan (100 mg/L) and without tryptophan (pH = 7). Three replicates were repeated for each IMOC. The samples were put on the orbital shaker and shaken with a speed of 90 rpm in the dark and under laboratory conditions for six days. The IAA production synthesized by microorganisms in liquid medium was determined after one, two, three, five and six days of incubation by the modified method described by Brick *el al*. (1991). One mL aliquot of fully grown cultures was centrifuged at 3,000 rpm for 30 minutes. The supernatant (2 mL) was mixed with two drops of orthophosphoric acid and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution). Development of pink color indicates IAA production. Optical density was taken at 530 nm with the help of spectrophotometer. Concentration of IAA produced by cultures was measured with the help of standard graph of IAA obtained in the range of 0-100 mg/mL.

2.3 Determining the biological nitrogen fixation capacity of IMOCs

2.3.1 Detection of the presence of functional nifH gene indicating for biological nitrogen fixation ability of collected IMOCs

The selected primers of PolF/PolR were tested on microorganism DNA extracted from $IMOC_2$ for searching *nifH* function gene. Firstly, DNA of each IMOC was extracted by MO BIO kit (Qiagen), then, primer polF/polR (Poly *el al*., 2001) were used for PCR reaction to amplify 360 bp sequences of *nif* H gene. The volume of 25 µL of PCR reaction included 12.5 µL Green mix (2X), 2 µL primer *polF* (10 µM), 2 µL primer *polR* (10 µM), 2 µL of pure DNA, and 6.5 µL deionized water. The reactions were carried out as follows: 5-min initial denaturation of DNA at 94°C, followed by 35 cycles of 1-minute denaturation at 94°C, 1-minute primer annealing 57ºC, and 1-minute extension at 72°C. Amplification was completed by a final extension step at 72°C for 10 minutes. To visualize the PCR products, 5 µL of the reactions were loaded into 2% of agarose gel, 5µL of 100 bp ladder was also loaded into gel as a molecular weight marker. Gels ran for 30 minutes at 150 volts and 500 milliamps and were then visualized and photographed by UV light from Gel Logic 1500 (Kodak) to find target sequences with the size of 360 bp.

2.3.2 Quantification of nitrogen fixation capacity of IMOCs

An aliquot of 10 grams of each IMOC sample was put into a 100 mL glass jar containing 90 mL sterilized distilled water, then 1 mL of the microbial solution was transferred to a 100 mL Erlenmeyer flask containing 50 mL of N-free Burks liquid medium. The composition of N-free Burks liquid medium (g/L) was sucrose (10 g), $K_2HPO_4.4H_2O$ (0.41 g), KH_2PO_4 (1.05 g), $CaCl_2.2\ H_2O$ (0.1 g), $MgSO_4.7H_2O$ (0.1 g), $FeSO_4.7H_2O$ (0.015 g), H_3BO_3 (0.0025 g), Mo (0.0025 g) (Mehata and Nautiyal, 2001). The samples were put on the orbital shaker at a speed of 90 rpm in the dark under the laboratory conditions for seven days. After seven days of incubation, an aliquot of 1mL culture solution was taken to a new 100 mL Erlenmeyer flask containing 50 mL fresh N-free Burks liquid medium, and the samples were shaken on the shaker for another seven days. Repeated the whole procedure for three times in total. After this step, the total nitrogen fixing microbes were determined on Burks agar medium. An assay to evaluate the nitrogen fixing capacity of IMOCs was done by adding 1 mL of the third enriched generation culture of each IOMC into 100 mL Erlenmeyer flask containing 15 mL fresh N-free Burks liquid medium. Each IMOC was repeated 18 times, and three replicates were scarified at each sampling time.

The samples were put on the orbital shaker at a speed of 90 rpm in the dark under the laboratory conditions for five days. The total nitrogen content biologically fixed by IMOCs was determined after zero, one, two, three, four and five days of incubation by a method described by Keeney and Nelson (1982). The microbial cultures were first digested with 5 mL of H_2SO_4 in the digestion chamber at 360°C for two hours. The color change was monitored from dark brown to greenish white by adding some drops of hydrogen peroxide. The contents were cooled and transferred the whole amount of sample into 50 mL volumetric flask, and 50 mL volume make-up was done with distilled water. This

solution was standed for 20 hours at room temperature. Optical density was taken at 650 nm with the help of spectrophotometer (Spectrometer Thermo Scientific, Multiskan Spectrum). The concentration of NH_4^+ produced was measured with the help of standard graph of NH_4^+ obtained in the range of 0-10 mg/L.

2.4 Data analysis

The data were analyzed by ANOVA and compared by DUNCAN test with MINITAB version 16 software.

3 RESULTS AND DISCUSSION

3.1 Collection of IMOCs from different ecosystem habitats

Fourteen IMO had been collected in Soc Trang province and one IMO was created from the mixture of all fourteen. IMOCs including bamboo, crop rotation, banana, shallot, vegetables, rice, watermelon, grassland, maize, salad, oranges, grapefruit, guava, sugarcane, were collected from farming systems in Soc Trang province, Vietnam.

3.2 Phosphate solubilizing and IAA synthesis ability of collected IMOCs

3.2.1 Phosphate solubility

The result of study on phosphate solubilizing ability of 15 different IMOCs from different ecosystem habitats was presented in Table 1 indicating that the capable of phosphate solubilization from tricalcium phosphate to form soluble phosphate by IMOCs widely varied among IMOCs. The time to reach the maximum values of soluble phosphate in liquid medium was different among them. Their phosphate solubilizing capacity was significantly different as compared with each other ($p<0.01$). Two out of 15 tested IMOCs released more than 2,000 mg/L P_2O_5 after 20 days of incubation, and eleven out of fifteen IMOCs had ability to liberate more than 1,000 mg/L P_2O_5 at the surveyed period. The phosphate solubilizing capacity of three IMOCs collected from mono watermelon, guava and sugarcane cultivation fields was impressively and significantly high on the second day of incubation as compared to that of others, and the value of soluble P_2O_5 was 1,247, 1,248 and 1,542 mg/L respectively. Especially, IMOCs from guava and sugarcane also were the earliest ones to reach the maximum peak of soluble P_2O_5 in liquid medium after two days of incubation, and afterwards the amount of soluble P_2O_5 was drastically reduced while the amount of soluble phosphate in the liquid medium of IMOCs collected from mono maize, vegetables, oranges, grapefruit cultivation fields and a mixed culture was maximally reached on the fifth day, varied between 1,439 and 1,738 mg/L and deepened thereafter. Although the phosphate solubilizing ability of IMOC from mono watermelon cultivation field in liquid medium was slower than the others during the first-nine incubation days, later it slightly increased and reached the highest point after day 20 with an amount of 1,901 mg/L. The same trend was observed for IMOC of grassland field with the highest amount of 2,011 mg/L of P_2O_5. Both IMOCs from mono watermelon and grassland fields together were top two phosphate solubilizing IMOCs among fifteen IMOCs surveyed. The highest phosphate soluble production belonged to IMOC from a crop rotation system field. At day 9, the value of soluble P_2O_5 was low (1,843 mg/L), slightly decreased on day 15 and reached the maximum peak at day 20 with an amount of 2,372 mg/L P_2O_5. The phosphate solubilizing abilities from tricalcium phosphate source in liquid medium of IMOCs from mono bamboo, banana and rice cultivation fields were found to be lowest with a ranging of 210 - 510 mg/L of P_2O_5.

As can be seen in Table 1, a big variation of soluble phosphate in the liquid medium among IMOCs varied from 210 mg/L to 2,372 mg/L was found. It means that some IMOCs owned lower soluble phosphate concentration in liquid medium as compared to others and vice versa since the higher soluble P level would indicate that the microbes have a better P-solubilizing and P-releasing ability. In this case, this type of microbes cannot be used to promote P uptake by plants since P is not released. However, a function of phosphate solubilization of a mixed IMOC was much better than that of some other single IMOCs. Therefore, a combination of several IMOCs from deferent ecosystem habitats is another approach and is very essential to have better phosphate solubilizing abilities of IMOC (Reddy, 2011).

In comparison with other previous studies, it was found that Wu *el al*. (2012) isolated a very high effective phosphate-solubilizing fungus from rhizosphere soil with the maximum amount of 1,252 mg/L of P_2O_5 for 40 hours in a liquid medium containing tricalcium phosphate (TCP) as sole phosphate source. Similarly, Tam *el al*. (2016) obtained 25 fungal strains from six ferrallisols samples of Tithonia and evaluated their solubilizing capacity for insoluble phosphate ($Ca_3(PO_4)_2$) in liquid medium. The result showed that these isolated fungi could dissolve phosphate from insoluble form with arrange of 37.25 mg/L to 494.41 mg/L of P_2O_5. Additionally, Walpola and Yoon (2013) isolated phosphate solubilizing bacteria in soil in South Korea which dissolved TCP with maximum P solubilization at 720.75 µg/L. Saikrithika *el al.* (2016) evaluated the amount of phosphorus solubility from TCP

of a bacterial strain isolated from vermi-compost applied soil. It was able to solubilize up to 125 mg/L of P_2O_5. For mineral phosphate solubilization capacity, Krishnaraj and Dahale (2014) concluded that 53 isolated strains including bacteria, fungi, actinomyces from many previous studies could solubilize and liberate phosphate with a range of 100 µg P_2O_5/mL to 500 mg P_2O_5/mL. Many studies have shown that P solubilizing microorganisms can secrete a variety of low-molecular organic acids during metabolism, such as malic acid, propionic acid, lactic acid, acetic acid and citric acid. These organic acid anions can react with calcium ions in the liquid medium to release P from modestly soluble phosphates (Lin *el al*., 2001). Besides, some extracellular enzymes, even ammonium salts and nitrate salts, etc. are released by microbes to release into liquid medium, leading to dissolve highly insoluble TCP (Krishnaraj and Dahale, 2014).

Table 1: Dynamic of soluble phosphate concentration in NBRIP liquid culture of 15 IMOCs within 20 days of incubation (n=3 and standard deviation)

Origin of samples	Soluble P_2O_5 (mg/L) concentration					
	Day 2	Day 5	Day 9	Day 15	Day 20	Highest value
Bamboo	110 ^{hi}	244^{ef}	451^{f}	510^{e}	462^{e}	510^{h}
Crop rotation	131 ^{gh}	436^{d}	1686^{b}	2018^{a}	2372^{a}	2372^{a}
Banana	119^{hi}	282^{def}	347^{g}	183^{g}	184^{f}	347^{i}
Shallot	164 ^{g}	357de	1160^{c}	34^{h}	19.2^{i}	1160^{g}
Salad	90 ^{i}	307de	1150^{c}	1148^{d}	1327^{d}	1327^{ef}
Rice	106^{hi}	133 ^{f}	211^{gh}	177^{g}	125^{fgh}	211^{j}
Watermelon	1249 ^{b}	1427^{b}	1662^{b}	1625^{c}	1901^{c}	1901^{b}
Grassland	766 ^{d}	1801^{a}	1843^{a}	1803^{b}	2011^{b}	2011^{b}
Maize	88^{i}	1499^{b}	780^{d}	119^{gh}	133^{fgh}	1499^{d}
Vegetables	602^{f}	1451^{b}	1155^{c}	385^{f}	157^{fg}	1451de
Oranges	858 ^{c}	1738^{a}	635^{e}	33^{h}	101^{ghi}	1738^{c}
Grapefruit	632 ^{f}	1439^{b}	153^{hi}	18^{h}	86^{ghi}	1439de
Guava	1248 ^{b}	1031^{c}	55ij	25^{h}	82^{ghi}	1248 ^{fg}
Sugarcane	1542 ^{a}	262^{ef}	15^{j}	38^{h}	97^{ghi}	1542^{d}
Mixed	672 ^{e}	1560^{b}	31^{ij}	33^{h}	65^{hi}	1560^{d}

**Note: Values in the same column with the same letters are not significant difference at 1% level (p<0.01)*

In short, it was clear that all collected IMOCs had a great potential in phosphorus solubility, and the solubilization efficacy of these IMOCs was similar to that of singly isolated fungal or bacterial strains.

3.2.2 IAA synthesis production

The result of the study on IAA synthesis ability of IMOCs from different ecosystem habitats was presented in Table 2. It can be seen that the amount of IAA produced by IMOCs obtained from different habitats varied significantly over the time period and was significantly different when compared with each other. The IAA producing capacity of IMOCs was synthesized very early even after one day of incubation. The synthesized IAA content of the IMOCs varied largely from 9.23 to 56.6 mg/L. The highest amount of IAA was observed after two days of incubation and found in IMOC originated from mono rice cultivated field with the value of 56.58 mg/L while the time for others IMOCs to reach their highest peak of IAA production was very different. The second position of IAA product belonged to the IMOC from mono maize cultivated field with an amount of 43.85 mg/L. The IMOC collected from oranges cultivated field and mixed IMOCs together shared the third place in synthesis of IAA with amount of 41.77 mg/L and 42.62 mg/L, respectively. The remaining IMOCs had IAA amount between 10.71 mg/L and 38.77 mg/L. The lowest IAA productions were found in IMOC from crop rotation system, mono banana and mono salad cultivated fields with 9.66 mg/L, 9.23 mg/L and 10.74 mg/L IAA produced, respectively. In general, the increase and reduction of IAA concentration in the liquid medium of almost collected IMOCs over the time period were gradually, except for IMOCs of grassland and mono salad cultivated fields, especially in case of salad IMOC which dropped strongly to 0.00 mg/L at the second day after inoculation.

Unlike behaviors of IMOCs in IAA producing capacity when cultivated in NBRIP medium supplied with tryptophan (100 mg/L), the behaviors of IMOCs in IAA producing capacity when cultivated in NBRIP medium supplied without tryptophan (100 mg/L) were different. There were only seven IMOCs showed their ability in IAA synthesis when cultivated in NBRIP liquid without tryptophan sup-

plementation although the amounts of IAA produced was very low and ranged between 0.46 mg/L and 3.11 mg/L. However, the IAA amounts in the liquid medium were extremely dropped on day 6 when almost IMOCs had 0 mg/L IAA, except for the case of IMOC from mono banana cultivated field where the amount of IAA was still maintained up to day 6 (data not showed). An amount of 3.09 mg/L and 3.11 mg/L IAA in NBRIP medium without tryptophan supplement was found to be on IMOCs from mono grapefruit and mono sugarcane cultivated fields, respectively as the highest IAA producers while IMOC collected from bamboo tub had the lowest amount of IAA (0.79 mg/L). Moreover, it was noteworthy that when mixing partly all the collected IMOCs together to have an integrated IMOC, the amount of IAA produced by this microbial community was quite good and stable over time period of 6 days.

Table 2: Concentration of synthesized IAA production of IMOCs by in NBRIP liquid medium added with tryptophan (100 mg/L) within 6 days of incubation (n=3, standard deviation)

Origin of samples	Synthesized IAA concentration (mg/L)					
	Day 1	Day 2	Day 3	Day 5	Day 6	Highest value
Bamboo*	18.57^{a}	28.77^{cd}	27.02^{d}	27.69^{b}	17.5^{fg}	28.77^{de}
Crop rotation*	6.54^{ef}	9.66^{g}	4.67^{hi}	8.27^{fg}	4.11^{hi}	9.66^{gh}
Banana*	3.07^{gh}	9.23^{g}	8.17^{gh}	2.33^{gh}	0.70^{i}	9.23^{h}
Shallot	17.31^{a}	17.16^{f}	13.44^{fg}	13.38^{ef}	7.15^{h}	17.31^{fg}
Vegetables	3.29^{fgh}	27.80^{cde}	33.06^{ab}	34.66^{a}	29.87^{bc}	34.66^{cd}
Rice	8.12^{de}	56.58^{a}	40.33^{a}	34.69^{a}	32.81^{bc}	56.58^{a}
Watermelon	17.29^{a}	30.53^{c}	24.56^{de}	35.93^{a}	28.48^{bcd}	35.93^{bcd}
Grassland	1.40^{h}	25.42^{cde}	24.17^{de}	1.33^{h}	3.63^{hi}	25.42^{e}
Maize	2.48^{gh}	23.72^{ef}	43.85^{a}	23.59^{bc}	23.81^{de}	43.85^{b}
Salad	10.74^{cd}	0.00^{h}	0.00^{i}	0.00^{h}	0.00^{i}	10.74^{gh}
Oranges	13.64^{bc}	24.86^{de}	22.50^{de}	36.37^{a}	41.77^{a}	41.77^{bc}
Grapefruit*	11.25^{cd}	23.59^{ef}	18.93^{ef}	20.98^{cd}	22.96^{ef}	23.59^{ef}
Guava*	16.18^{ab}	15.99^{f}	14.38^{f}	12.76^{ef}	15.04^{g}	16.18^{fgh}
Sugarcane*	5.79^{efg}	23.17^{e}	38.77^{ab}	15.52^{de}	17.00^{g}	38.77^{bc}
Mix IMO*	7.86^{de}	42.62^{b}	27.22^{cd}	25.05^{bc}	27.85^{cd}	42.62^{bc}

** IMOC was able to synthesize IAA in the absence of tryptophan; Values in the same column with the same letters are not significant difference at 1% level (p<0.01)*

The previous results study of Ahmad *el al*. (2005) tested for the production of IAA in a medium containing tryptophan (0, 1, 2 and 5 mg/mL) of 10 strains of *Azotobacter* sp., 11 strains of *Pseudomonas* sp, and the result showed that a low amount (2.68 - 10.80 mg/mL) of IAA production was observed in the treatments of *Azotobacter* strains in the liquid medium without tryptophan addition. Seven *Azotobacter* strains showed their high production of IAA (7.3 to 32.8 mg/ml) in the treatment added with 5 mg/mL of tryptophan while the value if IAA varied from 41.0 to 53.2 mg/mL for *Pseudomonas sp.* strains. Moreover, Ahmad *el al*. (2008) isolated free-living rhizospheric bacteria for their multiple plant growth promoting activities and quantified IAA amounts at different concentrations of tryptophan (0, 50, 150, 300, 400 and 500 µg/mL) for *Azotobacter* sp., *Pseudomonas* sp. and *Bacillus* sp. The results showed that these bacterial strains could not synthesize IAA properly in the condition without tryptophan, and they showed their highest ability in IAA production when the culture medium was added with 500 µg/mL tryptophan, and the amount of IAA was ranged from 7.03 µg/mL to 22.02 µg/mL.

During plant growth, in addition to endogenous IAA, plant growth is affected by a low amount of auxin outside of the plant from a IAA synthesis of microorganisms. Many bacterial species were found to be capable of producing phytohormones IAA (Saharan and Nehra, 2011). The effect of IAA on each plant variety depends on its concentration, and at low concentrations, IAA can stimulate the growth of plant, but it can also cause an inhibition at high levels (Frankenberger and Arshad, 1995). The numbers of specific bacteria around the roots determined the concentration of auxin. In the same way, different plants respond differently to variable concentrations of auxin (Sarwar and Frankenberger, 1994) and to kind of microorganisms (Ahmad *el al*., 2005). In the soil, the highest level of auxin synthesized by microorganisms was found for IAA and indole acetamide at normal conditions, resulting in maximum growth and yield of wheat (Khalid *el al*., 2004). Even slower and more stable IAA-producing strains

will also help to improve plant productivity (Tsavkelova *el al*., 2007).

3.3 Nitrogen fixation

3.3.1 Searching for functional nifH gene in collected IMOCs

The result of polymerase chain reaction showed that the primer amplified *nifH* gene successfully from DNA of all collected IMOCs, although there was no obvious variation in the size of *nifH* gene products between 14 collected IMOC (Figure 1). The size of target sequences of *nifH* gene was approximate 360 bp which matches the earlier study of Poly *el al*. (2001a and 2001b). When they used primer polF/polR to detect functional *nifH* gene and it is showed that these primers were sensitive with 5 referring N_2-fixing strains like *Azospirillum brasilense, Azospirillum lipoferum, Rhizobium leguminosarum, Sinorhizobium meliloti* and *Frankia alu* and 19 isolated strains from soil as well. It was also important to suggest that all IMOcs originated from different ecosystem habitats of this present study have a great potential and function for biological nitrogen fixation, no matter what strains or species they were and how many trains or species IMOCs had.

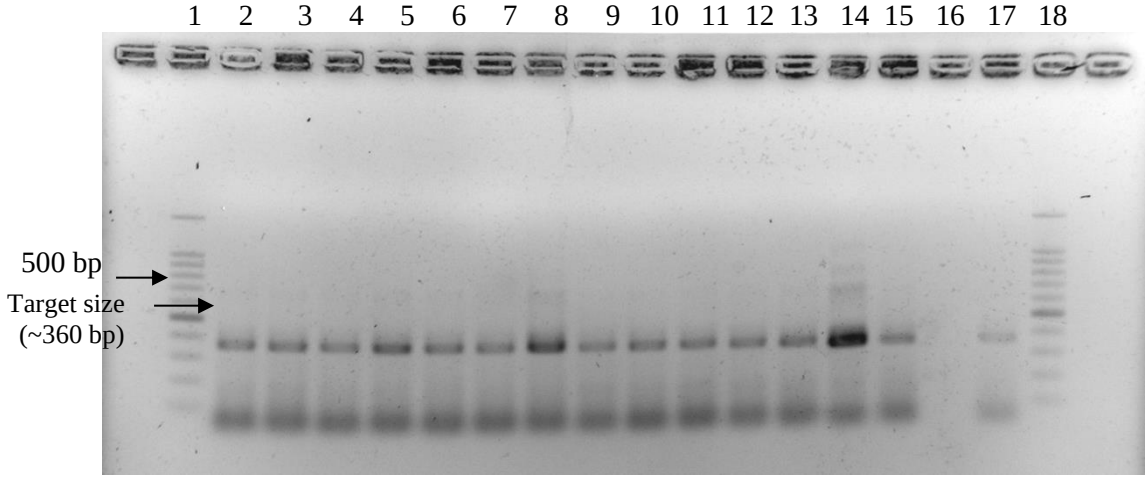

Figure 1: Functional *nifH* gene PCR products by polF/polR primer amplification of 14 collected IOCs

**Note: Land 1: 100 bp standard ladder; land 2: IMOC from bamboo; land 3: IMOC from crop rotation; land 4: IMOC from banana; land 5: IMOc from shallot; land 6: IMOC from vegetables; land 7: IMOC from rice; land 8: IMOC from watermelon; land 9: IMOC from grassland; land 10: IMOC from maize; land 11: IMOC from salad; land 12: IMOC from oranges; land 13: IMOC from grapefruit; land 14: IMOC from guava; land 15: IMOC from sugarcane; land 16: negative control (H_2O); land 17: positive control (a strain isolated from IMOCs of guava); Land 18: 100 bp standard ladder*

3.3.2 Quantification of nitrogen fixation ability of IMOCs

After three times of consecutive transferring the IMOCs liquid medium to enrich the growth of nitrogen fixers in Burks medium, the number of nitrogen fixing bacteria in Burks liquid medium was ranged from 10^5 to 10^7 CFU/mL (data not showed). The result of study on nitrogen fixing ability of IMOCs from different ecosystem habitats was presented in Table 3. It can be seen that there was a significant difference between IMOCs in nitrogen fixing capacity ($p<0.01$), and the time to appear maximum peak of NH_4^+ concentration in the Burks liquid medium was varied differently. In this present study, eight IMOCs showed their capacity in nitrogen fixation over 1 mg/L NH_4^+, and maximum amount of NH_4^+ fixed in the liquid medium was 6.48 mgN/L in the IMOC from bamboo tub after three days of incubation. The amount of nitrogen fixed by IMOCs was relatively low, and the nitrogen fixation ability of each IMOC was not only varied among the IMO but also dramatically fluctuated during the time period of incubation. The continuous ranks of ability in fixing nitrogen were found to belong IMOCs from shallot, banana, crop rotation, rice, watermelon, grassland, and grapefruit cultivated fields, respectively while other IMOCs had trivial N-fixed capabilities.

Table 3: Total NH_4^+ concentration in Burks liquid medium within five days of incubation (n=3 and standard deviation)

Origin of samples	Total NH_4^+ concentration (mg/L)				
	Day 0	Day 1	Day 2	Day 3	Day 5
Bamboo	5.60^{def}	11.81^{a}	6.02^{ab}	12.01^{a}	7.25^{a}
Crop rotation	5.75^{def}	6.84^{d}	4.99^{c}	10.07^{bc}	6.46^{abc}
Banana	5.51^{def}	7.81^{c}	5.63^{bc}	10.53^{bc}	6.59^{ab}
Shallot	5.80^{de}	9.40^{b}	5.14^{de}	11.23^{ab}	7.12^{a}
Vegetables	8.16^{a}	8.31^{c}	5.98^{ab}	8.39^{d}	6.42^{abcd}
Rice	6.98^{b}	8.07^{c}	5.40^{bc}	10.95^{ab}	5.72^{bcde}
Watermelon	6.36^{bcd}	6.45^{de}	5.12^{bc}	9.48^{de}	5.37^{e}
Grassland	6.85^{bc}	9.17^{b}	5.65^{bc}	9.30^{ef}	5.70^{bcde}
Maize	5.82^{de}	5.93^{ef}	5.20^{bc}	5.45^{e}	5.39^{e}
Salad	6.37^{bcd}	6.13^{def}	5.45^{bc}	4.97^{e}	5.53^{de}
Oranges	6.01^{cd}	6.44^{de}	6.76^{a}	5.72^{e}	5.27^{e}
Grapefruit	5.24^{def}	6.27^{f}	5.74^{bc}	5.40^{e}	5.19^{e}
Guava	5.34^{def}	5.53^{f}	5.62^{bc}	5.13^{e}	5.03^{e}
Sugarcane	4.90^{f}	5.83^{f}	5.82^{abc}	5.45^{e}	5.59^{cde}
Mix	5.93^{de}	6.21^{def}	5.56^{bc}	5.78^{e}	5.47^{e}

** Note: Values in the same column with the same letters are not significant difference at 1% level ($p<0.01$)*

Davis *el al*. (1964) reported that the bacterium *Pseudomonas methanitrificans* could utilize methane as a sole source of energy and could fix 70 mg/L nitrogen in an average for a period of two months. In large scale experiments, the maximum nitrogen fixation was 53 mg/L, and the higher nitrogen fixation observed in their study might be probably due to the autolysis process of cells during a longer incubation period. Thavasi *el al*. (2006) also revealed that the bacterium *Azotobacter chroococcum* isolated from crude oil contaminated marine environment could fix 4.2 mg/L of nitrogen in 96 hours. Mazumdar and Deka (2013) estimated that the amount of nitrogen fixed by free-living nitrogen fixing bacteria isolated from crude oil contaminated soil was recorded with a range of 9.74 mgN/L and 17.45 mgN/L over a period of two months. Similarly, Smita and Goyal (2017) estimated that the amount of nitrogen fixed by free-living nitrogen fixing bacteria from alkaline soils was found to be highest after 9 – 12 days of incubation, with the number ranged from 14.44 ppm/mL to 18.73 ppm/mL as total nitrogen content.

In comparison with other previous studies, one can see that the amount of nitrogen fixation of IMOCs was much lower than those of single isolated strains in N_2-fixing capacity from the previous researches. On the second day of incubation, the amount of total nitrogen fixed had been dropped deeply, even lower than that on the first incubation day. It could be explained that the groups of nitrogen consuming microbes, especially denitrifier groups in each IMOC could decelerate nitrogen by converting NH_4^+ or NO_3^- into other form of nitrogen like NO, N_2O and N_2 gases. This also explains for the strong fluctuation in the total nitrogen content in the Burks liquid medium during the time of incubation period, and there exist rival activities of two microbial groups: nitrogen fixers and nitrogen consumers in all IMOCs (Robertson and Groffman, 2015). Morover, although both groups of free-living organisms fix only small amounts of nitrogen, they can be important in sustaining plant communities in natural ecosystems (Hillel, 2007).

4 CONCLUSION

Fourteen collected IMOCs from different ecosystem habitats without isolation step earn many beneficial characteristics for plant growth and stimulation through phosphate solubilizing capacity, nitrogen fixing capacity and IAA, type of auxin synthesis capacity with a high extent. These good functions of all collected IMOCs can be exploited for enhancing soil fertility and plant growth. Moreover, to ensure the highest and stable efficacy of IOMCs for these good functions, a mixture of many IMOCs from many diverse origins of habitats is obviously needed and it is obvious to see that study on IOMCs for agricultural application is still lacking, and many outstanding results in this research field are still awaiting.

REFERENCES

Ahmad, F., Ahmad, I. and Khan, M.S., 2005. Indole acetic acid production by the indigenous isolates of Azotobacter and fluorescent Pseudomonas in the presence and absence of tryptophan. *Turk. J. of Biol.*, 29(1): 29-34.

Ahmad, F., Ahmad, I. and Khan, M.S., 2008. Screening of free-living rhizospheric bacteria for their multiple

plant growth promoting activities. *Microbiol. Res.*, 163(2): 173-181.

Brick, J.M., Bostock, R.M., and Silverstone, S.E., 1991. Rapid insitu assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. *Appl. Environ. Microbiol*. 57, 535–538.

Chiemela F. A., Serafin, L. N., Ricardo, L. I. and Joseph, L. N., 2013a. Isolation and Characterization of Indigenous Microorganism (Imo) from Ifugao Bamboo (Phyllostachys Aurea) Forest. *International Journal of Science and Research* (IJSR), ISSN (Online): 2319-7064.

Chiemela, F. A., Serafin, L. N., Ricardo, L. I., Joseph, L. N., 2013b. Application of Indigenous Microorganisms (IMO) for Bio-Conversion of Agricultural Waste. *International Journal of Science and Research* (IJSR) ISSN (Online): 2319-7064.

Cho, H.K., 1997. Korean nature farming. Chungchung book-do: Korean Nature Farming Association Publisher.

Davis, J.B., Coty, V.T. and Stanley, J.P., 1964. Atmospheric nitrogen fixation by methane oxidizing bacteria. *Journal of Bacteriology*. 88:468-472.

Frankenberger, W T., and Arshad, M., 1995. Phytohormones in soil: Marcel Dekker, Inc.

Hillel, D., 2007. Soil in the Environment, 1st Edition crucible of terrestrial life

Keeney and Nelson, 1982. Method in applied Soil microbiology and biochemistry. Edited by Kassem Alef and Paolo Nannipieri. Harcourt Brace and Company, pp79.

Khalid, A., Arshad, M., and Zahir, Z.A., 2004. Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. *Journal of Applied Microbiology*, 96(Suppl 3): 473-480(8).

Koon, H.W., Duponte, M. and Chang, K., 2013. Use of Korean Natural Farming for vegetable crop production in Hawai'i. *Hanai'ai/The Food Provider*.

Krishnaraj, P.U. and Dahale, S., 2014. Mineral phosphate solubilization: concepts and prospects in sustainable agriculture. *Proc Indian Natn Sci Acad*. 80(2): 389-405.

Kumar, B. L., and Gopal D.V.R, 2015. Effective role of indigenous microorganism for sustainable enviroment. 3 Biotech 5: 867-876.

Lin, Q.M., Wang, H., Zhao, X.R., and Zhao, Z.J., 2001. The solubilizing ability of some bacteria and fungi and its mechanisms. Microbiol. China 2001, 28, 26–30.

Mazumdar, A. and Deka, M., 2013. Isolation of free living nitrogen fixing bacteria from crude oil contaminated soil. *International Journal of Bio-Technology and Research*. 3: 69-76.

Mbouobda, H.D., Fotso, Djeuani, C.A., Fail, K. and Omokolo, N. D., 2013. Impact of effective and indigenous microorganisms manures on Colocassia esculenta and enzymes activities. *African Journal of Agricultural Research*. 8(12): 1086-1092.

Mehta, S. and Nautiyal, C. S., 2001. An Efficient Method for Qualitative Screening of Phosphate-Solubilizing Bacteria. current Microbiology. 43: 51-56.

Poly, F., Ranjard, L., Nazaret, S., Gourbière, F. and Monrozier, L.J., 2001. Comparison of *nifH* gene pools in soils and soil microenvironments with contrasting properties. *Applied and Environmental Microbiology*. 67(5): 2255-2262.

Poly, F., Monrozier, L.J. and Bally, R., 2001. Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res. Microbial*. 152: 95-103.

Reddy, R., 2011. Cho's global natural farming. South Asia Rural Reconstruction Association.

Robertson, G. P. and Groffman, P. M., 2015. Nitrogen transformations. *In:* E. A. Paul, (Ed.). Soil microbiology, ecology and biochemistry. Fourth Edition. Academic Press, Burlington, Massachusetts, USA. pp. 421-446.

Saharan, BS., and Nehra, V., 2011. Plant Growth Promoting Rhizobacteria: A Critical Review. *Life Sciences and Medicine Research*, Volume 2011: LSMR-21: pp. 1-30.

Saikrithika, S., Krishnaswamy V. G. and Sujatha B., 2016. A Study on Isolation of Phosphate Solubilizing Bacterial (PSB) Strain fromVermicomposted Soil and Their Phosphate Solubilizing Abilities. *International Journal of Advanced Biotechnology and Research* (IJBR) ISSN 0976-2612. 7(2): 526-535.

Sarwar, M., and Frankenberger, W.T., 1994. Influence of L-tryptophan and auxins applied to the rhizosphere on the vegetative growth of Zea mays L. *Plant and Soil*, 160 (Suppl 1): 97-104.

Sekhar, M. S., and Gopal, V. R. S., 2013. Studies on indigenous microorganisms (IMOs) increasing growth of leaves germination, chlorophyll content and differentiation between IMOs and chemical fertilizers in various crop plants. *International Journal of Emerging Technologies in Computational and Applied Sciences* (IJETCAS), 4(3): 313-318.

Smita M., and Goyal, D., 2017. Isolation and characterization of free-living nitrogen fixing bacteria from alkaline soils. *International Journal of Scientific World*, 5(1): 18-22.

Sumathi, T., Janardhan, A., Srilakhmi, D.V.R Sai Gopal and Narasimha R., 2012. Impact of indigenous microoganisms on soil microbial and enzyme activities. Archives of Applied Science Research, 4(2): 1065-1073.

Tam, H. T, Diep, C. N., Chau D.T.M., 2016. Isolation and identification phosphate-solubilizing fungi from ferralsols of tithonia (tithonia diversifolia (hamsl.) gray) in daknong and daklak province(s), Vietnam. *World Journal of Pharmacy and Pharmaceutical Sciences*. 5(9): 325-341.

Thavasi, R., Jayalaxmi, S., Balasubramanian, T., and Banat, I.M., 2006. Biodegradation of crude oil by nitrogen fixing marine bacteria *Azotobacter chroococcum*. *Research journal of microbial*. 5: 401-40.

Tsavkelova, E.A., Cherdyntseva, T.A., Klimova, S.Y., Shestakov, A.I., Botina, S.G., and Netrusov, A.I., 2007. Orchid-associated bacteria produce indole-3-acetic acid, promote seed germination, and increase their microbial yield in response to exogenous auxin. Archives of Microbiology, 188(Suppl 6): 655-664.

Walpola, B. C. and Yoon, M.H., 2013. Isolation and characterization of phosphate solubilizing. *African journal of microbiology research* 7: 266-275.

Yingben, W., Yuelin, H., Hongmei, Y., Wei, C., Zhen, W., Lijuan, X. and Aiqun, Z., 2012. Isolation of phosphate-solubilizing fungus and its application in solubilization of rock phosphates. *Pak J Biol Sci*. 15(23): 1144-1151.