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Effect of Plant Growth Promoting Rhizobacteria and Rhizofungus on the Growth of Hairy Basil (*Ocimum basilicum* L.f. var. citratum Back.)

Nuntavan Riddech[1,3,*], Tiptida Kidtook[2], Natthawat Sritongon[2], and Jindarat Ekprasert[1]

[1]Department of Microbiology, Faculty of Science, Khon Kaen University, Thailand 40002
[2]Graduate School of Microbiology, Faculty of Science, Khon Kaen University, Thailand 40002
[3]Salt-tolerant Rice Research Group, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand 40002

[*]Author for correspondence: nunrid@kku.ac.th; Tel & Fax: +66- 43202377

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In this study, plant growth promoting rhizobacteria (PGPR) and plant growth promoting fungi (PGPF) were isolated from soil adhered to the roots of herbs. PGPR and PGPF isolates were selected for producing microbial inoculant as a starter culture for bio-fertilizer production. The screening of PGPR and PGPF was performed using the spread plate technique on the selected medium. Total plant growth promoting microorganisms (PGPM) were composed of 72 isolates, nitrogen-fixing microorganisms: 39 isolates, phosphate-solubilizing: 11 isolates, and potassium-solubilizing: 22 isolates. Two bacterial isolates, S-K7-2 and S-P7-1, had the highest plant growth promoting abilities, and a fungus isolate, Di-K7-2, was able to produce the greatest amount of IAA, which was 45.17 µg IAA equivalent/ml. The isolates were tested on hairy basil seed germination. Treatment using microbial cell dissolved in sterile distilled water had the greatest potential for stimulating the growth of seed and presented 145.26% of GI, followed by 82.87% where the treatment was with IAA standard. The study of the effect of PGPR and PGPF on hairy basil growth found that the highest biomass was shown in treatment 3, peat supplemented bacteria (S-K7-2, S-P7-1), which indicated that rhizobacteria immobilized on peat was able to enhance the growth of hairy basil and had better potential for promoting plant development compared with chemical fertilizer treatment.

Keywords: plant growth promoting microorganisms, hairy basil, seed germination

Abbreviations: EC—electrical conductivity, FDA—fluorescein diacetate hydrolysis, PGPR—plant growth promoting rhizobacteria, PGPF—plant growth promoting fungi, PGPM—plant growth promoting microorganisms, PCR—polymerase chain reaction

INTRODUCTION

Herbs are plants which can be used for culinary, aromatic, and medicinal purposes (Ramalingum and Mahomoodally 2014). Hairy basil is an herb that is very popular as an ingredient in many Thai dishes; as such, it is widely cultivated. Eco-friendly products such as biofertilizers could be employed to aid herb growth; therefore, a study in this area could contribute to both agriculture and the environment. Moreover, as an important source of human food and medicine, it is important to preserve and promote the growth of herbs.

Plant growth promoting rhizobacteria (PGPRs) is a group of microorganisms such as *Pseudomonas*, *Agrobacterium*, *Azospirillum*, *Azotobacter*, *Enterobacter*, *Micrococcous*, *Caulobacter*, *Arthrobacter*, *Burkholderia*, *Bacillus*, and *Serratia* (Gray and Smith 2005) which are found in the soil around plant roots. Plant growth regulators such as nitrogen fixation activity, auxin (IAA) production activity, and nutrient (P and K) solubilization activity are used by PGPRs to regulate the growth of plants (Ahemad and Kibret 2014). Bio-control is another function of PGPRs which can help increase plant growth by preventing and/or eliminating phytopathogens.

PGPRs are able to convert organic soil matter into plant nutrients (N, P, and K), promote plant growth, and act as bio-fertilizer. The bio-fertilizer consists of microorganisms that can improve soil quality. PGPRs can colonize around the surface or at the inner root and enhance plant growth by direct and indirect mechanisms

including nitrogen fixation activity, phosphate and potassium solubilization activities, and IAA production activity.

PGPF is the heterogeneous group normally found at the root surface or the interior of the root or the rhizosphere. *Aspergillus, Fusarium, Penicillium, Piriformospora, Phoma,* and *Trichoderma* are well-known fungi reported as PGPF. The interactions between plant and PGPF show the effects of fungi on seed germination, seedling vigor, biomass production, root hair development, photosynthetic efficiency, flowering, and productivity. PGPF are able to improve plant biochemical composition, control plant pathogen, and help in nutrient uptake (Motaher et al. 2017). The aim of this study was to isolate and identify the PGPRs and PGPF from herb rhizosphere samples and to investigate the properties of these isolates for plant growth promotion. The ability of PGPM to stimulate herb seed germination in vitro was detected and the effects of PGPM inoculation on the growth of herb seedlings were examined. Finally, this study also aimed to propose the potential use of PGPM as microbial inoculant.

MATERIALS AND METHODS

Screening and Testing on Plant Growth Properties of The PGPRs

Rhizosphere Sampling

Fifteen rhizosphere soil samples together with wild herb plants (plant names are shown in Table 1) were collected from Chulabhorn Dam, Chaiyaphum province, Thailand. The samples were taken in plastic bags and carried to Khon Kaen University laboratories. The electrical conductivity and pH of each rhizosphere soil samples (tripicates) were measured in order to study the chemical properties of the sample.

Screening of Plant Growth Promoting Microorganisms from Herb Rhizosphere Samples

PGPM were screened according to a modified method described by Khalid et al. (2004) and Priya et al. (2013). Rhizosphere soil samples were air-dried at room temperature for 2 hours. Herb plants were shaken to remove the root zone soil, leaving the rhizosphere samples adhering to the surface of the roots. Rhizobacteria and rhizofungi were isolated using the spread plate technique on soil extract and were prepared by adding 1 g of peptone 0.5 g of K_2HPO_4, 1 g of yeast extract, 0.5 g of $(NH_4)_2HPO_4$, 0.1 g of $MgSO_4$, 0.01 g of $FeCl_3$, 0.1 g of $CaCl_2$, 15 g of agar, and 750 ml of distilled water. Finally, the medium was added to 250 ml of soil extract, obtained from the 1,000 g of extracted of soil, and supplemented with 0.5 g $CaCO_3$. After being filtered through filter paper No. 1, the agar plate was incubated at 30°C for three days. Then, the colonies with different morphologies were individually purified by re-streaking onto fresh media until single colonies were obtained. The purity of the isolates was checked under microscope (ECLIPSE E200 LED MV R, Nikon ECLIPES E200).

Study of Plant-Growth-Promoting Properties of Rhizobacterial and Rhizofungi Isolates

Determination of Nitrogen Fixing Activity

The fresh inoculum of microbes (24 h old) was prepared on nutrient agar. The isolated rhizobacteria and rhizofungi were tested for their ability to fix nitrogen by point inoculation onto Ashby's agar and Ashby's broth (free of nitrogen compounds). Ashby's agar was prepared by adding 15 g of mannitol, 0.2 g of $CaCl_2$, 0.2 g of K_2HPO_4, 0.2 g of $MgSO_4$, 0.1 ml solution of 10% MoO_3, 0.1 ml solution of 10% $FeCl_3$, and 15 g of agar in 1,000 ml of distilled water. The plates and tube containing Ashby's broth were incubated at 30°C for three days. The appearance of colonies on the nitrogen-free medium and turbid in culture broth (Xie et al. 2003) were indicative of positive results.

Determination of Phosphate Solubilizing Activity

Phosphate-solubilizing activity of the rhizobacterial and rhizofungi isolates was tested on Pikovskaya's agar (Pande et al. 2017) supplemented with tricalcium phosphate by point inoculation method. Plates were then incubated at 30°C for five days. The halo zone around the colony indicated a positive result.

Determination of Potassium Solubilizing Activity

Aleksandrov agar, prepared by adding 5 g of glucose, 0.5 g of $MgSO_4.7H_2O$, 0.005 g of $FeCl_3$, 0.1 g of $CaCO_3$, 0.1 g of $(NH_4)\ SO_4$, 2 g of Na_2HPO_4, 2 g of $AlKO_6Si_2$, and 15 g of agar in 1,000 ml of distilled water, was used to screen for potassium-solubilizing activity in the isolated rhizobacteria and rhizofungi. Plates were incubated at 30° C for five days. The colonies with positive phosphate-solubilizing activity showed clear zones. The SI was calculated using the method outlined in the equation below: (Collavino et al. 2010)

$$SI = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

IAA Production

In order to test for the ability of the rhizobacterial and rhizofungi isolates to produce IAA, the method of Bano

and Musarrat (2003) was used. Rhizobacteria and rhizofungi were inoculated into 10 ml of trypticase soy broth (TSB) supplemented with 500 µg/ml of tryptophan and incubated by shaking at 150 rpm for 48 h at room temperature. A hundred microliters of 10 mM orthophosphoric acid and 2 ml of Salkowski reagent were added into the bacterial culture and incubated at room temperature in the dark for 25 min. A positive result is obtained when the reaction in the test tube shows a pink color. The IAA positive isolates were selected for analyzing IAA content by using a spectrophotometer at the wavelength 520 nm and compared with IAA standard.

Hairy Basil Seed Germination Test

Hairy basil seeds were surface-sterilized by soaking in 70% alcohol for five minutes before being transferred to soak in 3% (v/v) of sodium hypochlorite for five minutes. The last step of seed surface sterilization was to rinse five times with sterile distilled water. The seeds were then transferred to soak in the mixture of PGPM suspension isolate Di-K7-2, S-K7-2, S-P7-1 in the ratio 1:1:1(v/v/v) for one hour. Ten hairy basil seeds were placed on moistened tissue paper in a petri dish and watered with sterile distilled water every two days. The seeds were incubated at room temperature for seven days to germinate. The germination index was calculated according to Kandil et al. (2012)'s equation:

$$GI = \frac{\text{Germination percentage in each treatment}}{\text{Germination percentage in the control}} \times 100$$

Effect of Microbial Inoculants on the Growth of Hairy Basil

PGPM Inoculant tested on the Growth of Hairy Basil Seedling

Microbial Inoculant Preparation

Two isolates of rhizobacteria (S-K7-2 and S-P7-2) and one isolate of rhizofungi (Di-K7-2) were inoculated on each agar plate for the antagonistic activity test. Only the three PGPM isolates that showed no inhibition activity were selected for inoculant preparation. A loop of each isolate was inoculated in NB and then incubated at 30°C for 24 h. The concentration of the bacterial suspension of 10^8 cell/ml was obtained by dilution with sterile distilled water. The fungus starter culture was prepared on potato dextrose agar which was incubated at 30°C for three days. Spore suspension was prepared in a saline solution to which 0.5% Tween-20 was added. After the particles were allowed to settle, the spore suspension was transferred to another tube, mixed using the vortex machine, and a 10-fold dilution was made. The spore was counted by using Hemacytometer and used as the inoculums at the density 1×10^8 spores/ml.

Pot Experiment Design

Production of Microbial Inoculants as a Bio-Fertilizer

An isolate of fungus (Di-K7-2) and two bacterial isolates (S-K7-2 and S-P7-1) isolated from herb rhizosphere soil samples were used as the mixed culture to prepare the bio -fertilizer. All microbes were tested for antagonistic activity before use in the mixed culture.

Treatment 2: three isolate were mixed in the ratio 1:1:1 (v/v/v) before being used as a starter culture.

Ten percent (v/w) of each isolate was inoculated into 100 g of carries (Kloepper 2009) at room temperature for 15 days before sowing into the bottom of the pot containing soil.

Completely Randomized Design (CRD) was used for this study with seven replications and seven treatments. The soil was mixed with biochar in the ratio of 1:0.25 (w/w). The 1.5 kg of the mixture of soil and biochar were then transferred to the plastic pot no. 3. The treatments were consisted of T1: herbs grown without adding fertilizer (control), T2: the soil supplement with the mixture of rhizofungus Di-K7-2 and rhizobacteria S-K7-2 and S-P7-1 that adhered on peat as a carrier, T3: the soil supplement with the mixture of rhizobacteria S-K7-2 and S-P7-1 that adhere on peat as a carrier, T4: the soil supplement with an isolate of rhizofungus Di-K7-2 that adheres on peat as a carrier, T5: the soil supplement with vermicompost, T6: the soil supplement with LDD (Pordor 12/ commercial bio-fertilizers), T7: the soil supplement with chemical fertilizer 16-16-16 and the hairy basil seeds were used and cultivated for 10 days until seedling grew. After that, three to five seedlings were transferred to the pot in each treatment. Basil seedling was cultivated in the greenhouse for 45 days. The herb was sampled at days 15, 30, and 45 to detect shoot and root fresh and dry weight, height, root length, chlorophyll, and total enzyme activity.

Measurement of Chlorophyll in Plant Leaves

This experiment utilized the method of Vivek et al. (2013). Leaves were randomly sampled from each treatment. The chlorophyll from 0.1 g of leaves was extracted by thorough grinding. Five milliliters of 80% acetone solution was added to the extracted leaves. Then, the mixture was filtered using filter paper No. 1 and the filtrate volume was measured. The absorption of chlorophyll solution was measured using 3 J2-0023, U-

5100 UV/VIS spectrophotometer at the wavelength of A663 and A645 for detecting chlorophyll A and chlorophyll B, respectively by using acetone as a blank. Then, the amount of pigment in the leaf was calculated via Vivek et al. (2013).

$$\text{Chlorophyll A (mg/ g)} = \frac{[12.7\ (A663) - 2.69\ (A645)] \times V}{(1000 \times W)}$$

$$\text{Chlorophyll B (mg / g)} = \frac{[22.9\ (A645) - 4.68\ (A663)] \times V}{(1000 \times W)}$$

$$\text{Total chlorophyll (mg / g)} = \frac{[20.2\ (A645) + 8.02\ (A663)] \times V}{(1000 \times W)}$$

where V is the total volume of the solution (ml); W is the fresh weight of the leaves (g).

Measurement of Total Microbial Activity in Soil Samples Using Fluorescein Diacetate Hydrolysis (FDA) Method

The FDA method was used for checking microbial activity associated with total enzyme activity in the soil sample (Adam and Duncan 2001). The moisture content of cultivated soil was measured before putting the sample into a 50 ml centrifuge tube. Then, 7.5 ml of 60 mM potassium phosphate buffer pH 7.6 and 0.1 ml of FDA were added into the tube. The mixture was incubated with shaking at 150 rpm, 30°C for 30 minutes. Chloroform: methanol (2:1 v/v) was added to the samples which were then centrifuged at 6,000 rpm for 10 minutes. One milliliter of the aliquot was taken to measure the absorbance at 490 nm using UV spectrophotometer. Calculation of total microbial activity in the samples was carried out by extrapolating with the values indicated in the FDA standard curve.

Quantification of Microorganisms in Cultivated Soil

The microorganisms in 1 g of soil samples were quantified by serial dilution and counted on nutrient agar plate. The experiment was carried out in triplicate for each sample. The number of microorganisms after 3 days of incubation was reported in the unit of CFU/g soil.

Measurement of pH, Electrical Conductivity (EC) and Moisture Content in Cultivated Soil

For the pH and EC analysis, the soil sample was added to a beaker containing distilled water in the ratio 1:5, and stirred for 5 min. Then, the soil suspension was left at room temperature for 10 mins. The pH and electrical conductivity of the soil solution were measured with pH and EC probes, respectively. Each analysis was carried out in triplicate.

For moisture content analysis, 1 g of soil sample was weighed as fresh weight. Then, the sample was dried in a hot air oven at 105°C for 24 h. The dried sample was cooled in a desiccator prior to measuring its dry weight. Percentage of moisture content was calculated.

$$\text{Moisture content, } w = \frac{Mw}{Md} \times 100\ \%$$

Mw = mass of wet soil; Md = dry mass of sample

Identification of Rhizobacterial Isolates and Rhizofungus Isolate

Bacterial isolates were identified based on their 16S rRNA gene sequences. The genomic DNA of each bacterial isolate was extracted using TIANAMP Bacterial DNA kit. The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using universal 8F primer (Weisburg et al. 1991): 5′-AGA GTT TGA TCM TGG CTC AG-3′ and universal 1512R primer (Kane et al. 1993): 5′-ACG GYT ACC TTG TTA CGA CTT-3′. The PCR product was sent for purification and nucleotide sequencing services to First BASE Laboratories Shd., Malaysia. The derived nucleotide sequences were subjected to assemble in BioEdit (biological alignment editor) using reverse complement function. The sequences were subjected to the BLASTN program (Altschul et al. 1997) on the NCBI website to compare the nucleotide sequences of type strains. The phylogenetic relationship of the bacterial isolates and their relatives was constructed using MEGA software version 7.0.9.

The fungal isolate was cultured on PDA, incubated at 30°C for 7 days, and sent to Macrogen (South Korea) for nucleotide sequencing. The type of fungi in GenBank (www.ncbi.nlm.nih.gov) was analyzed using nucleotide BLAST program and the phylogenetic relationship of the fungi isolates and their relatives constructed using MEGA.

In this study, the analysis of variance (ANOVA) of the values of fluorescein diacetate (FDA) in soil, IAA content, germination index of Hairy basil, total number of microorganisms, plant growth properties (included phosphate and potassium solubilization index), and plant growth parameters (included root and shoot length, biomass and chlorophyll contents) were analyzed by using Statistix 8 program. The significant difference among data was analyzed using the least significant difference (LSD) test.

RESULTS AND DISCUSSION

Screening of PGPM From the Herb Rhizosphere Samples

Seventy-two isolates of PGPM were selected by testing plant growth-promoting properties including nitrogen-fixing activity, phosphate and potassium-solubilizing activities, and IAA-producing activity. 72 microbial isolates were categorized as 39 isolates of nitrogen fixers, 11 isolates of phosphate solubilizers, and 22 isolates of potassium solubilizers, as presented in Table 1. PGPRs can promote plant growth in either direct or indirect ways. Direct promotion included nitrogen fixation, phosphorus solubilization, siderophore production, and the production of plant hormones such as auxins, cytokinin, and gibberellin. It also included the reduction of ethylene concentrations in plants. Meanwhile, the indirect mechanisms included antibiotic substance production for plant disease control, antagonistic microorganisms competing for iron around plant roots which causes a reduction of the growth of plant pathogen; finally, the production of hydrolytic enzymes such as cellulase, glucanase, protease, and chitinase which are also able to break down the cell wall of plant pathogens (Jadhav et al. 2017).

Tests for Plant Growth Promoting activity: Nitrogen Fixation, Phosphate, and Potassium Solubilization

PGPM possessing multiple properties to promote plant growth were selected to determine the activities and mechanism of these plant-growth-promoting properties. The results showed that 12 isolates possessed plant-growth-promoting activities. Isolates S-K7-2 and S-P7-1 showed the highest phosphate-solubilizing activity and potassium-solubilizing activity (Table 2). In this experiment, bacterial and fungal plant growth promoting microorganisms (PGPM) were screened from herb rhizosphere soils. Thirty-nine isolates of PGPM were able to grow on Ashby's agar, which is a nitrogen free medium. Eleven isolates and 22 isolates of PGPM were able to solubilize phosphate and potassium, respectively.

Table 1. The number of PGPM that showed plant growth properties and screened from herb rhizosphere samples.

Plant Growth Properties / Herb Rhizosphere Samples	Nitrogen Fixing (A Number of Isolates)	Phosphate Solubilizing (A Number of Isolates)	Potassium Solubilizing (A Number of Isolates)
Amomum xanthioides Wall	2	-	-
Barleria strigosa Willd	4	1	3
Calamus rotang Linn	5 (B:1+F:4)	-	2
Centotheca lappacea (L.) *Desv*	1	2	1
Croton cascarilloides Raeusch	2 (B:1+F:1)	-	1
Croton crassifolius Geiseler	2	-	3
Dillenia hookeri Pierre	2	2	2 (B:1+F:1)
Diospyros montana Roxb	1	-	4 (B:2+F:2)
Disocactus flagelliformis (L.) *Barthlott*	6 (B:5+F:1)	1	-
Osbeckia stellata Ham	2	1	-
Piper ribesioides Wall	1	2	1
Roureopsis stenopetala (Griff.) Schellenb	3 (B:1+F:2)	-	-
Strychnos axillaris Colebr	2	1	3
Sauropus androgynus (L.) Merr	4	1	1
Scleria levis Retz	2	-	1
Total	39	11	22

Table 2. Plant growth properties on herb rhizosphere isolates.

Isolates	Nitrogen Fixing Activity on Medium		Phosphate Solubilization (Solubilization Index/SI)	Potassium Solubilization (Solubilization Index/SI)
	Agar	Broth		
B-K7-2(B)	+	+	1.33±0.58^{bc}	1.49±0.28^{bc}
B-N7-1(B)	+	+	2.33±0.29^{ab}	2.07±0.22^{bc}
B-P7-1(B)	+	+	2.08±1.01^{abc}	2.27±0.21^{b}
Ce-P7-1(B)	+	+	-	1.10±0.17^{bc}
Cr-K7-1(B)	+	+	-	2.25±0.25^{b}
Di-K7-2(F)	+	+	1.05±0.00^{c}	1.16±0.00^{bc}
Dio-N7-1(B)	+	+	1.33±0.58^{bc}	1.85±0.13^{bc}
Dio-P7-1(B)	+	+	3.00±0.43^{a}	1.56±0.35^{bc}
Pi-K7-1(B)	+	+	2.61±0.90^{a}	1.08±0.14^{c}
S-K7-2(B)	+	+	2.53±0.18^{a}	3.38±1.08^{a}
S-P7-1(B)	+	+	2.22±0.96^{ab}	4.18±1.50^{a}
Sa-N7-3(B)	+	+	1.31±0.63^{bc}	1.52±0.12^{bc}
F-test			**	**
%CV			32.68	32.23

Different alphabets indicate results of significantly different values in each treatment.

** The differences of the results were statistically significant at 99% confidence interval ($P < 0.01$). Nitrogen-fixing activity (N), Phosphate-solubilizing activity (P), Potassium-solubilizing activity (K), Bacterial (B), Fungi (F).

This was in agreement with the report of Ahmad et al. (2008) which found 72 isolates of PGPRs such as *Azotobacter, Pseudomonas, Mesorhizobium*, and *Bacillus*, isolated from soil around the roots of different plants and Aligarh plants.

Measurement of Total Microbial Activity in the Rhizospheric Soils by Using Fluorescein Diacetate Hydrolysis (FDA)

The FDA activity measured from rhizospheric soil samples are shown in Figure 1. Total microbial activity was detectable in all soil samples. The highest FDA activity was clearly found in *Scleria levis Retz* (Sc) sample. Contrarily, the lowest FDA activity was seen in *Dillenia hookeri Pierre* (Di).

IAA Content in PGPM

The amount of IAA production in PGPM is presented in Figure 2. The isolates produced varying amounts of IAA at 99% confidence interval. The isolate Di-K7-2 produced the highest IAA content of 45.17 μg IAA equivalent/ml, followed by the isolates S-K7-2 and S-P7-1, respectively. The fungi had a high IAA production capacity, similar to the experiment by Kumar et al. (2017) fungi-*Trichoderma viride* VKF3, a mangrove isolate, used to produce auxin IAA under *in vitro* conditions yielded high IAA production of 115 μg/ml. All of these isolates exhibited plant-growth-promoting properties, such as IAA production activity that can stimulate the elongation and cell division in plants. In this experiment, isolate Di-K7-2 showed the highest IAA production of 45.17 μg IAA equivalent/ml, followed by S-K7-2 and S-P7-1 that had IAA production of 16.72 μg IAA equivalent/ml and 4 μg IAA equivalent/ml, respectively. This is similar to the report by Verma et al. (2013) which indicated that *P. aeruginosa* could produce the highest content of IAA of 25.13 μg/ml, followed by *Mesorhizobium* sp. (22.07 μg/ml) and *B. megaterium* (18.64 μg/ml). In this work, isolates S-K7-2 and S-P7-1 were able to fix nitrogen gas from the air and efficiently dissolve phosphorus and potassium, which are the primary macronutrients. Examples of phosphate-solubilizing bacteria were *Bacillus* sp., *Pseudomonas* sp., *Agrobacterium* sp., *Serratia* sp., *Erwinia* sp., and *Xanthomonas* sp. (Sharma et al. 2013), while potassium solubilizing bacteria were *Acidothiobacillus ferrooxidans*, *Paenibacillus* spp., *Bacillus mucilaginosus*, B. *edaphicus*, and *B. circulans* (Etesami et al. 2017).

The Effect of Plant-Growth-Promoting Rhizobacteria on Hairy Basil Germination

Results showed that the highest germination index of 145.26% was found in the treatment when seeds were treated with microbial suspension (cell). The seeds treated with standard IAA showed 82.87% GI. There was no germination of herb seeds when treated with sterile nutrient broth and the supernatant without microbial

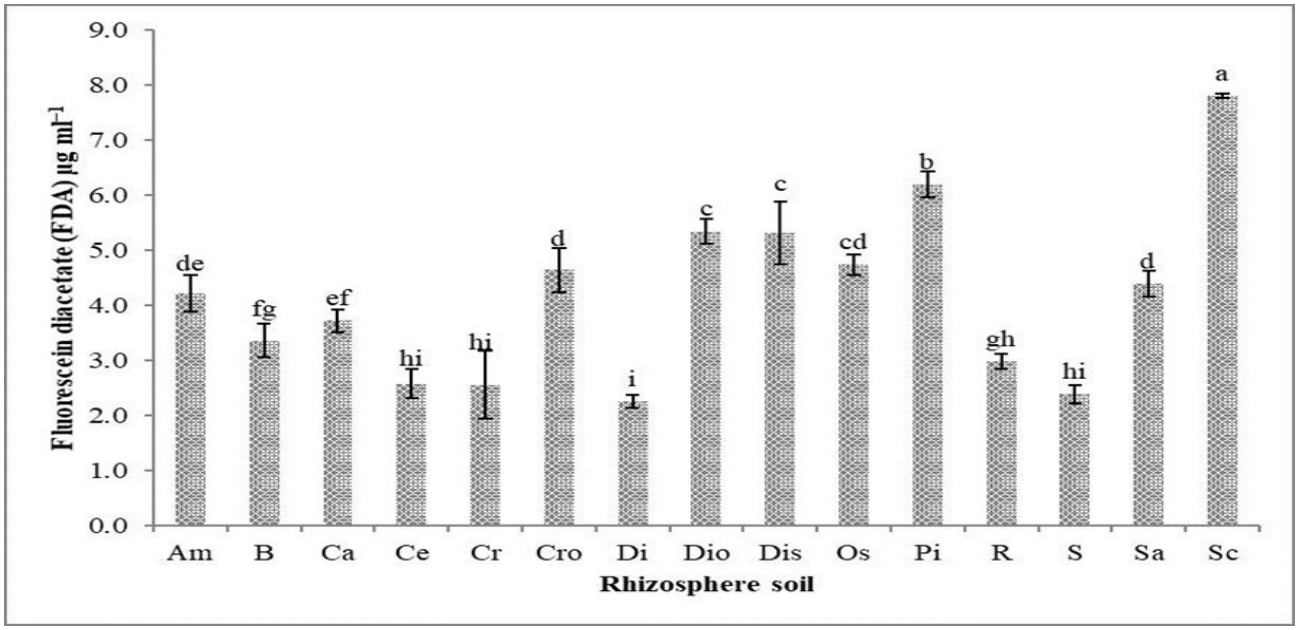

Fig. 1. The result of the measurement of total microbial activity using fluorescein diacetate (FDA) in soil samples around 15 plant roots types included *Amomum xanthioides Wall* (Am), *Barleria strigosa* Willd (B), *Calamus rotang* Linn (Ca), *Centotheca lappacea* (L.) *Desv* (Ce), *Croton cascarilloides* Raeusch (Cr), *Croton crassifolius* Geiseler (Cro), *Dillenia hookeri* Pierre (Di), *Diospyros montana* Roxb (Dio), *Disocactus flagelliformis* (L.) *Barthlott* (Dis), *Osbeckia stellata* Ham (Os), *Piper ribesioides* Wall (Pi), *Roureopsis stenopetala* (Griff.) *Schellenb* (R), *Strychnos axillaris* Colebr (S), *Sauropus androgynus* (L.) Merr (Sa), *Scleria levis* Retz (Sc).

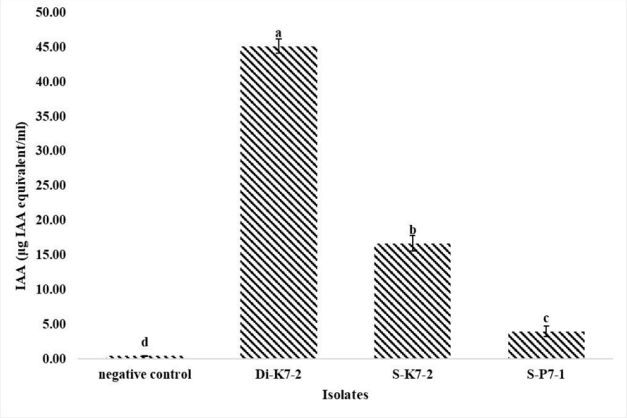

Fig. 2. The IAA (indole-3-acetic acid) content produced by the isolates Di-K7-2, S-K7-2, and S-P7-1. Error bars represent standard deviation of four replications.

inoculum. This might be due to the concentration of nutrient compounds and impure substances contained in the medium and supernatant inhibiting seed growth. Furthermore, it was found that the treatment where hairy basil was inoculated with PGPMs had the highest germination index. This means that the selected microorganisms had the potential to promote plant growth. Prathibha and Siddalingeshwara (2013) reported that PGPRs such as *Pseudomonas fluorescens* and *Bacillus subtilis* could be used as bacterial cells to promote the germination of sorghum seeds. Microbes were more effective in improving seed germination by showing the GI greater than 80%, which was higher than found in the treatment with only the supernatant. It should be noted that microbes can produce either toxic substances or secondary metabolites which are beneficial to plants.

Effect of Rhizobacteria and Rhizofungus as a Bio-Fertilizer to Promote Growth of Hairy Basil

The highest value root length of 29.88 cm was obtained after 30 days in Treatment 2, which was fungi and bacteria fixed on peat (Table 3). This result was comparable to that found in Treatment 7, where chemical fertilizer was used. On the other hand, the highest value of shoot height of 25.83 cm was obtained after 30 days in Treatment 3, which was a bacterium fixed on peat. This result was also comparable to that of Treatment 7 where chemical fertilizer was used. Moreover, the highest value of biomass (1.28 g) was obtained after 30 days and this data showed that Treatment 3 had most effect on the growth of hairy basil. There was no statistically significant difference between the growth parameters in all treatments in 45 days of planting, which is shown in Table 3. Our results also showed that microbes fixed in the carrier materials such as peat could protect bacterial and fungal suspension from changes in physicochemical parameters in the soil. Moreover, this supporting material allows the microbes to have a longer shelf life than those without protection. Therefore, in this work, the microbial cells were immobilized with peat. We found that hairy basil grew well in the treatment with bacteria fixed in peat. This is because the peat was a rich source of nutrients derived from the degradation of organic matter. Peat also has a transparent structure resulting in good ventilation, and suitability for microbial attachment. Furthermore, it also has water-holding properties, essential nutrients for plants in the form of decomposed organic matter for seedlings, and is free from various soil pathogens (Aube et al. 2015). The results revealed that at 30 days of cultivation, peat supplemented with bacteria could promote the highest amount of biomass, significantly different from other treatments. Peat is a good carrier as it can provide nutrients for the microbes. Therefore, it can reserve and extend the shelf life of bacterial and fungus cells for a long period of time when stored suitably.

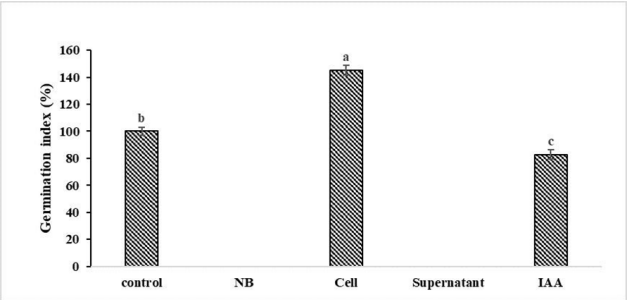

Fig. 3. Germination index (GI) on the seed of Hairy basil. Control: seed treated with sterile distilled water, NB: seed treated with nutrient broth, Cell/microbial suspension: seed treated with cell suspended in sterile distilled water, Supernatant: seed treated with aliquot without cell and IAA: standard IAA.

The Chlorophyll Content in Hairy Basil

The highest chlorophyll A content was found in Treatment 2 and 5 after 30 days of transplantation as shown in Table 4. In the case of chlorophyll B, the highest content was found in Treatment 2 after 30 days of transplantation. For the total chlorophyll, the highest content was found in Treatment 2 and 5. Our results show that rhizobacteria and rhizofungus immobilized on peat were able to convert the nitrogen in peat into nitrogen available (NH_4^+ and NO_3^-) for plant growth. Nitrogen is an important essential nutrient element for growth, protein synthesis and is the composition of chlorophyll in plant. It was found that the highest chlorophyll content in hairy basil could be obtained when it treated with peat supplemented with bacterial and fungal microbes (PGPM). This is indicative of healthy plants since chlorophyll is an important component that helps the photosynthesis process of plants.

Table 3. The growth of basil in the pot experiment at 15, 30, and 45 days after transplanted.

Treatments	Root Length (cm)			Shoot Height (cm)			Biomass (Dry Weight) (g)		
	15 DAT	30 DAT	45 DAT	15 DAT	30 DAT	45 DAT	15 DAT	30 DAT	45 DAT
T1: Control	24.13	26.38	24.88	7.75^{c}	19.50^{c}	27.50	0.14^{c}	0.89^{bc}	1.11
T2: peat + F + B	30.00	29.88	22.50	9.88^{b}	20.00^{c}	27.13	0.20^{bc}	0.71^{c}	1.08
T3: peat + B	30.75	27.88	23.38	10.83^{b}	25.83^{a}	28.75	0.22^{b}	1.28^{a}	1.09
T4: peat + F	25.90	24.38	19.63	9.25^{bc}	22.17^{bc}	29.00	0.24^{ab}	0.86^{bc}	1.12
T5: vermi-compost	30.50	24.88	24.25	13.33^{a}	24.83^{ab}	32.25	0.30^{a}	0.95^{bc}	1.35
T6: Bio-fertilizer PD 12	28.33	24.25	21.25	10.13^{b}	20.33^{c}	28.13	0.26^{ab}	0.75^{bc}	1.21
T7: Chemical fertilizer	26.5	29.5	24.63	11.28^{ab}	26.67^{a}	32	0.20^{bc}	1.00^{b}	1.16
F-test	ns	ns	ns	**	**	ns	**	**	ns
%CV	15.84	11.54	11.59	13.48	8..93	10.55	18.25	20.69	14.35

T1: without fertilizer, T2: peat supplemented with fungus (Di-K7-2) and bacteria (S-K7-2, S-P7-1), T3: peat supplemented bacteria (S-K7-2, S-P7-1), T4: peat supplemented with fungus (Di-K7-2), T5: vermicompost, T6: bio-fertilizer PD 12, T7: chemical fertilizer, F: fungus, B: bacteria. ns: non-significant, **: Mean values followed by the different letters in the same column are significant ($P < 0.01$), according to the least significant difference (LSD) test.

Fluorescein diacetate hydrolysis analysis (FDA) of soil samples collected from pot experiment. At 45 days of planting, Treatment 2, which was a mixture of fungi and bacteria, showed the highest microbial activity (Fig. 4). This activity increased further as the experiment went on. At the 15 days and 30 days of planting, the highest activity was found in Treatment 5 (vermicompost) and Treatment 3 (peat supplemented with bacteria), respectively. The results at 30 days and 45 days of planting showed highest microbial activity, which was also related to the highest total microorganisms count at 30 days and 45 days. FDA analysis for determination of total microbial activity was also performed to ensure the presence of active microbes in the soil, which might have played a role in promoting plant growth. This result was correlated to the other PGPM property analysis, finding the highest FDA activity (70%) in Treatment 2, which was peat supplemented with bacterial and fungal PGPM after 45 days of planting. High FDA activity is indicative of several microbial activities such as organic decomposition, soil organic matter dynamics and the release of inorganic nutrients for plant growth (Diallo-Diagne et al. 2016). pH and EC are appropriate, and do not affect plant growth. Based on all results combined, this newly-isolated bacterial and fungal PGPM is able to promote the growth of hairy basil.

The Number of Total Micro-organisms in Cultivated Soil

Total microorganisms in the cultivated soil were counted on agar plate as shown in Figure 5. After 45 days of planting, the number of microorganisms rose with the increasing time of experiment, except in Treatment 7. The highest microbial content (8.57 log CFU/g) was shown in treatment 6 (Bio-fertilizer PD 12), but it was not significantly different from treatments 2, 3, and 5 on the 45 days of planting. On the other hand, after 15 days of the planting, the highest number of microbes was found in Treatment 3 (peat supplemented with bacteria). On 30 days of planting, there was no significant difference in all treatments, as presented in Figure 5.

pH and Electrical Conductivity in Soil Samples

Neutral pH (approximately 6.7-7.8) was found in all treatments throughout 45 days of planting as shown in Figure 6. The electrical conductivity (EC) value of the

Table 4. The amount of chlorophyll content in hairy basil grown in the pot.

Treatments	Chlorophyll Content (mg/0.1g of leaf)								
	Chlorophyll A			Chlorophyll B			Total Chlorophyll		
	15 Days	30 Days	45 Days	15 Days	30 Days	45 Days	15 Days	30 Days	45 Days
T1	0.90	0.52^{b}	0.27	0.27	0.15^{c}	0.09	1.17	0.67^{b}	0.36
T2	0.86	0.71^{a}	0.42	0.26	0.31^{a}	0.11	1.11	1.02^{a}	0.53
T3	0.82	0.59^{b}	0.33	0.24	0.20^{bc}	0.14	1.06	0.79^{b}	0.46
T4	0.71	0.56^{b}	0.34	0.21	0.19^{bc}	0.1	0.92	0.74^{b}	0.43
T5	0.95	0.74^{a}	0.31	0.28	0.24^{ab}	0.09	1.23	0.98^{a}	0.47
T6	0.7	0.57^{b}	0.3	0.2	0.19^{bc}	0.09	0.9	0.76^{b}	0.36
T7	0.93	0.58^{b}	0.28	0.27	0.20^{bc}	0.07	1.2	0.78^{b}	0.35
F-test	ns	**	ns	ns	**	ns	ns	**	ns
%CV	16.48	8.27	25.9	19.37	20.52	35.9	17.15	9.44	26.57

T1: without fertilizer, T2: peat supplemented with fungus (Di-K7-2) and bacteria (S-K7-2, S-P7-1), T3: peat supplemented bacteria (S-K7-2, S-P7-1), T4: peat supplemented with fungus (Di-K7-2), T5: vermicompost, T6: bio-fertilizer PD 12, T7: chemical fertilizer,

ns: non-significant, **: Mean values followed by the different letters in the same column were significant ($P < 0.01$), according to the least significant difference (LSD) test.

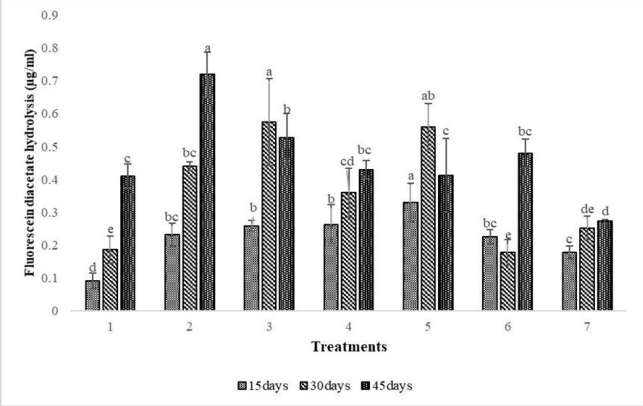

Fig. 4. The FDA activity of microorganisms in cultivated soil on day 15, 30 and 45 of planting. Error bars represent standard deviations of four replications. Seven treatments included T1: without fertilizer, T2: peat supplemented with fungus (Di-K7-2) and bacteria (S-K7-2, S-P7-1), T3: peat supplemented with bacteria (S-K7-2, S-P7-1), T4: peat supplemented with fungus (Di-K7-2), T5: vermicompost, T6: Bio-fertilizer PD 12, T7: Chemical fertilizer.

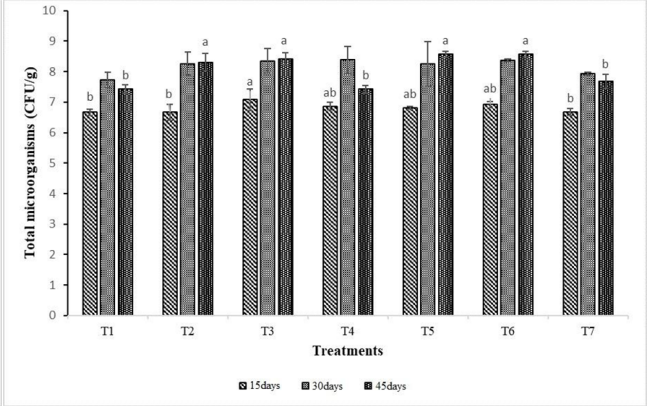

Fig. 5. The amount of microorganisms in the cultivated soil of basil at 15, 30 and 45 days of planting. Error bars represent standard deviation of four replications. Seven treatments included T1: without fertilizer, T2: peat supplemented with fungus (Di-K7-2) and bacteria (S-K7-2, S-P7-1), T3: peat supplemented with bacteria (S-K7-2, S-P7-1), T4: peat supplemented with fungus (Di-K7-2), T5: vermicompost, T6: Bio-fertilizer PD 12, T7: Chemical fertilizer.

soil was highest at 15 days of planting for all treatments, compared to levels at 30 and 45 days (Figure 7). The maximum EC value was in the range of 0.17 ± 0.01 - 0.24 ± 0.01 dS/m. Electrical conductivity (EC) indicates salinity in soil. The EC value in soil of > 4 dS/m negatively affects the growth of plant and biological activities in soil (Sonon et al. 2012) The results showed that the EC value in cultivated soil of all treatments was in the range of 0.17-0.24 dS/m which was not greater than 4 dS/m (Fig. 7). This indicated that the salinity in our cultivated soil did not have the effect on microbial activities as shown in the results of FDA activity and the amount of total microorganisms (Fig. 4 and 5). Moreover, after we applied the biofertilizer (peat + bacteria + fungal/Treatment 2) to the soil for cultivating hairy basil, it was found that the salinity was decreased when compared with another treatment. The similar profile was also found when the soil was treated with peat + bacteria (Treatment 3) or peat +fungal (Treatment 4). Peat can improve the chemical properties of soil and can be used as a buffering capacity in alkaline soil (Kida et al. 2005).

Molecular Identification of Rhizobacterial Isolates

Two different strains of rhizobacteria used as biofertilizer to enhance the growth of hairy basil were identified. The phylogenetic relationship between 16S rRNA gene sequences of our rhizobacterial isolates and those of their relatives is shown in Figure 8. The result showed that isolate S-P7-1 is 100% closely related to *Staphylococcus equorum* (Fig. 8), whereas isolate S-K7-2 is closely related

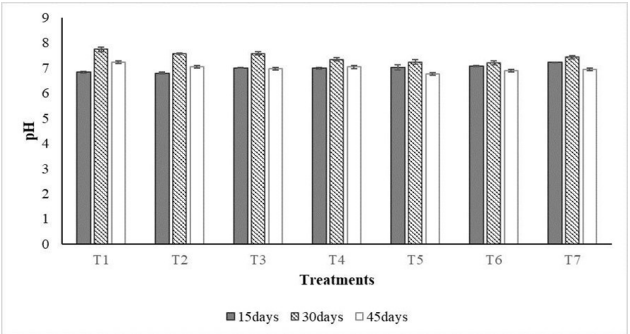

Fig. 6. *pH* of the cultivated soils of hairy basil at 15, 30 and 45 days of planting. Seven treatments included T1: without fertilizer, T2: peat supplemented with fungus (Di-K7-2) and bacteria (S-K7-2, S-P7-1), T3: peat supplemented with bacteria (S-K7-2, S-P7-1), T4: peat supplemented with fungus (Di-K7-2), T5: vermicompost, T6: Bio-fertilizer PD 12, T7: Chemical fertilizer.

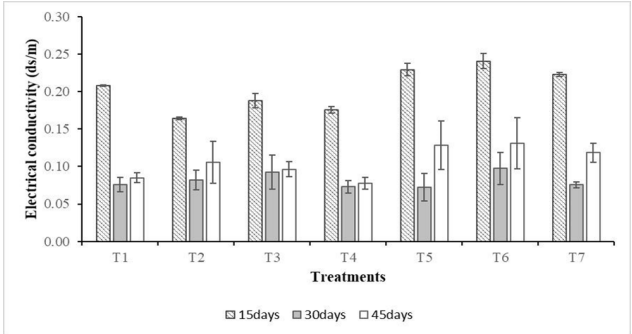

Fig. 7. Electrical conductivity of the cultivated soils of hairy basil at 15, 30, and 45 day of planting. Seven treatments included T1: without fertilizer, T2: peat supplemented with fungus (Di-K7-2) and bacteria (S-K7-2, S-P7-1), T3: peat supplemented with bacteria (S-K7-2, S-P7-1), T4: peat supplemented with fungus (Di-K7-2), T5: vermicompost, T6: Bio-fertilizer PD 12, T7: Chemical fertilizer.

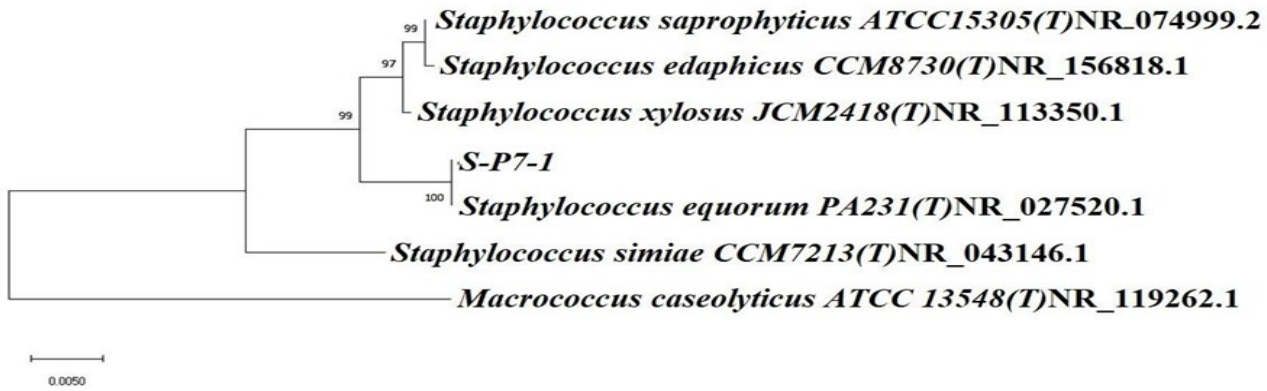

Fig. 8. Phylogenetic tree showing the species identification of the rhizobacterial isolates S-P7-1.

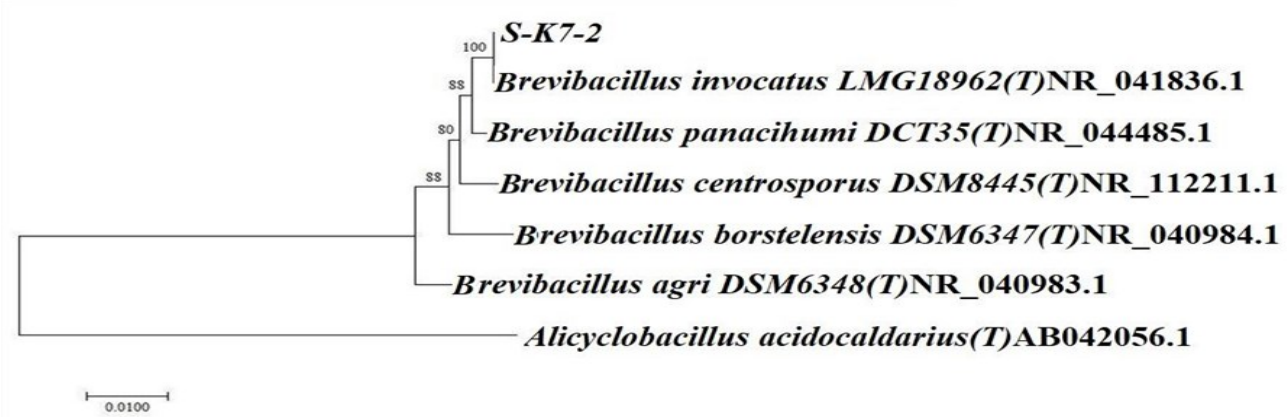

Fig. 9. Phylogenetic tree showing the species identification of the rhizobacterial isolates S-K7-2.

to *Brevibacillus invocatus* with 99.86% similarity (Fig. 9). Di-K7-2 is similar to *Aspergillus tubingensis* at 99.81% similarity (Fig. 10).

Nitrogen fixer is able to convert N_2 to ammonium and nitrate which are the available form of nitrogen for enhancing plant growth. One isolate of nitrogen fixing bacteria (NFB) was found in *Diospyros montana* Roxb (Dio) and two isolates of NFB in *Strychnos axillaris* Colebr (S) rhizosphere soil (Table 1). These three isolates (two bacterial strains and one fungal strain) were used as microbial inoculant for producing biofertilizer by immobilizing microbial cells on peat (Treatment 2). Also, the microorganisms in the cultivated soil from Treatment 2 (Fig. 5) could convert the nitrogen compound in peat to available forms of nitrogen, which can promote the growth of leaves and associate with the photosynthetic of plants (Marschner et al. 1995). Chlorophyll has an important role in the photosynthetic process. Results also showed that the highest total chlorophyll content was presented when hairy basil treated with these three isolates (peat +bacteria + fungal/ treatment 2) (Table 4). Finally, a single kind of microbe with the co-inoculum of bacteria isolates S-P7-2 and S-P7-1 (immobilized on peat/ Treatment 3) had higher potential on promoting plant growth than fungal isolate Di-K7-2. This can be seen in the results of the biomass which was significantly different from another treatment (Table 3).

The *Staphylococcus equorum* strain S-P7-1 showed multiple plant-growth-promoting properties, which were comparable to others in previous reports. For example, Amara et al. (2015) reported that *Staphylococcus equorum* subsp. *equorum* isolated from wheatgrass (*Triticum aestivum* L.) rhizosphere soil sample, possessed several properties, including IAA production, siderophore production, and phosphate solubilization, to promote plant growth.

Staphylococcus equorum strain EN21, isolated from the rhizosphere soil of *Salicornia hispanica*, showed the abilities in solubilization of phosphate, and ACC deaminase ammonia and siderophores production (Vega et al. 2020). The newly-isolated *Brevibacillus invocatus* strain S-K7-2 was able to cooperate well with *S. equorum* S-P7-1 in promoting hairy basil growth. It was previously known that *Bravibacillus* spp. could be used as PGPRs. Studies such as Nehra et al. (2016) reported that *Brevibacillus* spp. has PGP properties such as IAA,

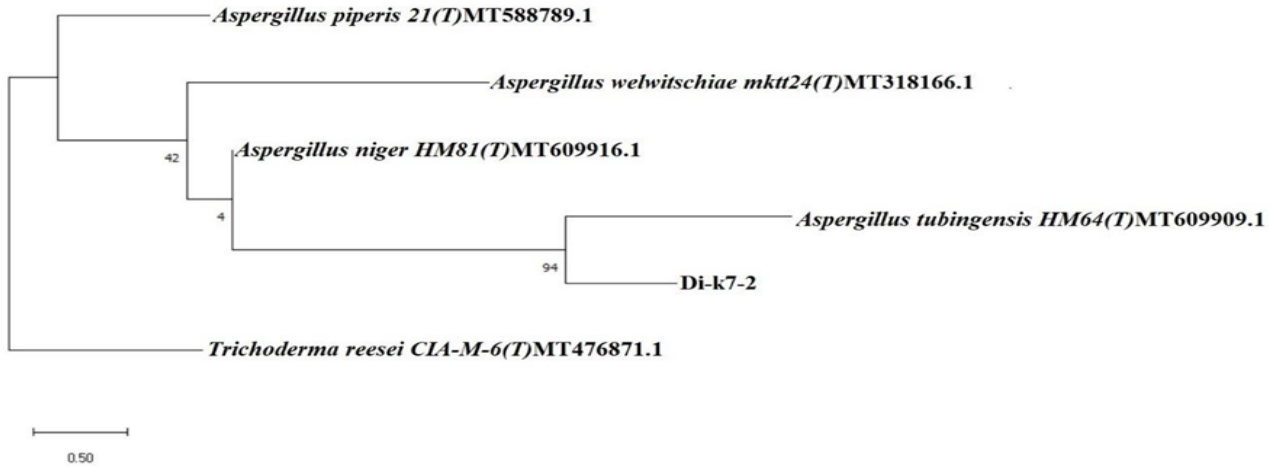

Fig. 10. Phylogenetic tree showing the species identification of fungus isolates Di-K7-2.

antifungal activity, and ammonia production. These bacteria had significant impact on growth and possessed many properties of PGP at high temperatures.

Additionally, a newly-isolated *Aspergillus tubingensis* strain Di-K7-2 was also applied together with bacterial PGPRs to aid plant growth enhancement in pot experiment. Previous reports indicated the successful use of fungal isolates to promote plant growth in many cases. For example, Kaur and Reddy (2017) studied a phosphate-solubilizing fungal strain, identified as *Aspergillus tubingensis* (PSF-4). This fungus could solubilize rock phosphate and significantly increase the yield of maize and wheat. Phosphate levels and soil enzyme activities were also significantly increased when treated with this inoculation. Pandya et al. (2018) reported that *Aspergillus* sp. strain NPF7 was a plant-growth-promoting fungi (PGPF, isolated from wheat rhizosphere), aiding the growth of wheat and chickpea. This fungus showed indole acetic acid (IAA) production, siderophore-producing activity, and the efficiency of phosphate solubilization.

Isolate S-K7-2, S-P7-1, and Di-K7-2 were identified as *Brevibacillus invocatus, Staphylococcus equorum*, and *Aspergillus tubingensis*, respectively. Toyota K (2015) reported that *Bacillus* sp. showed the nitrogen fixing activity and the expression of the nitrogenase reductase (nifH) gene when *Bacillus* sp. was inoculated on sweet potatoes. In the case of *Aspergillus*, it played the multifunctional roles on plant growth promotion especially, P solubilization, being a mycorrrhizal fungi helper and heavy metal bioleaching (Babu and Reddy 2011). In addition, Holguin et al (1992) reported that *Staphylococcus* sp. was able to increase the nitrogen fixing capacity of *L. Anguillarum*. This study is the first report on the nitrogen-fixing activity of *Aspergillus tubingensis*.

CONCLUSION

In this study, three isolates of microorganisms had the most outstanding PGPM properties. Di-K7-2 produced the highest IAA, whilst S-K7-2 and S-P7-1 were able to fix nitrogen from the air and showed the maximum solubilization on phosphorus and potassium in both isolates. Molecular identification of rhizobacterial isolates revealed that Di-K7-2 is similar to *Aspergillus tubingensis*, while S-K7-2 is similar to *Brevibacillus invocatus* and S-P7-1 similar to *Staphylococcus equorum*. In this experiment, it was found that these microbes, immobilized on carrier material such as peat, acted as a bio-fertilizer, enhancing the growth of hairy basil when compared to the control. Peat has a great ability to improve the quality of soil. The structure of peat consists of pores which is beneficial for plant roots to get sufficient air. Peat can help soil keep the moisture content, can act as a buffer, and is a rich source of organic nutrients. It is an environmentally-friendly product and is without toxic and harmful substances for plants and microbes. Peat is a nutrient for plants and microbes. In Thailand, the price of peat is around 20-25 THB per kg. It is a reasonable price when compared with the chemical nutrients which are high-cost and have negative effects on the soil. To enhance plant productivity and improve soil quality, peat is a suggested product for use in organic farms (Kitir et al. 2018).

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