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Potency of Bacterial Consortium From Apple Crops as Production of Indole Acetic Acid (IAA)

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Abstract : The chemical fertilizers cause a decrease in soil fertility and productivity of apple crops. Rhizosphere bacteria may be used as a biofertilizer agents due to its ability to secret produce metabolites required for the growth of plants. Three bacteria isolate SL4, SL5, and SL7 of apple crops rhizosphere (Apple crops in Batu, Indonesia), were observed through PGP (Plant Growth Promoting) activity traits that consist of cellulose activity, phosphate solubilization, and nitrogen fixation. Quantitative IAA production of three isolates was evaluated, and molecular identification base on 16S rDNA was conducted by MEGA 6.0 software. Results showed IAA concentration of consortium culture highest than the single culture of the isolate is 3,42 µg/mL after 5 days incubation. Bacterial isolates identification showed that SL4 has similarity 99,82% as *Bacillus subtilis* F3-7, SL5 has similarity 99,43 % as *Staphylococcus arlettae* 5H7a, and SL7 has similarity 99,94 % as *Bacillus methylotrophicus* CL12.
Keywords : Consortium, IAA, 16S rDNA.

Introduction

Plant Growth Promoting PGP activity is an activity that can support plant growth. The activity carried out by bacteria through natural metabolism to produce a compound used by plants as nutrients. Therefore, bacteria can serve as agents of biofertilizer. Biofertilizer is basically responsible for maintaining the availability of the essential nutrient elements required by plants, as well as produce metabolite compounds that act as enzymes or phytohormones that can stimulate plant growth[1]. Biofertilizer has direct implications for the growth and development of plants that relies upon its production of growth hormones such as IAA hormone that can support each stage of plant growth and development[2]. IAA-producing bacteria related physiological processes in plants by entering IAA produced by bacteria to plants. The root is one of the most sensitive plant tissues to fluctuations in IAA. IAA-producing bacteria are able to increase the number of exogenous IAA which is beneficial in primary root growth process, the formation of lateral roots and advent root.

The rhizosphere bacteria around rooting plants get the energy intake of the compound metabolites secreted by the plant through the roots. The amino acid tryptophan is a precursor of Auxin biosynthesis in plant or bacteria[3]. Biosynthesis of IAA from the soil by bacteria can stimulate in the presence of tryptophan derived from the root exudate cells or damaged plant cells. The amount of tryptophan in the media decided the production of IAA. Some bacteria known to trigger the growth of PGP plant roots commonly belong to genus *Azospirillum, Pseudomonas dan Xanthomonas* sp.[4], few Gram-positive of Bacillus strain[5] and *Staphylococcus arlettae*[6].

Mixed cultures of soil bacteria have PGP activity that increases the production of metabolites compound to support plant growth. The advantages of employing mixed cultures compare with pure cultures in PGP activity, have been demonstrated by synergistic interactions among members of the association which is considered effective and efficient[7]. The aims of this research are to evaluate the potency of consortium culture in the production of IAA compared to a single culture and molecular identification of SL4, SL5 and SL7 isolates based on 16S rDNA.

Material and Methods

Maintenance Isolates and Antagonism Test

In this research three bacteria isolates SL4, SL5 and SL7 were studied. These isolates were isolated in the rhizosphere of Apple Crops in Junggo Batu Indonesia. Screening of biofertilizer properties of these isolates was previously conducted that consisted of cellulolytic activity in CMC (Carboxymethyl Cellulose) agar medium, phosphate solubilizing in Pikovskaya agar medium, and nitrogen fixation in N-Free Semi Solid Malate provided by Microbiology Laboratory, Mathematics and Natural Science Faculty, Biology Department, Brawijaya University. Morphological characteristics, such as gram reaction, cell shape were examined using an optic microscope (Olympus, CX 21). Antagonistic test of three isolates was performed to evaluate the synergistic interaction among these bacteria that can be employed in the consortium.The isolates were cross-streaked and in the middle of the plate. There is a growth of colonies between isolates that blends, incubated for 24-72 h at 30°C and inhibition profiles were observed. The clear zone between the growth of the isolates indicated that these three isolates cannot be in the consortium[8].

Consortium Inoculum Preparation

In order to obtain a consortium inoculum, the isolates were grown separately in LB (Luria Bertani) Broth contain 5 µg/mL Tryptophan for 18 h at 30 ˚C in 150 rpm (adjust to 10^8 CFU/mL). Each bacterial inoculum added in LB (Luria Bertani) with the same volume and OD (Optical Density) to get consortium inoculum. The consortium inoculum added 10% (v/w) of the total volume into specific medium tested.

Quantitative of IAA Production Assay

Total production of IAA was measured with the colorimetric assay based on the Salkowsky reagent (7,5 mL 0,5M $FeCl_3$, 150 mL H2SO4, aquades 250 mL)[9]. Single culture inoculum SL4, SL5, SL7 and consortium inoculum added 10% (v/w) into 100 mL (Flasks 250 mL) LB (Luria Bertani) Broth contain 5 µg/mL Tryptophan incubated for 144 h at 30 ◦C in 150 rpm in the dark. Total production of IAA measured every 24 h. The reaction between Salkowsky reagent and culture supernatant was performed in 2 : 1 ratio for 30 min in the dark. Indolic compounds of IAA were spectrophotometrically determined at 535 nm. A standard curve was determined using indol-3-acetic acid synthesis.

Molecular Identification of Bacterial Isolates

Bacteria isolates SL4, SL5 and SL7 grown in LB (Luria Bertani) Broth for 24 h. Bacteria DNA was isolated following published protocols[10]. Amplification of 16S rDNA by PCR was carried out using the universal primers 27f (5' AGAGTTTGATCMTGGCTCAG 3') dan 1492r (5' TACGGYTACCTTGTT ACGACTT 3'). 10 µL reaction mixture consisted of 5 µL (2x i-Taq DNA Polymerase), 1 µL of 10pmol each primer (27F and 1492R), 1 µL of 450 ng DNA template and 2 µL ddH_2O . The thermocycling profile was carried out with 35 cycles and predenaturation at 94 ˚C (5 min), denaturation at 94 ˚C (20 s), annealing at 52 ˚C (30 s), extension at 72 ˚C (5 min) and a final extension at 72 ˚C (5 min). Aliquots (30 µl) of PCR products were electrophoresed and visualized in 1,5 % agarose gels using standard electrophoresis procedures. Partial 16S rRNA gene of were sequenced by 1stBASE, Malaysia. Finally, 16S rRNA sequence of the isolate was compared with that of other microorganisms by way BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The results were compared to sequences of GenBank based on partial 16S rDNA sequences and phylogenetic tree was constructed by the Maximum-Likelihood method using the MEGA software version 5.01 based on 1000 bootstraps.

Statistical Analysis

Statistical analysis was performed with SPSS version 20.0. Data (three replicates) were analyzed using Analysis of Variance (ANOVA) and the Games-Howell. All tests were subjected to a 95% confidence limit ($P < 0.05$).

Results and Discussion

Quantitative of IAA Concentration

The result showed consortium culture produced higher IAA compared to single culture. The concentration of IAA produced by SL4 increased from day 1 to day 5 in the range from 0.41±0.09 to 1.97±0.60 µg/mL. The concentration of IAA produced by SL5 increased from day 1 to day 5 in the range from 0.14±0.24 to 1.76±0.47 µg/mL. The concentration of IAA produced by SL7 increased from day 1 to day 5 with a range from 0.20±0.23 to 2.87±0.94 µg/mL. The concentration of IAA produced by consortium increased from day 1 to day 5 with a value ranging from 0.51±0.31 to 3.42±0.33 µg/mL. The concentration of IAA produced by SL4, SL5, SL7 and consortium decreased on day 6 (**Fig. 1**).

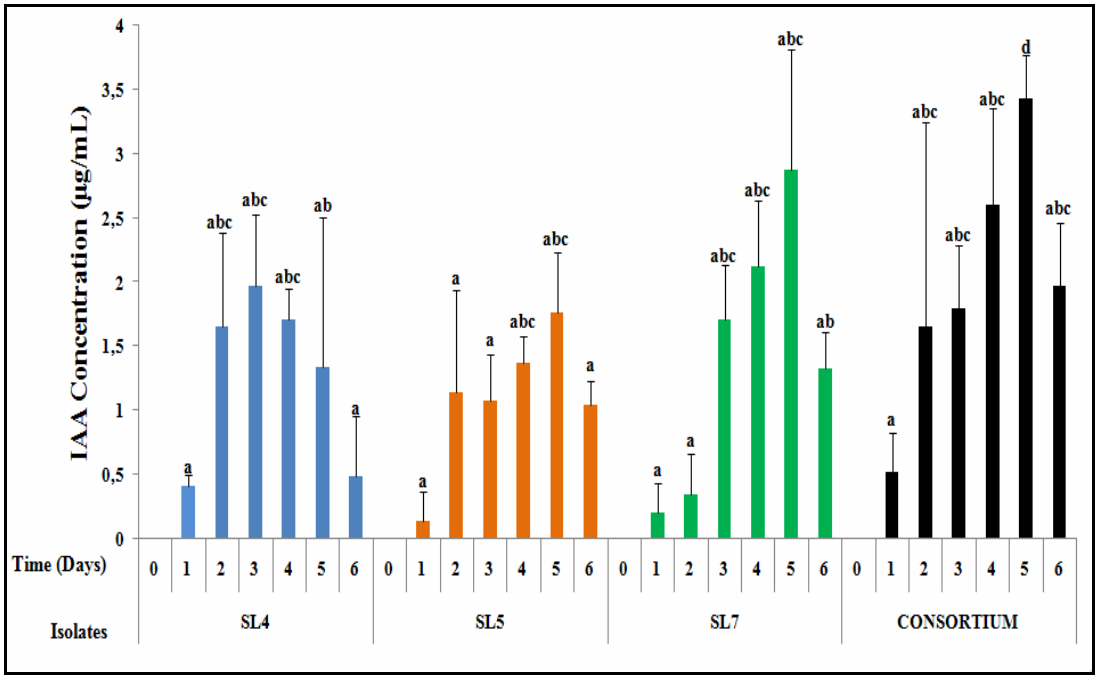

Fig. 1. IAA concentration was produced by single and consortium culture. Each value is the mean of three replicates. Error bars represent ± standard deviation. Different letters represent significant statistical differences based on Games-Howell test ($P < 0.05$).

Single culture (SL5 and SL7) and consortium culture of these isolates produced highest IAA after incubation 5 days, but SL4 produced a concentration of IAA highest after incubation 3 days. IAA is synthesized as a secondary metabolite by bacteria which is induced by tryptophan as precursor during log phase until stationary phase in which optimum phase of IAA[11]. As shown in (**Fig.1.**), IAA production started to increase since day 1 until day 5 incubation due to cell growth in a medium that utilizes tryptophan as a precursor in the IAA synthesis process. IAA is highly produced after 48 hours incubation due to the end of the logarithmic phase that generates enzymes used for IAA biosyntheses such as tryptophan-2-monooxygenase, indole-3-pyruvate decarboxylase, amine oxidase, nitrile hydratase and amidase[12]. Production of IAA will progressively increase in stationary phase but later decrease in death phase, which explain decrease IAA after 6 days incubation. In death phase, IAA oxidase and peroxidase are present to degrade IAA hormone[13].

Among the single culture of three isolates, SL7 showed highest IAA than the other isolates. The amount of IAA concentration by species and strain of bacteria depend on the condition of the cultivation, including the

presence of tryptophan, oxygenation level, pH, growth phase and nutrition in medium[14]. Similarly, the result showed the addition of 5 µg/mL tryptophan in the medium culture can produce a concentration of IAA until 3,42 µg/mL. IAA production by bacteria increased in the presence of IAA precursor, such as L-tryptophan, in the culture medium. IAA production in both strains increased with increases in tryptophan concentration[15]. Three isolates mixed in the consortium showed increase IAA that indicates the synergistic relationship among isolates in IAA production.

Each bacteria through different pathway in the production of IAA [16]. Biosynthesis of IAA by bacteria with tryptophan as the main precursor compounds divided into indole-3-pyruvate, indole-3-acetonitrile (IAN), tryptamine, and indole-3-acetamide[17]. Association of many bacteria producing IAA can increase its production rate from the different pathway that enables exogenous IAA to be utilized by the plant to support the growth. Inoculation of combination bacteria producing IAA can show the significant effect of germination rates on Tomato[18] and increase N, P, K, Ca and Mg uptake in sweet potato cultivar[19] due to the stimulatory effect of bacteria through interaction between the exogenous signal of IAA with the plants roots.

Molecular identification of the SL4, SL5 and SL7 isolates

Partial sequence of 16S rDNA of SL4, SL5 and SL7 were identified that showed SL4 has similarity of 99,82% to *Bacillus subtilis* F3-7 with fragment lengths of 1410bp, SL5 has similarity 99,43 % (**Fig.2.**) to *Staphylococcus arlettae* 5H7a with fragment lengths of 1430bp (**Fig.2.**), and SL7 has similarity of 99,94 % to *Bacillus methylotrophicus* CL12 with fragment lengths of 1415bp (**Fig.3.**).

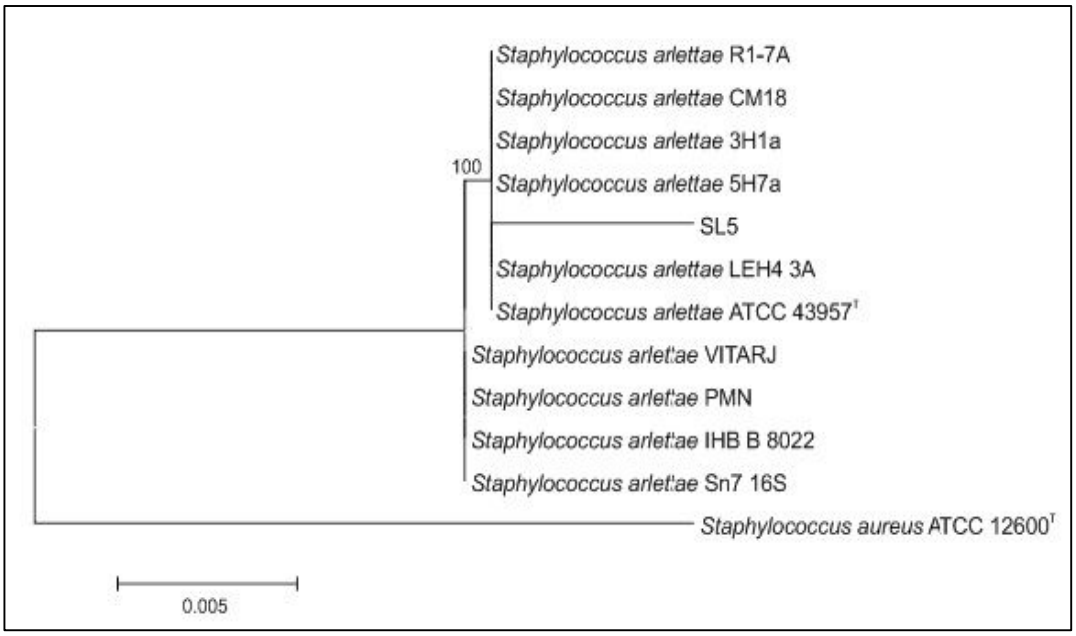

Fig. 2. Phylogenetic tree of isolates based on 16S rDNA analysis SL5 compare references isolates constructed by the Maximum-Likelihood method using the MEGA software version 5.1 based on 1000 bootstraps.

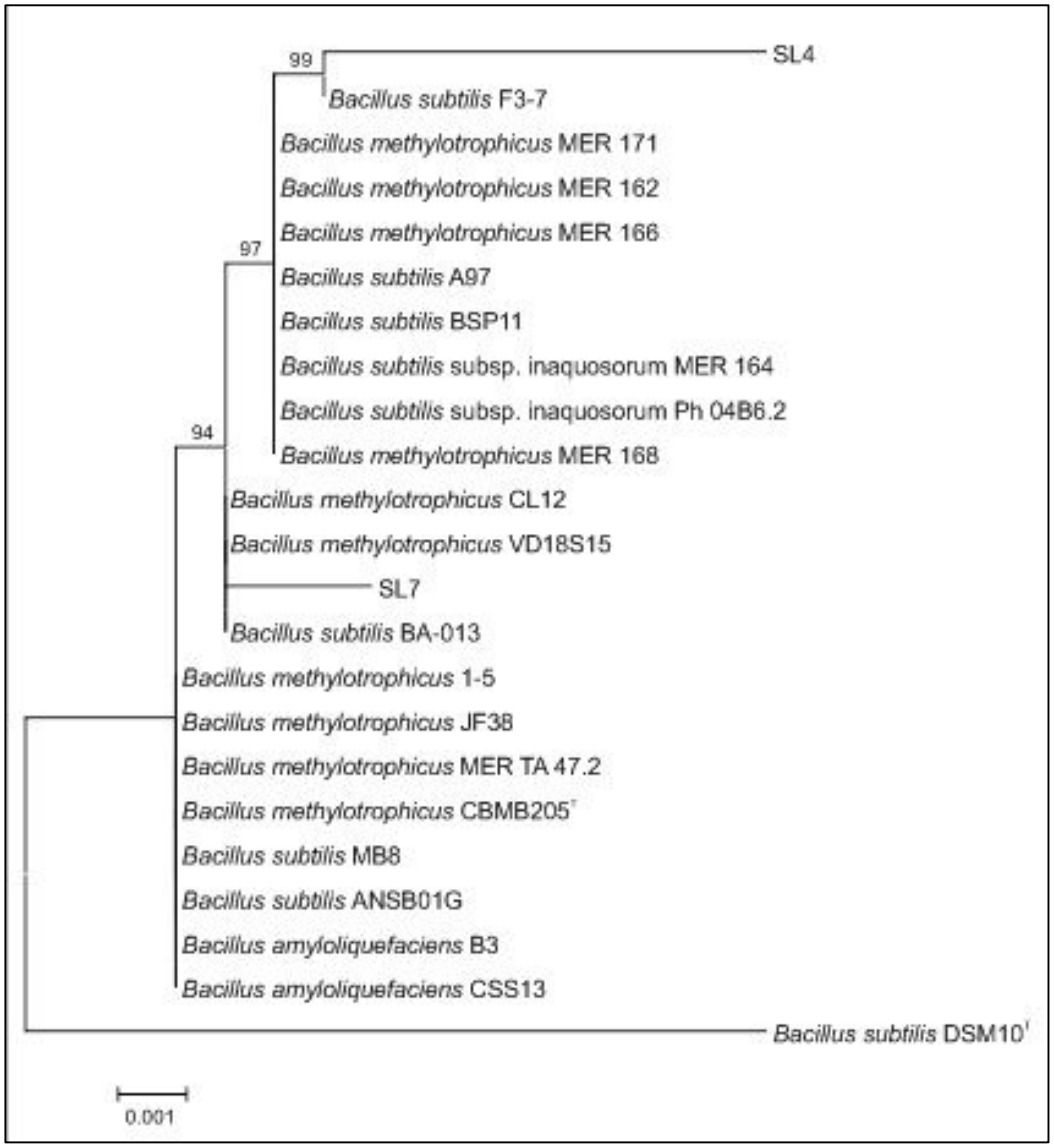

Fig. 3. Phylogenetic tree of isolates based on 16S rDNA analysis SL4 and SL7 compare references isolates constructed by the Maximum-Likelihood method using the MEGA software version 5.1 based on 1000 bootstraps.

Bacillus subtilis obtained from the rhizosphere of paddy, were tested for efficiency of IAA production, and showed the maximum IAA production of 4- 5 µg/mL[20]**.** *Staphylococcus arlettae* obtained from the arsenic-contaminated soil showed IAA efficiency production of 41,07 µg/mL[21]**.** *Bacillus methylotrophicus* obtained from endophytic bacteria alpine grasslands showed IAA efficiency of 7,76 µg/mL[22]. The ability of *Bacillus subtilis* in producing IAA indicates the presence of similar genes involved in the IAA biosynthesis such as trp, ysnE, and yhcX gene[23]. yhcX gene is similar to nitrilase which directly catalyze the conversion of indole 3-acetonitrile IAA[24]. YsnE is similar to IAA acetyltransferase involved in the activity of PGP on pro-α teobacterium bacteria *Azospirillum brasilense*[25]. YsnE along with trp play its role to synthesize IAA in tryptophan-dependent manner [25].

Conclusion

The concentration of IAA by consortium culture (SL4, SL5 and SL7) was highest than single culture of isolate after 5 days incubation. The bacterial consortium can increase IAA production synergistically. Bacteria

isolate SL4 has similarity to *Bacillus subtilis* F3-7, SL5 to *Staphylococcus arlettae* 5H7a, and SL7 to *Bacillus methylotrophicus* CL12.

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