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POTENTIAL OF INDOLE ACETIC ACID PRODUCING RHIZOBACTERIA TO PROMOTE THE GROWTH AND INCREASE THE YIELD OF EDAMAME, A VEGETABLE SOYBEAN (*GLYCINE MAX*)

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ABSTRACT

Five isolates of indole acetic acid (IAA) producing rhizobacteria have been isolated from rhizospheres of plants grown in Bali, Indonesia. Based on 16s rRNA gene analysis those isolates were identified as *Stenotrophomonas maltophilia* Sg3 *Proteus mirabilis* BjB17, *Providencia rettgeri* AlDp5, *Bacillus thuringiensis* TNJbx.3.3, *Bacillus cereus* GR12. All of these isolates significantly ($P<0.05$) increased relative growth rate (RGR), net assimilation rate, leaf size, chlorophyll content of leaf, dry weight of roots and shoots, number of root nodules of edamame, a vegetable soybean. Treatment with rhizobacteria was also significantly ($p<0.05$) increased number of pod plant^{-1}, weight of pods plant^{-1}, pod weight, and protein content in the seed. Five isolates of IAA producing rhizobacteria treatment increased the number of pod plant^{-1} ranged from 42.06% to 54.48%, weight of pods plant^{-1} ranged from 69.62% to 91.08%, pod weight ranged from 13.91% to 28.53% when compared to control. In general, *Stenotrophomonas maltophilia* Sg3 showed the highest ability to promote the growth and increase the yield of edamame. These results suggested that this isolate is the most promising bio-stimulant to increase productivity of edamame in Indonesia.

KEYWORDS: *edamame, indole acetic acid, rhizobacteria, growth promotion*

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INTRODUCTION

Edamame is a vegetable soybean which is commonly consumed freshly. This type of soybean is harvested when the reproductive stage has reached R6, where pods have developed maximally but still immature and the color is green, and the seed has filled about 80-90% of pod's space.[1] The average productivity of edamame in Indonesia ranged from 3 to 3.5 ton ha^{-1}.[2]which is much lower than those of Japan, China and United State of America which are respectively 19.7 ton ha^{-1}, 18 ton ha^{-1}, and 16.3 ton ha^{-1}.[3] These data indicated that there is a possibility to increase the productivity of edamame in Indonesia. One of the efforts to increase plant productivity is through the use of indole acetic acid (IAA) producing rhizobacteria.[4] Effectiveness of IAA producing rhizobacteria to promote the growth and increase the yield of several plants have been reported by several researchers.[5-10] The level of growth promotion and yield increment resulted from rhizobacteria treatment have been known to vary depending on the species or strain of rhizobacteria, and the species or cultivar of the plants. Other studies on the use of rhizobacteria to promote the growth and increase the yield of soybean have also been done.[11-12], however very limited information available on the use of rhizobacteria to promote the growth and increase the yield of edamame. This study was undertaken to find and indentify IAA producing rhizobacteria of local Bali, Indonesia that can be used to promote the growth, increase the yield of edamame. As the significance of IAA-producing rhizobacteria in promoting the plant growth is specific to certain host plant, it is necessary to find strains of rhizobacteria that suitable to edamame. The mechanism by which IAA-producing rhizobacteria promote the growth of edamame should be studied in further research. In addition, the field trial under different localities and seasons is needed to ensure the efficacy and stability of IAA-producing rhizobacteria. We isolated IAA-producing rhizobacteria from rhizospheres of several plants grown in Bali, Indonesia. By using IAA-producing rhizobacteria in the cultivation of edamame as biostimulant is expected to increase the efficiency of the use of fertilizer and to some extent reduce the production cost and increase the income of producers. Results of this study will give a solution to increase edamame productivity in Indonesia to approach edamame productivities in Japan, China and the United State of America.

MATERIALS AND METHODS

Test for IAA Production[13]

Five isolates of rhizobacteria were isolated from rhizospheres of several plants grown in Bali, Indonesia were used in this study. Those isolates are isolate Sg3, isolated from rhizosphere of *Arachis hyphogea*, isolate BjB17, isolated from rhizosphere of *Impatiens balsamina*, isolate GR12, isolated from rhizosphere of *Calliandra haematocephala*, isolate AlDp5, isolated from rhizosphere of *Imperata cylindrica*, and isolate TNJbx.3.3, isolated from rhizosphere of *Pterospermum javanicum*). Ability of rhizobacteria to produce IAA was tested according to the method developed by Patten and Glick.[13] Isolates of rhizobacteria were grown for 48 h in nutrient broth medium enriched by 2 mg ml^{-1} L-tryptophan. The cultures were then centrifuged at 1610x *g* for 30 min, and the supernatant was collected and passed through Millipore filter (0.45 □m diameter). Content of IAA in the filtrate was detected using Salkowski reagent (1 ml 0.5 M $FeCl_3$ and 49 ml 35% $HClO_4$). Salkowski reagent and filtrate respectively 0.5 ml were put into Eppendorf tube (volume 1.5 ml), and then was incubated in the dark at room temperature for 30 min. When the color of solution turned into pink, indicated that rhizobacteria produced IAA.

Identification of IAA-Producing Rhizobacteria[6,9]

Five isolates of rhizobacteria, namely Sg3, BjB17, GR12, AlDp5, and TNJbx.3.3 were grown in tryptic soy broth medium (casein peptone : 17 g , soya peptone : 3 g, NaCl : 5 g, 2.5 g K_2HPO_4 : 2.5 g, glucose : 2.5 g and distilled water to make 1 liter) and incubated in a orbital shaker in the dark under room temperature (28±2°C) for 24 h. Extraction and purification of DNA was done using Gene JET Genomic DNA Purification Kit procedure (Thermo Fisher Scientific Inc., USA). Two primers viz. 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') were used to amplify the 16S rRNA gene. The reaction was performed in PCR machine GeneAmp® PCR system 2700 (Applied Biosystem, California USA) under the following conditions: pre-denaturation at temperature 94°C (2 min 2 sec) followed by 30 cycles each of denaturation at 94°C (15 sec), annealing at 50°C (30 sec), extension at 72°C (1 min 30 sec), and final extension at 72°C for 10 min. Pure DNA of KtB3 was sequenced using Genetic Analyzer machine (Applied Biosystem ABI PRISM310, California, USA). The sequence was aligned with GenBank using BLAST-N program from the National Center for Biotechnology Information (NCBI) to determine the similarity of tested isolates with other previously identified isolates. Phylogeny analysis was performed using MEGA 6.0 version neighbor joining method with a bootstrap 1000x. Phyllogenetic tree was developed using TreeGraph 2.0.

Evaluation in a Green House[14]

All five isolates of rhizobacteria were tested for their efficacy to promote the growth and increase the yield of edamame in a green house. Six treatments were tested in this experiment viz. treatment with rhizobatreia Sg3, BjB17, GR12, AlDp5, TNJbx.3.3 and control. Each treatment was replicated four times and allocated in randomized block design. Cultural medium for edamame cultivation consisting of soil, compost, and ash of rice husk (3:1:1, w/w/w) and put into plastic pots (30 cm diam, and 27 cm height). Each pot was filled with 8 kg of cultural medium, and 10 pots were prepared for each experimental unit. Treatment with rhizobacteria was done through seed treatment prior to sowing. Rhizobacteria was cultured in a nutrient broth medium for 48 h under room temperature. Sterile distilled water was used to prepare rhiozobacteria suspension and the density of rhizobacteria was adjusted to 10^6 CFU ml^{-1}. The edamame seeds were dipped in rhizobacteria suspension for 60 min. Several growth parameters were measured such as leaf chlorophyll content, relative growth rate (RGR), and net assimilation rate (NAR). Leaf chlorophyll content was measured using a

chlorophyll-meter SPAD-502 (Konica Minolta, Japan) at 30, 45, and 60 days after sowing (DAS). The Relative Growth Rate (in mg day^{-1}) and Net Assimilation Rate (NAR) in mg dm^{-2} day^{-1} were calculated according to Hasanah [14] as follows:

$$RGR = \frac{Ln\ W_2 - Ln\ W_1}{T_2 - T_1}$$

$$NAR = \frac{W_2 - W_1}{T_2 - T_1} \times \frac{Ln\ LA_2 - Ln\ A_1}{LA_2 - LA_1}$$

W1 = total dry matter at time T1
W2 = total dry matter at time T2
LA1 = total leaf area at time T1
LA2 = total leaf area at time T2
Ln = natural logarithm

Several yield components were also determined such as number of pods $plant^{-1}$, weight of pods $plant^{-1}$, and protein content of edamame seed (75 DAS)

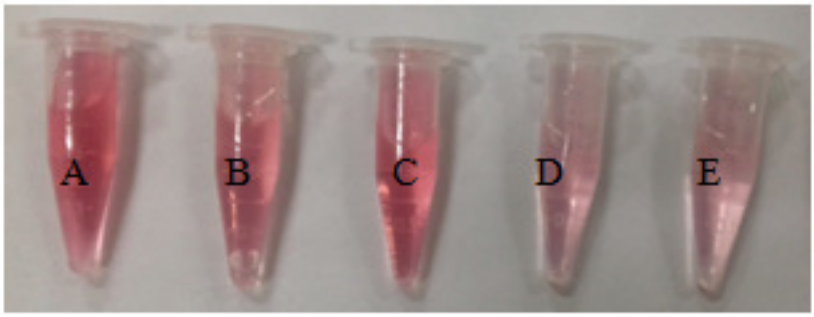
STATISTICAL ANALYSIS

All data for growth parameters and yield components were subjected to the analysis of variance followed by Duncan's multiple range test at 5% level of significance using SAS software version 6.12 (SAS Institute, Gary, NC., USA).

RESULTS AND DISCUSSION

IAA Production

All five isolates of rhizobacteria namely isolates TNJbx3.3, Sg3, BjB17, AlDp5, and GR12 was proven to produce IAA in the cultural filtrates. The color change from colorless to pink was observed in the cultural filtrates when they were reacted with Salkowski reagent. The appearance of pink color in the cultural filtrates of rhizobacteria indicated the presences of IAA. The IAA was oxidized by $HClO_4$ and then reacted with $FeCl_3$ and formed tris-(indole-3-acetato) iron complex with pink color.[15,16] as shown in Figure 1.

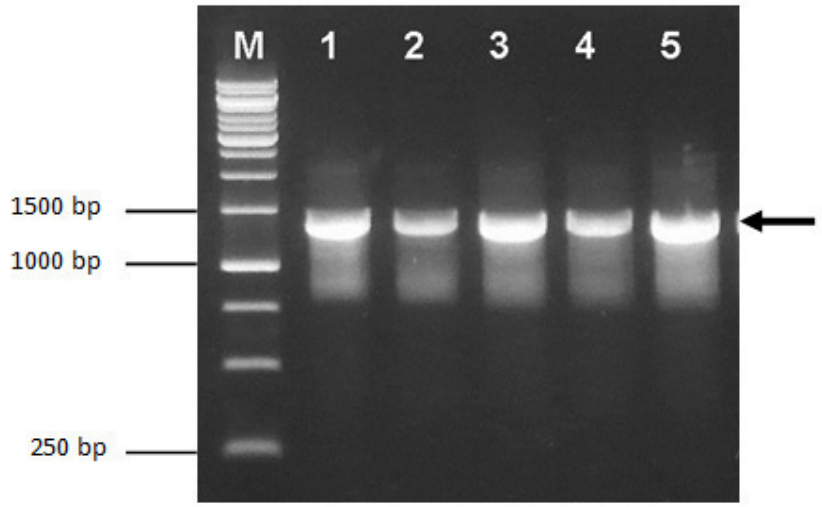


A. isolate TNJbx3.3; B. Isolate Sg3; C. Isolate BjB17; D. Isolate AlDp5; E. Isolate GR12.

Figure 1
Cultural filtrates of five isolates of rhizobacteria in Eppendorf tubes show pink color after they were reacted with Salkowski reagent.

Species of IAA-producing Rhizobacteria

Aplification of 16S rRNA genes of five isolates resulted in fragments respectively of 1,500 as shown in Fig. 2. The size of amplified 16S rRNA gene is in agreement with the result of study done by Ahmed[17] that the size of amplified 16S rRNA gene was 1,500 bp. Based on the analysis of 16S rRNA gene sequence aligned with data base of GenBank using BlastN program, isolate AlDp5 belong to the *Providencia rettgeri* due to its homolog with *P. rettgeri* strain OK-NRB-DRDO/MP (KX289656.1), *P. rettgeri* strain MSS2 (KF923809.1), and *P. rettgeri* strain ALK420 (KC456550.1) with similarity by 99%. This is supported by phyllogenetic tree analysis using Maximum Parsimony (MP) method 1,000x replicates of Bootstrap, that isolate AlDp5 is *Providencia rettgeri*, because it is in the same clade with sequences of *P. rettgeri* with 100% Bootstrap Support (BS) (Fig.3).



M: 1 Kb DNA marker; 1.Isolate Sg3; 2.Isola BjB17; 3. Isolate TNJbx3.3; 4. Isolate AIDp5; 5.Isolate GR12.

Figure 2
Agarose gel electrophoreses shows 16S rDNA bands (arrow) of 5 isolates of rizhobacteria.

Species of isolate BjB17 is *Proteus mirabilis*. It has homolog with *Proteus mirabilis* strain C27DMVR (KR140188.1), *P. mirabilis* strain 2115 (JF947362.1), and *P. mirabilis* strain Z37 (KC212059.1) with a similarity of 99%. Isolate BjB17 located in the same clade with *P. mirabilis* based on phyllogenetic tree analysis with 1,000x Bootstrap replicates and 100% Bootstrap Support (BS). Isolate GR12 belong to the species of *Bacillus cereus* and showed homolog with *B. cereus* strain HYM81 (KT982238.1), *B. cereus* strain ClCC10041 (KJ675635.1), and *B. cereus* strain R5 339 (JQ659737.1) with a similarity of 99%. Result of phyllogenetic tree analysis using MP method with 1,000x replicates of Bootstrap showed that species of isolate GR12 *Bacillus cereus*, because it is in the same clade with sequences of *B. cereus* 100% BS. Isolate TNJbx.3.3 belongs to the species *Bacillus thuringiensis* and showed homolog with *B. thuringiensis* strain RII2-100 (LT604457.1), *B. thuringiensis* strain RII2-97 (LT604455.1), and *B. thuringiensis* strain RII2-66 (LT604435.1) with similarity of 99%. Based on phyllogenetic tree analysis using MP method with 1,000x replicates of Bootstrap showed that isolate TNJbx.3.3 is *B. thuringiensis*, because it is in the same clade with sequences of *B. thuringiensis* with 58.4% BS. Isolate Sg3 belongs to the species *Stenotrophomonas maltophilia* and showed homolog with *S. maltophilia* strain S4B (LC168839.1), *S. maltophilia* strain M83 (LN890169.1), dan *S. maltophilia* strain CYJ (KP185140.1) with similarity of 99%. Based on phyllogenetic tree analysis using MP method with 1,000x replicates of Bootstrap showed that isolate Sg3 is *S. maltophilia*, because its is in the clade with sequences of *S. maltophilia* with 100% BS.

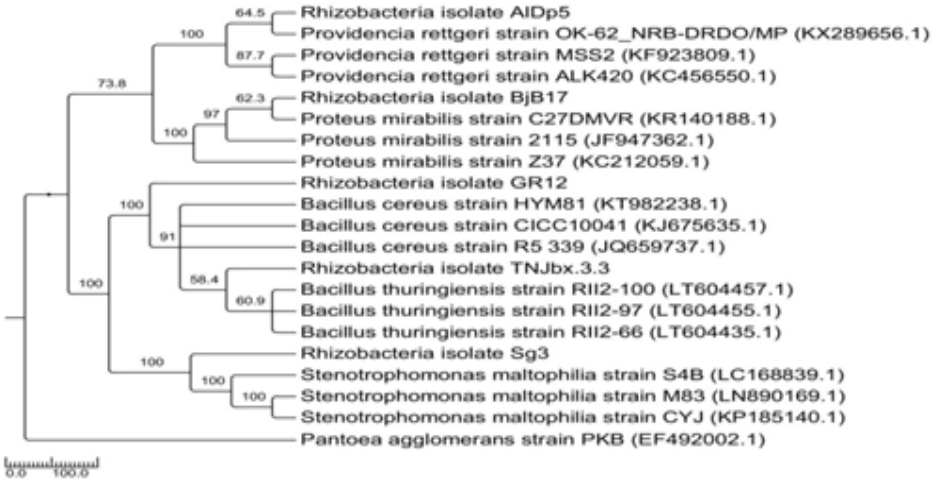


Figure 3
Maximum Parsimony Phylogenetic Tree of 16S rDNA shows genetic relationship between 5 isolates of IAA-producing rhizobacteria with previously identified rhizobacteria available in Genbank Database. Value of Bootstrap derived from 1,000x replicates.

Effectiveness of IAA producing rhizobacteria to promote the growth

Treatment with isolates of IAA producing rhizobacteria significantly (P<0.05) increased relative growth rate (RGR) and net assimilation rate (NAR) measured at 30, 45, and 60 days after planting (DAP) as presented in Table 1. The increase of RGR on 30 DAP varied between 23.02% to 94.32% when compared to control, while the increase of RGR on 45 and 60 DAP respectively 33.15% to 42.12% and 40.51% to 46.57%. Similar trend was aslo observed on NAR in which NAR on 30 DAP increased 25.73% to 59.06%, while on 45 and 60 DAP the increase respectively ranged from 31.70% to 40.65%, and 27.45% and 40.19% (Table 1).

Table 1
Effect of IAA producing rhizobacteria to the relative growth rate (RGR) and net assimilation rate (NAR) of edamame

Treatment	RGR (mg day^{-1})			NAR (mg dm^{-2} day^{-1})		
	30 HST	45 HST	60 HST	30 HST	45 HST	60 HST
Control	50.77 a*	34.56 a	26.56 a	1.71 a	1.23 a	1.02 c
Sg3	63.97 c (25.99%)**	46.82 c (35.47%)	37.32 b (40.51%)	2.15 b (25.73%)	1.62 b (31.70%)	1.30 b (27.45%)
BJB17	98.66 f (94.32%)	47.91 d (38.62%)	38.93 e (46.57%)	2.24 c (30.99%)	1.69 c (37.39%)	1.41 a (38.23%)
TNJbx3.3	68.88 d (35.67%)	49.12 f (42.12%)	38.24 c (43.97%)	2.36 d (38.01%)	1.72 d (39.83%)	1.32 b (29.41%)
AIDp5	73.66 e (45.08%)	48.05 e (39.03%)	38.88 e (46.38%)	2.57 e (50.29%)	1.62 b (31.70%)	1.32 b (29.41%)
GR12	62.46 b (23.02%)	46.02 b (33.15%)	38.58 d (45.25%)	2.72 f (59.06%)	1.73 d (40.65%)	1.43 a (40.19%)

**** Values followed by the same letters in the same column are not significantly different (p>0.05) according to Duncan's Multitle Range Test at 5% level. ** Values in the parenthesis indicated the percentage of increase when compared to control.***

All five isolates of IAA producing rhizobacteria significantly (p<0.05) increased the leaf area plant^{-1}, leaf chlorophyll content, number of nodules, dry weight of root and dry weight of shoot (Table 2). Treatment with IAA producing rhizobacteria increased the leaf areas per plant by 23.32% to 51.32%. Isolate Sg3 showed the highest ability to increase the leaf areas per plant, leaf chlorophyll content, dry weight of root, and dry weght of shoot (Table 2). The leaf chlorophyll content increased between 13.61% to 21.26% when compared to control, while number of nodules, dry weight of root and dry weight of shoot respectively increased by 30.07% to 142.85%, 20.0% to 215.0%, and 21.15 to 29.78%. Several researchers reported that treatment with rhizobacteria obviously promoted the growth of plants through the increase of leaf area, leaf chlorophyll content, number of nodules, dry weight of root and dry weight of shoot.[8,18,19,2021,22] Seed coating of maize seed with the susspension of *Pseudomonas putida* DSM291 could increase the leaf area by 190.56%[18] when compared to control, while treatment as seed coating on maize seed with IAA producing rhizobacteria isolate MA-11 increased the leaf chlorophyll content by 13.56%.[21] Other study showed that treatment with *Azospirillum brasilense* in combination with *Bradyrhizobium* spp. as seed coating on soybean seed increased the number of nodules by 5.42%[20], while treatment of *Bacillus thuringeinsis* KR1 in combination with *Rhizobium leguminosarum* PR1 as seed coating on seed of pea (*Pisum sativum*) increased the number of nodules by 84.61% when compared with the treatment of *R. leguminosarum* PR1 alone.[19] Treatment with *Bacillus thuringiensis* increased the dry weight of shoot of tomato by 35.3% to 201.9%[22], while treatment with *Bacillus subtilis* increased the dry weight of root of *Allium cepa* by 15.47%.[8]

Table 2
Effect of IAA producing rhizobacteria on leaf area per plant, chlorophyll content of leaf, the number of nodules, and dry weight of root and shoot.

Treatment	Leaf area per plant 60 DAS(dm^2)	Chlorophyll content of leaf 60 DAS (SPAD unit)	The number of nodules	Dry weight of root(g)	Dry weight of shoot (g)
Control	1490.8 a*	33.20 a	33.25 a	2,00 a	58,77 a
Sg3	2256.0 c (51.32%)**	40.26 c (21.26%)	70.50 d (112.03%)	6,30 d (215.0%)	76,27 f (29.78%)
BJB17	1875.9 b (25.83%)	37.72 b (13.61%)	80.75 e (142.85%)	3,20 b (60.0%)	71,20 b (21.15%)
TNJbx3.3	2154.1 b (44.49%)	38.54 b (16.08%)	51.00 cb (53.38%)	2,43 c (21.5%)	74,17 e (26.20%)
AIDp5	2123.6 b (42.44%)	38.58 b (16.20%)	43.25 b (30.07%)	2,40 c (20.0%)	73,53 d (25.11%)
GR12	1838.5 b (23.32%)	37.81 b (13.88%)	56.50 c (57.14%)	3,47 b (73.5%)	73,16 c (24.48%)

**** Values followed by the same letters in the same column are not significantly different (p>0.05) according to Duncan's Multiple Range Test at 5% level. ** Values in the parenthesis indicated the percentage of increase when compared to control.***

Effectiveness of IAA producing rhizobacteria to increase the yield

Treatment with IAA producing rhizobacteria significantly ($p<0.05$) increased the number of pod per plant, the weight of pod per plant and average weight of pod (Table 3). The number of pod per plant increased by 42.06% to 54.48%, while the weight of pod per plant increased by 69.62% to 91.08%. Isolate Sg3 showed the highest ability to increase the number and weight of pod per plant. This data is suppoted by the growth promotion data in which isolate Sg3 showed the highest ability to increase the leaf areas per plant, leaf chlorophyll content, dry weight of root, and dry weight of shoot. Affectiveness of IAA producing rhizobacteria to increase the yield of plants varied in accordance with species and strain of rhizobacteria and the type or cultivar of plants. Previous studies on soybean showed that treatment with IAA producing rhizobacteria increased the number of pod per plant by 16.12% to 105.37%.[23.24] On other plants (mungbean (*Vigna radiate*) and peanut (*Arachis hypogaea*) treatment with IAA producing rhizobacteria increased the number of pod per plant respectively by 32% and 28.40%.[25,26]

Table 3

Effect of IAA producing rhizobacteria on the number of pod per plant, the weight of pod per plant, pot weight, and protein contents of edamame seeds.

Treatment	The number of pod per plant (g)	The weight of pod per plant (g)	Pod weight (g per pod)
Control	36.25 a	90.73 f	2.502 f
Sg3	56.00 b (54.48%)**	173.37 a (91.08%)	3.095 b (23.70%)
BJB17	54.00 b (48.96%)	153.90 e (69.62%)	2.850 e (13.91%)
TNJbx3.3	55.50 b (53.10%)	171.10 b (88.58%)	3.082 c (23.18%)
AlDp5	51.50 b (42.06%)	165.67 c (82.59%)	3.216 a (28.53%)
GR12	52.25 b (44.13%)	160.52 d (76.92%)	3.072 d (22.78%)

**** Values followed by the same letters in the same column are not significantly different ($p>0.05$) according to Duncan's Multiple Range Test at 5% level. ** Values in the parenthesis indicated the percentage of increase when compared to control.***

In our study we proved that five isolates of IAA producing increased the RGR, NAR, leaf area, leaf chlorophyll content, dry weight of roots and shoot, the number of nodules, number of pods per plant, weight of pods per plant, and average weight of pod. This occurs because the IAA produced by rhizobacteria in the rhizosphere of plants contribute to regulating physiological processes, especially in the roots of plants. IAA in regulating physiological processes of plants refers to the acid growth hypothesis and the gene activation hypothesis.[27] Based on the acid growth hypothesis, the IAA is responsible for regulating the process of cell elongation, cell enlargement, and the development of organs[28], while based on the gene activation hypothesis, the IAA is responsible for regulating the process of cell division, cell differentiation, and protein synthesis.[29] Tsavkelova *et al*.[30] report that the IAA is not only responsible for the division, elongation, and differentiation of cells and tissues of plants but also responsible for stimulating seed and tuber germination, increase of the rate of xylem and root formation, control processes of vegetative growth, tropism, florescence, fructification of plants, photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stress factors. Increased physiological processes in the root can increase root growth and organ development. Teale *et al*.[31] report that the high concentration of IAA can inhibit root elongation and stimulate cell division in root meristem. Cell division in meristem root causes root length, number of roots, the number of lateral roots and root hairs increase.[32] Increasing the number of lateral roots and root hairs can increase koinokulasi Rhizobium so as to increase the number of nodules[33], and also increase the absorption of nutrients.[34] Increasing the number of nodules and absorption of nutrients, especially nitrogen can increase the protein, amino acids, amides, nucleic acids, nucleotides, and chlorophyll. Nitrogen will increase the green color of leaves and can encourage the growth of stems and leaves.[35] Nitrogen deficiency can reduce the nitrogen content of leaves, leaf chlorophyll content, and carbon assimilation in plants that will reduce plant dry weight.[36] Therefore, an increase in nitrogen content can affect the photosynthesis of plants that can ultimately increase vegetative growth and yield.[37] Among five species of IAA producing rhizobacteria we found and identified in the present study, *Stenotrophomonas maltophilia* Sg3 showed the best performance in promoting the growth and increase the yield of Edamame. This strain may be use as promising biostimulant in the edamame cultivation in Indonesia.

CONCLUSION

Five species of IAA producing rhizobacteria namely *Providencia rettgeri* AlDp5, *Proteus mirabilis* BJB17, *Bacillus cereus* GR12, *Bacillus thuringiensis* TNJbx.3.3, and *Stenotrophomonas maltophilia* Sg3 effectively promoted the growth and increased the yield of edamame, a vegetable soybean. Among them, *S. maltophilia* Sg3 showed the best performance in promoting the growth and increase the yield of edamame, thus may be use as biostimulant to increase the yield of edamame. Although many studies have been intensivley done to isolate IAA-producing rhizobacteria as bio-stimulant for crop yield increment, a considerable work is still required to prove its potential

use under field condition. The field trials with larger areas and different localities is needed to ensure the stability and effectivenss of *S. Maltophilia* Sg3 as bio-stimulant in edamame cultivation in Indonesia.

CONFLICT OF INTEREST

Conflict of interest declared none.

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