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Production of IAA by *Bradyrhizobium* sp.

Nisa Rachmania Mubarik, Irni Mahagiani, and Aris Tri Wahyudi

Abstract**—**The objective of this research was to determine the potency of indigenous acid-aluminium tolerant *Bradyrhizobium japonicum* as producer of indole acetic acid (IAA) and applied it as nitrogen fixation on local soybeans viz Anjasmoro, Tanggamus (yellow soybean seeds), and Detam (black soybean seed). Three isolates of acid-aluminium tolerant *Bradyrhizobium japonicum* (BJ) were used in this research, i.e. BJ 11 (wt), BJ 11 (19) - BJ 11(wt) mutant, and USDA 110 as a reference isolate. All of isolates tested to produce the IAA by using Salkowsky method. Effect of IAA production by each of *B. japonicum* was tested on growth pouch and greenhouse using three varieties of soybean. All isolates could grow well and produce IAA on yeast mannitol broth (YMB) medium in the presence of 0.5 mM L-tryptophan. BJ 11 (19) produced the highest of IAA at 4 days incubation compared to BJ 11 (wt) and USDA 110. All tested isolates of *Bradyrhizobium japonicum* have showed effect on stimulating the formation of root nodules in soybean varieties grown on Leonard bottle. The concentration of IAA on root nodules of soybean symbiotic with *B. japonicum* was significantly different with control, except on the treatment using Tanggamus soybean.

Keywords**—**Acid-aluminium tolerant isolate, *Bradyrhizobium japonicum*, indole acetic acid, soybean.

I. Introduction

MANY efforts have been developed to increase the productivity of soybean such as, cultivating the plant on acid soil which is supplemented with acid-tolerant root nodule bacteria producing indole-3- acetic hormone (IAA).

Acid-aluminium tolerant *Bradyrhizobium japonicum* is one of root nodule bacteria that can contribute on plant growth by providing fixed nitrogen in nodules of soybean grown in acid soil [1]. Some strains of *B. japonicum* were tolerant on an acid condition, even at the pH level 4.0-4.5 [2]. Twenty five strains of *B. japonicum* had been selected for acid tolerance using either solid and broth medium. The results showed that BJ 11 isolate has the highest tolerance on acid and had a good ability to grow on pH 4.5 media [1]. One of the indigenous isolate, BJ 11 (wt), has been shown to increase the growth and production of soybean grown in acidic soil (pH 5.0-5.5) [3].

Furthermore, Wahyudi *et al.* [4] constructed several mutants of *B. japonicum* using transposon Tn5 mutagenesis. One of the mutant of BJ 11, i.e. BJ 11(19), besides of the wild-type were able to form root nodules on soybean could increase plant height, shoot- and root- weight, number of flowers, pods, seeds, seeds dry weight, and shoot and seed nitrogen content [5]. In the Leonard bottle experiment, BJ 11 (19) significantly could increase dry weight of the upper crop and nitrogen uptake of soybean cultivar Slamet higher than standard strain from USA, USDA 110 [6].

Fuhrmann [7] described the diversity of *Bradyrhizobium* strains dividing them into two groups, *B. elkanii* and *B. japonicum*, according to IAA production. The aim of this study was to determine the potency of plant growth promoting *Bradyrhizobium japonicum* as producer of IAA and its application on three varieties of soybean, i.e. Anjasmoro, Tanggamus, and Detam. Anjasmoro and Tanggamus are including yellow soybean seeds (*Glycine max*), whereas Detam is a black soybean seed (*Glycine soja*). Tanggamus is one of leading variety which is adapted in dry acid soil.

II. Materials and Methods

A. Materials

Indigenous isolate of *B. japonicum*, i.e. BJ 11 (wt) and BJ 11 (19) one of BJ 11(wt) mutant, and reference isolate, USDA 110 were maintained at culture collection of Microbiology Laboratory and Institut Pertanian Bogor Culture Collection (IPBCC), Biology Department, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University (IPB). Three varieties of soybean seeds, i.e. Anjasmoro, Tanggamus (yellow soybean seeds) and Detam (black soybean seed) were obtained from Research Institute for Beans and Tubers, Malang- Indonesia.

B. Inoculants Preparation

Production of IAA by *B. japonicum* was assayed as described by Patten and Glick [8]. A number of 10^8 cells/ml *B. japonicum* of each isolates was grown on Yeast Mannitol Broth (YMB) that consist of mannitol 10 gL^{-1}, K_2HPO_4 0.5 gL^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 gL^{-1}, NaCl 0.2 gL^{-1}, yeast extract 0.5 gL^{-1}, rifampicin 50 μg ml^{-1} [4] supplemented with 0.5 mM L-tryptophan or without (control). The isolates were incubated for about 8 days at 125 rpm shaker and room temperature. Bacterial cells were removed by centrifugation at 8400 g for 10 minutes at 4^0C to obtain the crude extract of Indole acetic acid (IAA). One ml of the supernatant was mixed with 4 ml of Salkowski's reagent in the ratio of 1:4 and incubated at room temperature for 20 min. Development of a pink colour indicated indoles. The absorbance of supernatant mixture (supernatant + Salkowski's reagent) for IAA production was measured at 520 nm The quantity of indoles was determined

N.R. Mubarik is corresponding author and with the Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Jalan Agatis, IPB Dermaga, Bogor 16680, Indonesia (phone: +62-251-8622833; fax: +62-251-8622833; e-mail: nrachmania@ipb.ac.id).

I. Mahagiani is a graduate from Major of Microbiology, Postgraduate School of Bogor Agricultural University, Bogor 16680, Indonesia.

A.T. Wahyudi is with the Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Jalan Agatis, IPB Dermaga, Bogor 16680, Indonesia.

by comparison with a standard curve using an IAA standard graph.

C. Treatments with B. japonicum on Soybean Seedlings on Growth Pouch

Soybean seeds were selected based on size and healthiness (able to produce shoot). In a growth pouch study [9], soybean seeds were surface sterilized by using 95% ethanol for ten seconds, 5% H_2O_2 for five minutes and they were rinsed seven times using sterilized water. After 24 hours incubation, germinated seeds were selected based on 2-3 mm radicula length and put on growth pouch. Seed growth pouches sterilized at 121ºC for 15 to 20 min were filled with sterile N-free Alva (pH 4.5) solution, respectively [6]. Each 100 µl culture of *B. japonicum* (10^9 Cell/ml) was *inoculated* into germinated seeds and incubated for 7 days at room temperature and dark conditions. Seeds treated without culture served as controls. At 8 days after inoculation, *B. japonicum* inoculation, parameters of primary root length and number of lateral roots were measured as indicators of early growth promotion.

D. Greenhouse Experiments

Two days germinated seeds were sown on Leonard bottle [5].Three soybean seedlings were grown charcoal-sand media on Leonard bottle containing nitrogen-deficient sterile N-free Ahmed-Evans (pH 6.9) and Alva (pH 4.5) solution, respectively. The solution provided by capillary watering. Seedlings were maintained for 35 days after planting (DAP) based on vegetative phase growth of soybean. The following parameters were measured: (a) shoot- and root- dry weight, (b) primary root length, (c) number of nodules per plant and (d) concentration of IAA on nodules. Extraction of IAA from nodule was used by Unyayar *et al*. method [10].

E. Statistical Analysis

Experiments were performed in triplicate. Values shown represent mean _ standard error of mean (SEM). Data were analyzed for variance by ANOVA followed by Duncan test (α= 0.05). Analyses were performed using SPSS 16 programme for Windows.

III. Results and Discussion

A. Growth and IAA Production

USDA 110 showed less growth than BJ 11 (wt) and BJ 11 (19) on YMB medium supplemented with 0.5 mM L-tryptophan (Fig. 1). At 8 days incubation, the number of cell was achieved by BJ 11 (wt) and BJ 11 (19) viz 6.0 and 5.8, respectively. Whereas USDA 110 only achieved log 3.4. All isolates could not produce IAA on YMB medium without supplemented with 0.5 mM L-tryptophan (data not showed). At 8 days incubation, in the presence of 0.5 mM L-tryptophan, BJ 11 (19) produced significantly higher IAA than BJ 11 (wt) and USDA 110 (Fig. 2). BJ 11 (19) produced IAA maximum at day 4, while BJ 11 (wt) at day 2, and USDA 110 at day 7.

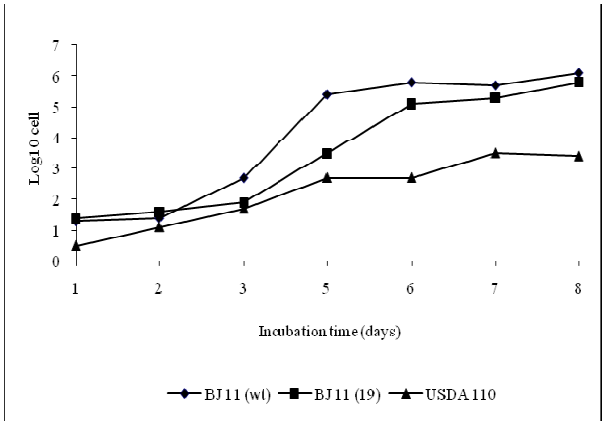

Fig. 1 The growth of acid-aluminium tolerant *Bradyrhizobium japonicum* strains on YMB medium supplemented with 0.5 mM L-tryptophan

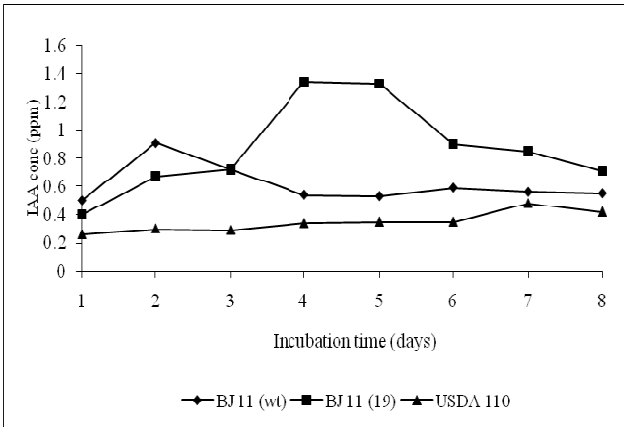

Fig. 2 IAA production of acid-aluminium tolerant *Bradyrhizobium japonicum* strains

B. Treatments with B. japonicum on Soybean Seedling in Growth Pouch

Treatment of soybean seeds inoculated with isolates BJ 11 (wt), BJ 11 (19) and USDA110 showed no significant effect to induce primary root elongation (Table I, II and III).

Treatments with isolates BJ 11 (wt), BJ 11 (19), and USDA110 on soybean seedlings showed significant effect to induce the formation of lateral roots better than control (without inoculation) on Detam soybean. The highest number of lateral root on each soybean variety was found on the seeds inoculated with USDA 110 on Anjasmoro soybean (Table I) and BJ 11(wt) on Tanggamus (Table II). Patten and Glick [8] reported that IAA-producing bacteria can stimulate the growth of the host root system. Lateral root growth of plants induced by a high concentration of IAA, while the main root was stimulated by low concentrations of IAA, between 10^{-9}-10^{-12} M.

TABLE I
PRIMARY ROOT ELONGATION AND NUMBER OF LATERAL ROOT OF ANJASMORO VARIETY SOYBEAN INOCULATED WITH *B. JAPONICUM* AT PH 4.5, 8DAYS AFTER INOCULATION IN GROWTH POUCH

Treatments	Primary root length (cm)	Number of lateral root
USDA 110	3.07±0.75a	17.00±1.15 c
BJ 11(wt)	3.17±0.29a	11.00±0.58 ab
BJ 11(19)	8.00±0.5ba	9.00±2.00ab
Control (without inoculation)	2.73±0.25a	8.00±3.78a

Numbers on the same column followed by the same letter were not significantly different based on Duncan Multiple Range units in parentheses. Do not label axes only with units. Test (α = 0.05). Data were the mean value of three replicates ± deviation standard.

TABLE II
PRIMARY ROOT ELONGATION AND NUMBER OF LATERAL ROOT OF TANGGAMUS VARIETY SOYBEAN INOCULATED WITH *B. JAPONICUM* AT PH 4.5, 8 DAYS AFTER INOCULATION IN GROWTH POUCH

Treatment	Primary root length (cm)	Number of lateral root
USDA 110	2.67±0.29a	9.00±2.31a
BJ 11(wt)	4.17±1.26a	16.00±4.04b
BJ 11(19)	3.33±1.76a	8.00±0.58a
Control (without inoculation)	2.17±1.15a	8.00±3.51a

Numbers on the same column followed by the same letter were not significantly different based on Duncan Multiple Range Test (α = 0.05). Data were the mean value of three replicates ± deviation standard.

TABLE III
PRIMARY ROOT ELONGATION AND NUMBER OF LATERAL ROOT OF DETAM VARIETY SOYBEAN INOCULATED WITH *B. JAPONICUM* AT PH 4.5, 8 DAYS AFTER INOCULATION IN GROWTH POUCH

Treatment	Primary root length (cm)	Number of lateral root
USDA 110	15.00±0.5b	10.00±1.53b
BJ 11(wt)	16.50±1.32b	13.00±1.53c
BJ 11(19)	16.83±2.75b	10.00±1.53b
Control (without inoculation)	14.67±1.16b	5.00±1.00a

Numbers on the same column followed by the same letter were not significantly different based on Duncan Multiple Range Test (α = 0.05). Data were the mean value of three replicates ± deviation standard.

C. Greenhouse Experiments

At 35 DAP, treatments on soybean inoculated with isolates BJ 11 (wt), BJ 11 (19) and USDA110 showed no significant effect on shoot dry weight, root dry weight, and primary root length (Table IVa, b and c). The number of nodules formed on the roots of plants treated with isolate BJ 11 (wt) was significantly different when compared with control plants of the three varieties of soybean (Fig. 3, Table IV a, b and c). Treatments with BJ 11 (wt) and BJ 11 (19) showed better effect compared to treatment with USDA 110 on Tanggamus and Detam soybean, respectively. While the control plants were inoculated only with YMB (control without inoculation) just not able to form root nodules. This suggests that IAA also played an important role in the formation of legume nodule [11].

The concentration of IAA on nodules was significantly different with control (Table IV), except on the treatment using Tanggamus soybean (Table IVb). This is presumably due to the complexity of the IAA influence on plant growth. The number of enzymes produced by microbes plays a role in activating of the IAA production, while other enzymes can inhibit its production [12].

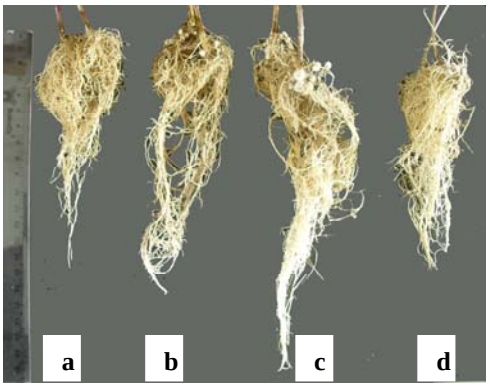

Fig. 3 Treatment of *B. japonicum* inoculation on the growth of Anjasmoro soybean roots at 35 DAP on Leonard bottle under greenhouse condition. (a) Control (without inoculation), (b) USDA 110, (c) BJ 11 (wt), and (d) BJ 11 (19)

TABLE IV
B. JAPONICUM EFFECTS ON GROWTH OF (A) ANJASMORO, (B) TANGGAMUS, AND (C) DETAM SOYBEAN SEEDLINGS GROWN FOR 35 DAP UNDER GREENHOUSE CONDITIONS

a. Anjasmoro

Parameter	BJ 11(wt)	BJ 11(19)	USDA110	Control (without inocula-tion)
Shoot dry weight (g)	2,31±0,31 a	2,24±1,12 a	2,61±0,72 a	1,93±0,88 a
Root dry weight (g)	0,47±0,01 ab	0,40±0,07 a	0,47±0,09 ab	0,59±0,04 b
Root length (cm)	19,75±2,47 a	17,25±0,35 a	21,00±0,00 a	20,00±5,66a
Nodule number (plant $^{-1}$)	33,50±0,71 b	29,00±5,66 b	34,50±12,02 b	0,00±0,00 a
Concentra-tion of IAA on nodules (ppm)	10,81±3,39 b	9,77±1,91 b	7,69±0,00 ab	0,00±0,00 a

Numbers on the same column followed by the same letter were not significantly different based on Duncan Multiple Range Test (α = 0.05). Data were the mean value of three replicates ± deviation standard.

b. Tanggamus

Parameter	BJ 11(wt)	BJ 11(19)	USDA110	Control (without inocula-tion)
Shoot dry weight (g)	1,60±0,69a	1,65±0,36a	1,65±0,08a	1,63±0,01 a
Root dry weight (g)	4,00±0,00a	3,50±0,71a	4,00±0,00a	4,50±0,00 a
Root length (cm)	17,75±1,77a	16,65±0,49a	18,50±4,95a	34,75±2,1 5a
Nodule number (plant $^{-1}$)	21,00±0,00b	24,00±6,00a b	14,00±5,66 ab	0,00±0,00 a
Concentra-tion of IAA on nodules (ppm)	4,58±6,48a	0,00±0,00a	7,04±9,96ab	0,00±0,00 a

Numbers on the same column followed by the same letter were not significantly different based on Duncan Multiple Range Test (α = 0.05). Data were the mean value of three replicates ± deviation standard.

c. Detam

Parameter	BJ 11(wt)	BJ 11(19)	USDA110	Control (without inocula-tion)
Shoot dry weight (g)	2,13±0,58a	1,95±0,07a	2,07±0,02a	2,01±0,21 a
Root dry weight (g)	0,55±0,00a	0,52±0,68a	0,46±0,00a	0,56±0,17 a
Root length (cm)	21,00±2,83a	21,75±1,77a	22,50±2,83a	18,75±3,1 8a
Nodule number (plant $^{-1}$)	25,50±3,53 bc	34,00±8,48 c	13,00±8,48 ab	0,00±0,00 a
Concentra-tion of IAA on nodules (ppm)	12,15±0,89 b	28,68±0,00c	17,37±0,00 b	0,00±0,00 a

Numbers on the same column followed by the same letter were not significantly different based on Duncan Multiple Range Test (α = 0.05). Data were the mean value of three replicates ± deviation standard.

Treatment plants inoculated with *Bradyrhizobium* strains showed trend the same influences on the shoot- and root- dry weight in all three soybean varieties compared to control (Table IV a, b and c). There were no significant effects. Husen *et al.* [12] reported that the roots are inhibited by the high concentration of IAA which can activate the 1-aminocyclopropane-1-carboxylate (ACC) aminase to synthesize ACC which is a precursor of the hormone ethylene. If isolate produces ACC deaminase, such as produced by *Pseudomonas*, this enzyme will able to stimulate the growth of soybean by pressing the ethylene biosynthesis.

IV. Conclusion

Bradyrhizobium japonicum isolates tested, i.e. BJ 11 (wt), BJ 11 (19), and USDA 110 could grow well and produce indole acetic acid (IAA) on yeast mannitol broth (YMB) medium in the presence of 0.5 mM L-tryptophan. Indole acetic acid produced by *Bradyrhizobium japonicum* have showed effect on stimulating the formation of root nodules in soybean varieties, i.e. varieties Anjasmoro, Tanggamus, and Detam grown on Leonard bottle.

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