

The Use of Acid-Aluminium Tolerant *Bradyrhizobium japonicum* Inoculant for Soybean Grown on Acid Soils

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Land with low pH soil spread widely in Indonesia can be used for soybean (*Glycine max*) cultivation, although the production is low. The use of acid tolerant soybean and acid-Al tolerant nitrogen-fixing bacteria was an alternative way to increase soybean productivity on acid soils. This research was conducted to study the influence of acid-Al tolerant *Bradyrhizobium japonicum* on growth of Slamet cultivar soybean planted on acid soils in greenhouse. Three strains of acid-Al tolerant *B. japonicum*, i.e. BJ 11 (19), BJ 11 (5), and BJ 11 (wt), were used in this experiment. The result showed that inoculation of all acid-Al tolerant *B. japonicum* strains could increase plant height, shoot and root weight, number of flowers, pods, seeds, seeds dry weight, and shoot and seed nitrogen content.

Key words: *Bradyrhizobium japonicum*, acid-aluminium tolerant, soybean, Slamet cultivar

INTRODUCTION

In 2007, Indonesian demand on soybean (*Glycine max*) was 1.8 million tons. However, domestic production was 608.263 kg (<http://www.bps.go.id> [30 August 2008]). Extensification of the agricultural program on low pH land is an effort to increase the agricultural production especially soybean.

Legume such as soybean could increase soil fertility and plant productivity. Inoculation of root nodule bacteria, such as *Bradyrhizobium japonicum*, on soybean plantation could enhance soybean quality and productivity (Somasegaran & Hoben 1994; Abbasi *et al.* 2008). A wide variation of *B. japonicum* tolerance to acid soil conditions have been reported on many agriculturally important legumes from various countries (Ayanaba *et al.* 1983; Indrasumunar *et al.* 2000). Bacteria can increase plant productivity because its ability to fix nitrogen and provide ammonium for plant. Atmospheric nitrogen (N_2) was fixed by the bacteria into ammonium (NH_3) in a nodule, so that the fixed nitrogen can be used by the plant for its growth (Atlas & Bartha 1998).

Bradyrhizobium japonicum is one of root nodule bacteria that can contribute on plant growth by providing fixed nitrogen in nodules of soybean plants (Somasegaran & Hoben 1994). Some strains of *B. japonicum* were tolerant on acids condition, even at the pH level of 4.0-4.5 (Denarie *et al.* 1992). Twenty five strains of *B. japonicum* had been selected for their acid tolerance using either solid and broth medium (Endarini *et al.* 1995). The results showed that BJ 11 isolate had the highest tolerance on acid and had a good ability to grow on pH 4.5 media.

Wahyudi *et al.* (1998) constructed several mutants of *B. japonicum* using transposon Tn5 mutagenesis. Two mutants, i.e. BJ 11 (5) and BJ 11 (19) strains, were able to form root nodules on siratro and soybean. Dry weight of the nodules

formed by the mutant was higher than that of the wild type (BJ 11) (Monasari 2007). This research was conducted to study the influence of acid-Al tolerant *B. japonicum* on growth of Slamet cultivar soybean planted on acid soils in a green house. Slamet cultivar is a hybrid of Wilis and Dempo cultivars. The advantages of the hybrid were high protein content on the seed (34%), could grow better on acid soil, and also resistant to rust disease. The growth and productivity of Slamet cultivar grown on acid soils was higher than Sumbing, Singgalang, Tidar, Wilis, and Kipas Putih cultivars (Harun & Ammar 2001).

MATERIALS AND METHODS

Materials. Acid tolerant isolates *B. japonicum* (BJ 11 (19), BJ 11 (5), and BJ 11 (wt)) used for this study were bacterial collections of Microbiology Laboratory of Biology Department, Faculty of Science and Mathematics, Bogor Agricultural University. Soybean seeds were obtained from Research Institute for Food Crops and Genetic Resources, Bogor. Acid soils were taken from Jasinga, Bogor with pH 4.7. Peats were obtained from Indonesian Biotechnology Research Institute for Estate Crops. Physical and chemical analysis of soils and peats was done in Indonesian soil Research Institute, Bogor.

Experimental Design. All data collected in greenhouse were analyzed using SPSS 13.0 software and *Duncan Multiple Range Test* (DMRT). The experiment was arranged into five treatments using inoculant of *B. japonicum* and nitrogen addition. Negative control did not use inoculant and nitrogen addition. Each treatments were made in six replicates.

Medium and Inoculants Preparation. *Bradyrhizobium japonicum* isolates were grown on *Yeast Mannitol Agar* (YMA) for 7-8 days at room temperature (25-27 °C). The YMA media consist of mannitol (10 g/l), K_2HPO_4 (0.5 g/l), $MgSO_4 \cdot 7H_2O$ (0.2 g/l), NaCl (0.2 g/l), yeast extract (0.5 g/l), added with congo red (0.0025%) and rifampicin (50 µg/ml).

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The isolates were resistant to rifampicin (Wahyudi *et al.* 1998; Monasari 2007). Then they were subcultured into *Yeast Mannitol Broth* (YMB) and incubated for five days on a shaker at 125 rpm.

Preparation of Plant Cultivation Media. Medium for greenhouse experiment was mixed composition of 1,200 g soil and 800 g peat in a polybag. Acid soils were firstly prepared by drying and filtered using with 2 mm pore diameter. The media were sterilized using autoclave at 121 °C and 15 psi for one hour. Positive control media were added with 0.05% KNO₃. The media were inoculated with 20% (v/w) bacterial culture (10⁸ cells/ml, OD_{620nm} = 0.7).

Cultivation of Plant. Soybean seed were sown five seeds per polybag. Soybean seeds were selected based on size and healthiness (able to shoot). Seed surface were sterilized using 2% NaOCl for two minutes and they were rinsed five times using sterile water (Habibah 2008). Fourteen days after planting (DAP), the shoots were plucked and left two seeds sown up to 30 DAP. Watering was carried every day. The plants were harvested two times, i.e. at the 50 DAP for crop nodules and 75-108 DAP for crop pods (Habibah 2008).

Plant Response Observation. Plant height were measured every 10 days from 20 DAP up to 70 DAP. Number of flowers, effective root nodule percentage, shoot and root weight, percentage of nitrogen on shoot, and nitrogenase activity on root nodule were measured at 50 DAP. Number of pods and seeds, seed dry weight per 100 seeds, and nitrogen content on seeds were measured at 75 and 108 DAP, respectively. Nitrogenase activity was measured by acetylene reduction assay (ARA) methods according to Anas and Muluk (2003) at Soil Biotechnology Laboratory, Faculty of Agriculture, Bogor Agricultural university, Bogor, Indonesia. Nitrogen content on shoot and seeds were measured using Kjeldahl method (Sulaeman *et al.* 2005).

Isolate Viability Test. Determination of viable cells of tested isolate was done using plate count method (Habibah 2008). One gram soil of each polybag was diluted into a tube filled with 9 ml NaCl 0.85%. Appropriate dilution were plated on YMA media and incubated at room temperature for 5 days. Viability of bacterial cell on soil was counted at 15, 30, 50, and 70 DAP.

RESULTS

Bacterial Growth. Isolates were able to grow on YMA added with 0.0025% congo red and 50 µg/ml rifampicin incubated on room temperature for 7 days. Morphology of

B. japonicum colonies was mucoid, not quite able to adsorp congo red, and curve elevation (Figure 1).

Plant Growth Response. Plant height data showed that inoculation of *B. japonicum* was able to increase plant height (p<0.05). The plants inoculated with isolate BJ 11 (19) were significantly different on plant height from control at 20 DAP. There were three inoculated treatments were significantly different from the control on the plant height at 60 and 70 DAP (Table 1).

Inoculation BJ 11 (19) and BJ 11 (5) isolates showed better response on shoot weight than other treatments. The highest root weight were found on plants inoculated by BJ 11 (5) isolate (Figure 2). The highest numbers of flowers, effective root nodule percentage, and percentage of nitrogen on shoot were found on the plants inoculated with BJ 11 (19) and BJ



Figure 1. Colony of *B. japonicum* BJ 11 (19) after 7 days of incubation on YMA + 0.0025% congo red + 50 µg/ml rifampicin.

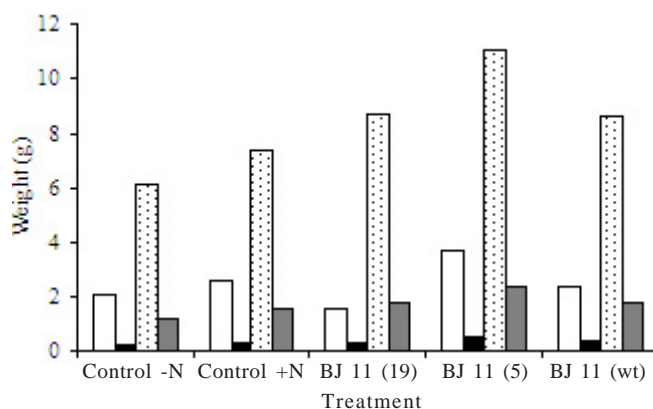


Figure 2. Effects of *B. japonicum* inoculation on wet and dry weight of root and shoot of plants at 50 days after planting. □: wet root weight, ■: dry root weight, ▨: wet shoot weight, ▩: dry shoot weight.

Table 1. Effects of *B. japonicum* inoculation on plant height at 20-70 days after planting (DAP)

Treatment	Height (cm)					
	20 DAP	30 DAP	40 DAP	50 DAP	60 DAP	70 DAP
Control (-N)	16.6 ± 2.19b	31.4 ± 4.42ab	57.8 ± 4.07b	81.5 ± 5.39b	93.3 ± 4.62c	105.1 ± 2.09c
Control (+N)	17.2 ± 2.44b	32.3 ± 3.79ab	59.5 ± 6.43b	96.5 ± 12.8a	104.7 ± 8.96b	115.2 ± 4.60b
BJ 11 (19)	20.5 ± 1.45a	34.5 ± 2.16a	65.1 ± 9.30ab	105.2 ± 3.77a	114.9 ± 2.22a	126.4 ± 1.44a
BJ 11 (5)	18.8 ± 2.39ab	34.9 ± 2.38a	69.5 ± 5.35a	104.3 ± 2.88a	113.9 ± 1.95a	124.8 ± 2.42a
BJ 11 (wt)	18.3 ± 0.92ab	29.5 ± 1.62b	63.6 ± 4.93ab	98.4 ± 5.07a	112.3 ± 3.03a	126.0 ± 1.62a

Numbers on the same column followed by the same letter were not significantly different based on Duncan Multiple Range Test ($\alpha = 0.05$). Data were the mean value ± deviation standard (n = 6).

Table 2. Effects of *B. japonicum* inoculation on number of flowers and root nodules, nitrogenase activity, percentages of effective root nodule and nitrogen content in shoot of plant at 50 days after planting

Treatment	Number of flowers	Number of nodules	Effective nodules (%)	Nitrogenase activity ($\mu\text{mol}/\text{hour}/\text{mg}$ root)	Nitrogen contain in shoot (%)
Control (-N)	11	-	-	-	2.21
Control (+N)	15	3	-	-	2.93
BJ 11 (19)	19	16	31.30	0.730	4.25
BJ 11 (5)	18	23	17.39	0.357	3.45
BJ 11 (wt)	15	13	15.38	0.294	3.45

Table 3. Effects of *B. japonicum* inoculation on numbers of pods and seeds, weight of 100 seeds, and percentages of nitrogen contain in seeds

Treatment	Number of pods	Number of seeds	Weight 100 seeds	Nitrogen content in seed (%)
Control (-N)	22.4 \pm 1.52d	43.6 \pm 3.65c	8.9 \pm 0.65c	6.704 \pm 0.15b
Control (+N)	26.8 \pm 1.30c	50.4 \pm 4.83c	10.1 \pm 0.37b	6.882 \pm 0.39ab
BJ 11 (19)	35.4 \pm 3.13a	70.6 \pm 6.50a	13.5 \pm 0.97a	7.132 \pm 0.08a
BJ 11 (5)	31.8 \pm 1.64b	63.0 \pm 3.16ab	13.3 \pm 0.39a	7.104 \pm 0.07a
BJ 11 (wt)	30.2 \pm 1.64bc	59.0 \pm 2.35b	12.9 \pm 0.59a	7.164 \pm 0.02a

Number on the same column followed by the same letter were not significantly different based on Duncan Multiple Range Test ($\alpha = 0.01$). Datas were the mean value \pm deviation standard (n = 5).

11 (5) isolates (Table 2). Plants inoculated with BJ 11 (19) and BJ 11 (5) isolates also had the highest nitrogenase activity. Number of nodules were found higher on plants inoculated with BJ 11 (5) than that of BJ 11 (19) and BJ 11 (wt) isolates. No nodules were found at control. However a few number of uneffective nodules were found at control supplemented with nitrogen fertilizer.

Inoculation of *B. japonicum* showed higher response on weight of 100 seeds, percentages of nitrogen on seeds, and numbers of pods (Table 3). Inoculation of BJ 11 (19) isolate could increase number of seeds and pods that was higher than other treatments.

Isolate Viability. Cells density in the soil of soybean inoculated by BJ 11 (19) on 15 DAP was 3.1×10^6 cells per gram of soil and it was increased up to 5.4×10^6 cell on 30 DAP (Table 4). Cell density of BJ 11 (wt) and BJ 11 (5) isolates was lower than a million cell per gram of soil at 15 DAP. Three isolates [BJ 11 (wt), BJ 11 (5), and BJ 11 (19)], showed increasing of cell quantity on 30 DAP (Table 4) and it was decreased at 50 DAP. At the final observation (70 DAP), the quantity of viable cell was lower than 3.0×10^4 cell per gram of soil.

DISCUSSION

Bradyrhizobium japonicum was slowly growing bacterium. Its colony on solid medium was seen after 7 days of incubation. *Bradyrhizobium japonicum* is difference to other bacterial colony due to their ability to unabsorb congo red (Somasegaran & Hoben 1994). Exopolysaccharides was secreted in a large amount by *B. japonicum* (Parveen *et al.* 1997), and the colony was appeared sticky and mucoid colony on yeast mannitol agar. The exopolysaccharide was resistance to acid condition (Lounch & Miller 2001).

Inoculation of root nodule bacteria could increase plant height. There was an association between soybean and root nodule bacteria which played an important role in increasing the plant growth. Effects of *B. japonicum* inoculation were also seen on root and shoot weight. The highest root and

Table 4. Isolates viability in soil samples

Treatment	Cell density ($\times 10^6$ CFU $\cdot\text{g}^{-1}$ soil)			
	15 DAP	30 DAP	50 DAP	70 DAP
Control (-N)	-	-	-	-
Control (+ N)	-	-	0.3	-
BJ 11 (19)	3.1	5.4	2.1	*
BJ 11 (5)	*	4.5	2.9	*
BJ 11 wt	*	3.4	2.7	*

-: no colony of *B. japonicum*, *: less than 30 colony forming unit (CFU).

shoot weight were found on plants inoculated with BJ 11 (5) isolate. This was caused by the ability of the plants to form root branches and longer in order to reach minerals or nutrition. Wide root system help plants to grow on low or limited inorganic nitrogen (Srivastava & Singh 1999).

The bacteria supplied fixed nitrogen to the plants and then support growth and development of plants. The highest number of flower was found on the plants inoculated with BJ 11 (19) isolate. This result was supported by the percentages of effective root nodule on the plants inoculated with isolate BJ 11 (19).

Plants inoculated by BJ 11 (19) had the highest shoot nitrogen percentages. Root nodule bacteria were able to fixed nitrogen so that the nitrogen on inoculated plants were higher than the plants with addition of inorganic nitrogen (Elfiati 2006). Different activity of *nif* gene expression on root nodule bacteria could be indicated by different activity of nitrogen fixation rate (Harun & Ammar 2001).

Numbers of pods and seeds, weight of 100 seeds, and seeds nitrogen percentages were also affected by inoculation of *B. japonicum*. According to Saraswati (1999), inoculation of *Rhizobium* effectively influenced the formation and development of pods. Higher number of pods also had higher numbers of seeds (Harun & Ammar 2001).

Three isolates used in this experiment showed the same ability to increase weight of 100 seeds. The plants inoculated with BJ 11 (19) isolate had a relatively higher of weight of 100 seeds than that of other treatments. Slamet cultivar commonly had 12.5 g/100 seeds (Sunarto 1995), and in low pH condition

at Jasinga soid on plants without *B. japonicum* inoculation was 9.64 g/100 seeds (Widodo 2008). In this experiment, Slamet cultivar inoculated with *B. japonicum* on acid soils had seeds weight of 13.5 g/100 seeds. *Bradyrhizobium japonicum* isolates could increase the percentage of nitrogen contain in seeds, similar result was also reported by Bertham (2006).

Effectivity of root nodule bacteria on N_2 fixation were affected by the compatibility between bacteria and the soybean cultivar. A single *Rhizobium* strain is generally able to infect certain species of legumes (Madigan et al. 2003). Previous study (Habibah 2008) showed that the growth of soybean Slamet cultivar inoculated by isolate BJ 11 (19) was better than that of other treatments.

In conclusions, inoculation of acid-Aluminium tolerant *Bradyrhizobium japonicum* BJ 11 (19), BJ 11 (5) and BJ 11 (wt) strains were able to promote the growth and productivity of Slamet cultivar grown on acid soil.

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