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Selection and Bioassay of *Azotobacter* sp. Isolates to Improve Growth of Chili (*Capsicum annum* L.) on Entisols in Ambon

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Leafy vegetables contributes to the inflation rate in Ambon City due to low productivity in rainy season. Some vegetables are imported from other islands while importantvegetables such as local petsai (*Brassica chinensis* L.) and chili (*Capsicum annum* L.) are cultivated in low nitrogen soil, Entisols. Lack of nitrogen could be overcome by using inorganic fertilizeras well as biofertilzer. The soil can be inoculated with rhizobacteria, such as *Azotobacter*, to increase the nitrogen uptake and improve the quality of vegetables. This research was conducted to isolate and select *Azotobacter* from rhizosphere of vegetables and to examine the effect of *Azotobacter* inoculation on chili-seedling growth and nitrogen uptake by using bioassay method. *Azotobacter* sp. was isolated in nitrogen-free Ashby's Media. The bioassay was held in the green house with randomized block design experiment, which examined the combination of isolates and population of *Azotobacter* sp. on chili. Two best isolates which was selected based on pH, nitrogen content and cell viability were s2a10 (from petsai's rhizosphere) and c2a9 (from chili's rhizosphere). Bioassay showed that *Azotobacter* inoculation followed by reduced NPK fertilizer doses had no effect on transplant dry weight and nitrogen uptake. All *Azotobacter* inoculation except 10^8 CFU mL^{-1} s2a10 maintain soil nitrogen although *Azotobacter* population in soil was slightly reduced. This showed that *Azotobacter* sp. potentially reduce the use of inorganic biofertilizer.

Keywords: *Azotobacter*, chili, Entisols, nitrogen fixation

Komoditas sayuran di Kota Ambon adalah salah satu penyumbang inflasi. Berbagai jenis sayuran masih diimpor dari luar pulau padahal Kota Ambon memiliki sawi (*Brassica chinensis* L.) dan cabai (*Capsicum annuum* L.) lokal yang telah dibudidayakan di tanah Entisols dan kekurangan nitrogen. Untuk memperkuat produksi benih kedua sayuran lokal tersebut, selain pupuk anorganik, inokulasi rizobakteri *Azotobacter* dapat meningkatkan serapan unsur hara nitrogen dan meningkatkan kualitas tanaman. Penelitian ini dilakukan untuk mengisolasi dan menseleksi isolat *Azotobacter* dari rizosfer kedua komoditas dan menguji kapasitasnya untuk meningkatkan pertumbuhan dan serapan N bibit tanaman cabai (*Capsicum annuum* L.). Bakteri *Azotobacter* sp. diisolasi dengan metode pengayaan dan dilanjutkan dengan metode gores pada media selektif Ashby bebas N. Uji hayati di rumah kaca dirancang dalam rancangan acak kelompok yang menguji kombinasi isolat dan kepadatan inokulan cair *Azotobacter* sp. Berdasarkan N tersedia, kepadatan sel dan pH kultur, maka dua isolat terbaik adalah s2a10 dari rizosfer sawi dan c2a9 dari rizosfer cabai. Uji hayati memperlihatkan bahwa inokulasi *Azotobacter* disertai penuruan dosis pupuk NPK tidak mempengaruhi berat kering dan serapan nitrogen cabai. Seluruh perlakukan inokulasi *Azotobacter* kecuali 10^8 CFU mL^{-1} s2a10 menjaga kadar nitrogen tanah meskipun populasi *Azotobacter*di tanah menurun. Percobaan ini memberikan gambaran bahwa *Azotobacter* sp. berpotensi menurunkan penggunaan pupuk anorganik.

Kata kunci: *Azotobacter*, cabai, Entisol, fiksasi nitrogen

Indonesia has 76,022,000 ha potential dry land from overall 148,000,000 ha of cultivation land. Entisols is a soil order in which vegetable production has been carried out. In Indonesia, Entisols constitute up to 14,540,000 ha (Balai Penelitian Tanah 2006). Low amount of nutrient in Entisols may lead to the limited crop production. Inorganic fertilizer has been used for years to supply the nutrient to the crops to improve the yield. However, excess amount of inorganic fertilizer will reduce the soil fertility and it is hazardous to the environment (Gruhn *et al*. 2000). In sustainable agricultural system, part of the inorganic fertilizer can be replaced by plant growth promoting rhizobcteria (PGPR). Instead of using merely inorganic fertilizer, a combination of both fertilizer and PGPR should be used.

One of the well known PGPR is aerobic non symbiotic nitrogen fixer *Azotobacter* sp., which also produces phytohormones (Tripathi *et al.*2015). *Azotobacter* sp. release the phytohormones such as Indole Acetic Acid (IAA) to induce cell development (Kumar *et al*. 2014). Nowadays, the ability of

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Azotobacter to produce exopolysaccharide (EPS) has been well documented. The EPS is extracellular structure that protects nitrogenases in the nitrogen fixation process (Sabra *et al*. 2000). *Azotobacter* has been found in the rhizosphere of various plants.Tarigan *et al*. (2013) has isolated the *Azotobacter* from soybean (*Glycine max* L.) rizosphere. Widiastuti *et al*. (2010) has also isolated *Azotobacter* from the rhizosphere of palm(*Elaeis guineensis*), coffee (*Coffea arabica*), rubber tree (*Hevea brasiliensis*), cashew tree (*Anacardium occidentale*), and corn (*Zea mays*).

In attempt to develop local *Azotobacter* biofertilizer to support leafy vegetable production in Ambon city, indigenous isolates were needed. Indigenous *Azotobacter* biofertilizer is expected to increase vegetable yield as well as its quality. This study has been carried out to isolate and select *Azotobacter* from the rhizosphere of chili (*Capsicum annuum*) and petsai (*Brassica chinensis)* and to examine the effect of *Azotobacter* inoculation on chili-seedling growth and nitrogen uptake by using bioassay method.

MATERIALS AND METHODS

This experiment was conducted from September 2015 to March 2016 at the Faculty of Agriculture, Universitas Padjadjaran. *Azotobacter* was isolated from petsai's rhizosphere grown in Entisols in Desa Waiheru, Kecamatan Teluk Baguala, Ambon and chili rhizosphere grown in the same soil order in Desa Hative Besar, Kecamatan Teluk Ambon, Ambon. Bioassay was performed in Entisols from Negeri Hative Besar, Kecamatan Teluk Ambon, Kota Ambon (pH 5.8; 1.67% organic carbon, 0.12% total nitrogen, 9.08 mg 100 g^{-1} available P, 7.86 ppm total P, 0.44 cmol kg^{-1}available K, and 35.01 mg 100 g^{-1} total K) to grow chili Unpad-CK5.

***Azotobacter* Isolation.** Isolation of *Azotobater* has been carried out using nitrogen free Ashby's media (KH_2PO_4 0.2 g; $MgSO_4.7H_2O$ 0.2 g; Mannitol 10 g; NaCl 0.2 g; $CaSO_4.2H_2O$ 0.1 g; $CaCO_3$ 5 g per liter distilled water). One g of rhizosphere were poured into 50 mL autoclaved nitrogen free Ashby's broth and incubated for five days at 30 °C until the pellicle was appeared. One loop of pellicle was streaked on Ashby's agar plate and incubated for 2 d at 30 °C. Single colony were transferred to slantsof the same medium. Isolates were subcultured several times to finally get five pure cultures, which were maintained in slant agar at room temperature. The *Azotobacter* isolates were characterized based on the colony morphology, Gram and capsule staining.

Isolates Selection. All isolates were cultured in 100 mL Ashb's broth in 200 mL glass bottle placedon 115 rpm gyratory at room temperature (25-27 °C) for three days. At the end of the incubation, Ashby's broth acidity was determined and the available nitrogen was analyzed by Kjeldhal method after 9 000 rpm centrifugation at 4 °C. The number of cells was counted by using haemocytometer under light microscope. Two best isolates were selected based on rank.

Bioassay on Chili Seedling. The final stage of the experiment was plant inoculation with different *Azotobacter* isolates and determination of the best inoculum cell density using chili (*C. annuum* L.) as the indicator plant. This bioassay was set up in randomized block design consisting of five treatment combinations:

Control (without *Azotobacter* sp. inoculation) + 100% of NPK fertilizer;
Azotobacter sp.s2a10 inoculation (10^6 CFU mL^{-1}) + 75% of NPK fertilizer;
Azotobacter sp.s2a10 inoculation (10^8 CFU mL^{-1}) + 75% of NPK fertilizer;
Azotobacter sp.c2a9 inoculation (10^6 CFU mL^{-1}) + 75% of NPK fertilizer;
Azotobacter sp.c2a9 inoculation (10^8 CFU mL^{-1}) + 75% of NPK fertilizer.

Each treatment was performed five times. Before experiment, *Azotobacter* population in bulk soil was counted by using dilution plate method in free-N Ashby medium. The initial population of indigenous *Azotobacter* has been used as a reference to determine the effect of treatments on *Azotobacter* population in the rhizosphere of chili.

Chili seeds were soaked in *Azotobacter* liquid inoculum and 1% gum Arabic (m:v) for 30 sec. Single seed was planted in growth media containing100 g of Entisols and 100 g cow manure in black polybag; and maintained in green house for 21 d. The dose of NPK fertilizer for early growth of chili was 200 kg ha^{-1}, which is applied 7 d after planting. Dry weight of 21 d old transplants, units of *Azotobacter* in Ashby's plate agar, and the amount of available nitrogen in soil were analyzed using analysis of variance (F test) and Duncan multiple range test, to determine if F test were significant. The count of *Azotobacter* population and the amount of available nitrogen in untreated soil were also analyzed and later used as a reference to determine the effects of *Azotobacter* and NPK treatments to the soil

RESULT

Indigenous *Azotobacter* Isolates. Based on morphological observation and Gram staining, five isolates were identified as *Azotobacter* sp. *Azotobacter* had been cultivated in free nitrogen Ashby's broth (pH 7) for three days and at the end of incubation, we found that available N ($NH_4^+$and No_3^-), cell density and broth acidity differed from one isolate to another (Table 1). The observation results, however, showed that there was no great acidity difference between these liquid cultures. The acidity ranged between 7.22 and 7.79. The populations of *Azotobacter* also varied between 5×10^6 CFU mL^{-1} and 43×10^7 CFU mL^{-1}. The highest number of cells by direct counting using haemocytometer was shown by s2a10 inoculum. Regardless of the isolates, NH_4^+content was always lower than NO_3^- content, indicating that nitrification had taken place.

Bioassay on Chili Plant. Statistical analysis showed that the biomass, indicated by dry weight, of control plants without *Azotobacter* were not significantly different from that of the other plants which received the treatment (Table 2). However, inoculation of *Azotobacter* with reduced dosage of NPK significantly affected either the population of the *Azotobacter* in the rhizosphere or the soil nitrogen content.

The population of *Azotobacter* in bulk soil before experiment was 1.83×10^5 CFU mL^{-1}. The population was increased after inoculation of *Azotobacter*. Rhizosphere containing the highest *Azotobacter* population (1.6×10^6 CFU mL^{-1}) were found in control plant. Plant inoculated with 10^8 CFU mL^{-1} c2a9 in combination with 75% of NPK gave similar *Azotobacter* population count (1.51×10^6 CFU mL^{-1}) with control.Total nitrogen in Entisols untreated soil was 0.12%, which is categorized as low according to the Indonesian Soil Research Institute (2009). All treatments enhanced the total nitrogen in soil up to 0.48%. The amount of total available nitrogen in control soil with NPK 100% was equal with that of soil inoculated with 10^6 CFU mL^{-1} *Azotobacter* sp. s2a10 + 75% NPK as well as 106 CFU mL and 10^8 CFU mL^{-1} *Azotobacter* sp. c2a9 + NPK 75%. The lowest total nitrogen was shown by plant treated with 10^8 CFU mL^{-1} *Azotobacter* sp. s2a10 + NPK 75%.

Due to limited transplant biomass, we combined all five replicates prior to analyzing N uptake (Table 3). Without analytical statistics, the results showed that N uptake of control plant was the lowest. Seedling inoculation followed by lower level of inorganic fertilization enhanced N uptake.

Table1. Available nitrogen, *Azotobacter* cell density and acidity of liquid culture after 3 d incubation

Isolates	NH_4^+ (ppm)	NO_3^- (ppm)	Cell density (10^7 CFU mL^{-1})	Acidity
c2a8	15.6	364.5	0.5	7.71
c2a9	20.0	508.7	12.1	7.47
s 1a	26.2	423.9	22.1	7.22
s 2a10	22.4	288.2	43.0	7.79
s2a9	10.2	457.8	15.0	7.72

Isolates were selected based on four aspects, i.e.NO_3^- and NH_4^+ concentrations, cell density, and acidity. Considering these parameters (Table 1), two isolates, s2a10 from petsai rhizosphere and c2a9 from chili rhizosphere, were selected.

Tabel 2 Effect of *Azotobacter* and NPK fertilizer on plant biomass, *Azotobacter* population, and soil nitrogen of chili transplant

Combination	Dry weight (g)	Population of *Azotobacter* (10^6 CFU $gram^{-1}$)	Soil total nitrogen (%)
Control (without *Azotobacter*) + NPK 100%	0.02	1,60 c	0.44 b
Azotobacter sp. s2a10, 10^6 CFU mL^{-1}+ NPK 75%	0.04	0,98 b	0.39 b
Azotobacter sp. s2a10, 10^8 CFU mL^{-1}+ NPK 75%	0.03	1,10 b	0.31 a
Azotobacter sp. c2a9, 10^6 CFU mL^{-1} + NPK 75%	0.02	0,33 a	0.48 b
Azotobacter sp. c2a9, 10^8 CFU mL^{-1} + NPK 75%	0.02	1,51 c	0.42 b

Note: The same letter (a, b or c) in a column indicates that the correponding values were not significantly differentaccording to the Duncan multiple range test (p>5%).

Tabel 3. Nitrogen uptake by chili at 21 d after transplanting

Combination	N uptake (mg/plant)*
Control (without *Azotobacter*) + NPK 100%	1,02
Azotobacter sp. s2a10, 10^6 CFU mL^{-1} + NPK 75%	1,44
Azotobacter sp. s2a10, 10^8 CFU mL^{-1} + NPK 75%	1,52
Azotobacter sp. c2a9, 10^6 CFU mL^{-1} + NPK 75%	1,62
Azotobacter sp. c2a9, 10^8 CFU mL^{-1} + NPK 75%	2,56

*Data from two nitrogen analysis (duplo).

DISCUSSION

Azotobacter obtained from the rhizosphere of vegetables could be formulated as biofertilizer to enhance the quality of vegetable production while reducing the use of NPK fertilizer. The first step of this experiment was carried out to screen the isolates based on pH, the population density of the isolates, and the available nitrogen produced, then, the selected bacteria were used in bioassay. The acidity of inoculum is one of the quality parameters in standardized biofertilizer, since each microbe lives and proliferates within specific pH level. According to the Government Regulation no 70, the Indonesian Ministry of Agriculture (Peraturan Menteri Pertanian no. 70/2011) in Indonesia the value ranges between 5.0-8.0. The pH of the liquid culture of all isolates after three days fermentation were slightly around the range of 6.25-7.44, which was optimum for the growth of *Azotobacter* species (Becking 2006).

Azotobacter sp. is empirically known for its ability to fix nitrogen. Slight increase of pH inhibits the *Azotobacter* proliferation in the inoculum, since alkaline condition clearly reduces nitrogen fixation. Acidic growth environment influences the *Azotobacter* population density, and, generally, *Azotobacter* is uncommonly found in the lower pH. Previous research showed that all soils within pH range 7.07-8.56 contained *Azotobacter* (Mazinani *et al*. 2012).Based on the measurement of the selective parameters (NO_3^- and NH_4^+content, cell density, and acidity) as presented in Table 1, two isolates, s2a10 from petsai's rhizosphere and c2a9 from chili's rhizosphere, were selected.

The population density of the isolates represents their ability to adapt and to perform their activity in soil (Schmidt *et al.* 2014). *Azotobacter* has an instinctive ability to fix dinitrogen to NH_3 catalyzed by nitrogenase. Once NH_3 is formed, it will be transformed to available nitrogen N-NH_4^+ and N-NO_3^-. Both forms of ionic nitrogen are detectable in nitrogen-free Ashby's broth following *Azotobacter* application. The presence of these ionic nitrogens after incubation proves the capability of nitrogen fixation (Hoffman *et al*. 2014).

The results of the bioassay involving inoculation of chili seedling with *Azotobacter* sp. indicated that the PGPR can potentially reduce inorganic fertilizer dosage. The combination of *Azotobacter* (*Azotobacter* sp. c2a9, 10^6 CFU mL^{-1} or 10^8 CFU mL^{-1}) with reduced dose of inorganic fertilizer (NPK 75%) produced equal amount of biomass as the plant treated with 100% inorganic fertilizer (without *Azotobacter*). Similar experiment by Jarak *et al*. (2010) showed that chili plants inoculated with *Azotobacter* produced the same average biomass (0.03 g) as the plant without *Azotobacter* inoculation.

The number of *Azotobacter* cells in rizosphere indicates the capacity of the plant roots to provide nutrient for bacterial growth. It also indicates the survival rate of *Azotobacter*, which will be used as biofertilizer. All treatments increased the population of *Azotobacter* in soil, including the control treatment, compared to population before experiment. Indigenous *Azotobacter* from either liquid inoculum or crude exopolysaccharide is able to proliferate in rhizosphere by using plant-derived small organic molecules which is excreted from roots as carbon aand nitrogen sources (Ahemad and Kibret, 2014). The composition and population density of *Azotobacter* in rhizosphere should be equivalent to the condition in either soil or plant. In this experiment, mixture of Entisols and cow manure (1:1; v:v) were shown to support plant growth and root exudates, serving as carbon and other nutrition sources for heterotrophic *Azotobacter*.

Except soil with 10^6 CFU mL^{-1} *Azotobacter* sp. c2a9 and NPK 75%, the amount of total nitrogen inoculated soil with NPK 75% was similar to that of control which indicated that *Azotobacter* fixed nitrogen in low

nitrogen soil. *Azotobacter* inoculation potentially decreased the use of NPK fertilizer to 25%. Untreated soil contained 0.12% total nitrogen, low nitrogen give benefit to nitrogen fixation process since nitrogenase system is repress when high rate nitrate or nitrite taken up by the *Azotobacter*'s cells (Cejudo and Peneque 1987) and cellular level of fixed nitrogen is sufficiently high (Halbleib and Ludden, 2000).

The increase of available nitrogen was also reported by Hindersah *et al*. (2014) in sorghum inoculated with indigenous *Azotobacter* in bioassay experiment using inceptisol and entisols soil orders. Plants stimulate microbial growth by secreting root exudate that functions as a source of nutrient (Hamilton and Frank 2001). This mechanism might have something in common with the relation between s2a10 isolate, which had been isolated from petsai rhizosphere, and chili plant. Different hosts might have negative effect to the *Azotobacter* nitrogenase activity (Swain and Abhijita 2013).

Seedling inoculation followed by lower level of inorganic fertilization enhanced N upatake. This finding indicated that local indigenous *Azotobacter* was able to increase N uptake by chili plant but did not yet play a significant role in dry matter enhancement. It is generally assumed that N uptake correlates with partitioning of dry matter but based on our bioassay, increased N uptake did not significantly improve the dry matter content. However, in long term, mixed agricultural input in certain commodities increased N uptake,which, in turn, is correlated with the yield (Kubat *et al*. 2003).

Two *Azotobacter* isolates, s2a10 and c2a9, which were isolated from petsai's and chili's rhizosphere, respectively,were selected based on their pH, cell density, and available nitrogen. Bioassay showed that *Azotobacter* inoculation followed by reduced NPK fertilizer doses had no effect on transplant dry weight and nitrogen uptake. All *Azotobacter* inoculation except 10^8 CFU mL^{-1} s2a10 maintain soil nitrogen although *Azotobacter* population in soil was slightly reduced.

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