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RESEARCH ARTICLE

SELECTION OF *AZOTOBACTER* FROM DRY LAND AND ITS EFFECT ONMAIZE SEEDLING

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ABSTRACT

The productivity of important local food crop maize in dry land of Nusa Tenggara Timur Province, Indonesia was low since it cultivated by using conventional method without appropriate plant nutrition system. A way to enhance maize yield is inoculation of beneficial microbes, *Azotobacter*. The purpose of this research was to isolate and select *Azotobacter* to be used as biofertilizer based on N_2 fixation, cytokinin as well as exopolysachharide (EPS) production from six indigenous *Azotobacter* isolates; and their capacity to promote maize seedling growth. The nitrogen fixation capacities of native *Azotobacter* in N-free media varied from 0.01 to 0.39 µM C_2H_4/g/h. Cytokinin production of these strain in liquid culture was 0.11 to 40.04 mg/L while EPS content in liquid culture with nitrogen varied from 0.4 to 27.3 g/L. Bioassay on maize seedling performed in nitrogen-free liquid showed that *Azotobacter* AS4 promote root growth of maize seedlings.

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INTRODUCTION

A significant change has taken place in plant nutrition system in food production. In the past, the important nutrition source to maximize plant productivity was inorganic fertilizer. Nowadays, enhance the productivity was drive by using natural fertilizer such as compost, biomass-based organic fertilizer and biofertilizer. In sustainable agriculture, biofertilizer widely used is non-symbiotic N_2 fixing bacteria *Azotobacter* which colonize rhizosphere of important crops. Members of the genus *Azotobacter* are known to be obligate aerobes, gram negative, pleomorphic in shape, non-spore forming, mesophilic and heterotrophic bacteria (Holt *et al*., 1994; Dadook *et al.,* 2014, Yu *et al*., 2017).

Azotobacter belong to the group of Plant Growth Promoting Rhizobacteria, beneficial bacteria that colonize rhizosphere and enhance plant growth. The mechanisms by which *Azotobacter* increase plant growth and yield is asymbiotic dinitrogen fixation, phytohormone production, especially cytokinin (Aquilanti *et al*., 2014; wani *et al*., 2013). and exopolysaccharides production (Emtiazi *et al*., 2004; Hindersah *et al*., 2006). Increasing in growth and yield of important food crops due to inoculation with *Azotobacter* have been reported elsewhere. Pot as well as field trials carried out in different environmental and soil showed that inoculation important food crops with *Azotobacter* has positive effects on plant growth and yields.

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The effect of *Azotobacter*on vegetative growth and yields of maize has been studied by numerous authors (Nieto and Frankenberger, 1991; Hajnal-Jafari *et al*., 2012). Application *Azotobacter*at 3 L/ha gave a same maize growth with application of inorganic fertilizers (Katriani *et al*., 2011). For a number of years, In Indonesia research of *Azotobacter*as biofertilizer is increased significantly. Nowadays some *Azotobacter* inoculum in form of liquid as well as carrier-based biofertilizer is commercialized to support sustainable production of important food crops.

Maize in some region in Nusa Tenggara Timur Province bordering Republic Democratic of Timor Leste is important local food crop and commonly cultivated using conventional method without appropriate plant nutrition system so that productivity is still low. A way to enhance local corn yield is adding biofertilizer containing nitrogen (N_2) fixing bacteria. The purpose of this research was to isolate and select Azotobacter for biofertilizer formulation based on N_2 fixation, cytokinin as well as exopolysachharide (EPS) production from six indigenous *Azotobacter* strains; and their capacity to promote maize seedling growth. This research will be important to collect effective local strain of *Azotobacter* in order to produce certain biofertilizer with better adaption to soil and climate condition of dry region.

MATERIALS AND METHODS

Isolation Method: *Azotobacter* were isolated from soil surrounding roots of local maize grown in local farmer agricultural land in Alas Selatan Village, Nusa TenggaraTimur

Province, Indonesia. Soil which collected in dry season was slighty alcaline Entisols (pH $_{H2O}$ 7.84) with C-organic 0.67% (very low), total N 0.12 % (low), $P_2O_{5Potensial}$ 92.01 mg/100 g (very high), K_2O $_{Potensial}$ 24.19 mg/100 g. Research has been carried out at Soil Biology Laboratory Universitas Padjadjaran, Indonesia. *Azotobacter* was isolated in two steps by using nitrogen-free Ashby's medium (10 g manitol, 0.2 g KH_2PO_4, 0.2 g$MgSO_4.7H_2O$, 0.2 g NaCl, 0.1 g $CaCO_3$, 10 g Na_2MoO_4, 1 L aquadest; pH 7). At first step, 5 g soil was poured into 50 mL autoclaved liquid medium and incubated at 30^{o}C for 5 days until biofilm appearance in the medium surface. The second step was isolation of *Azotobacter* from biofilm by streaking one loop of liquid in Ashby's plate agar. After a period of 3 days of incubation at 30 C, colonies on the plates were observed for presence of specific characteristics appearance of *Azotobacter*. Strains were characterized by cell morpholgy (Gram stain, presence of spores and capsule) as well as colony morpholofy (form, elevation,margin, appearance, optical property, pigmentation and texture). Each strain showed morphological characteristics of *Azotobacter* were then tested for its N_2 Fixing ability as well as phytohormone (cytokinin) and exopolysaccharide production.

Nitrogen Fixation Measurement: Capacity of *Azotobacter* isolates were tested by determining the concentration o nitrogen in liquid culture and by using acetylene reduction assay in slant. *Azotobacter* were grown in 50 mL Ashby's liquid medium on gyratory shaker (115 rpm) at 30 ^{0}C for 3 days prior to centrifuge 10,000 rpm at 4^{o}C for 15 minutes. Concentration of nitrogen in supernatant was measured by acid digestion and subsequent measurement by the Kjeldahl method (Bremner, 1965). For determination of acetylene reduction, *Azotobacter* isolates were streaked on slant with Ashby's medium, incubated at 30^{0}C for 3 days. Acetylene equivalent to 10% of the net air volume of the test tube was injected to slant. Gas samples of 0.5 ml were withdrawn every 30 min and Chromatographed immediately.

Cytokinin Analysis: *Azotobacter* pure culture in slant of Ashby were streaking onto plate agar of modified N–free medium which contained $K_2HPO_4$0.5 g, $MgSO_4.7H_2O$0.2 g, NaCl0.2 g, $CaCO_3$; 3.0 g, Mannitol5.0 g,sucrose 5.0 g, 1 mL/L n Fe-Mo solution, agar 15 g, aquadest 1000 mL. Plates were incubated 3 days at 30^{o}C. A total of one mL of bacterial suspension containing 10^8 CFU/mL was transferred into 50 ml of free-N liquid media described above and incubated for 3 days at room temperature on gyratory shaker. Quantification of cytokinins in bacterial culture was performed by high performance liquid chromatography operated at room temperature by using μ-Bondapak C_{18}/280 nm.

Exopolysaccharide Analysis: Culture were streaking onto plate agar medium described by Vermani *et al*. (1997) (10 g Sukrosa, 1,0 g KH_2PO_4, 1,0 g $MgSO_4.7H_2O$, 0,5 g NaCl, 0,1 g $CaCO_3$, 0,1 g $NaNO_3$, 0,1 g $FeSO_4$, 10 mg Na_2MoO_4, 15 g agar, 1 L aquadet) to induce exopolysaccharide production. The bacteria were maintained on the basal medium for 3days at 30^{o}C. A total of one mL of bacterial suspension containing 10^8 CFU/mL was trasnferred into 50 ml of Vermani's liquid media and incubated for 3 days at room temperature on gyratory shaker. Liquid cultures were centrifuged at 4,000 rpm at 4^{o}C for 15 minutes. The supernatant was removed, two volume of cold acetone (97%) was added, and stand one night at 4^{o}C. The EPS was collected on pre-weight Whatman no 1 filter paper by centrifugation at 4,000 rpm at 4^{o}C for 15 minutes. The weight of EPS was measured after heating at 35^{o}C for 1 hours and desiccating for 20 minutes.

Bioassay on Maize Seedling: An in vitro experiment had been performed in liquid culture of N-free Murphy media formulated for maize (Murphy, 1996). The experiment has been set up in randomized block design which evaluate the effectiveness of *Azotobacter* sp. AS4 to increase the seedling growth. *Azotobacter* AS4 had selected based on their capacity to fix nitrogen, and produce either cytokinin or EPS in first experiment. Liquid inoculant of *Azotobacter* has been prepared in liquid N-free Ashby's media; and the cell density each isolate were 10^6 CFU/mL and 10^8 CFU/mL. *Azotobacter* liquid culture (1%) was mixed with N-free Murphy media in 100 mL Erlenmeyer before single three-days old maize seedling were placed on the surface of media by using sterile strainer. The in vitro cultures were maintained in green house for three weeks when plant height, N uptake as well as total N content and *Azotobacter* count in liquid media were measured. All data was subjected to analysis of variance (5% F-test) by using SPSS v.17. If the F-test was significant then Duncan's multiple range test was conducted.

RESULTS AND DISCUSSION

Nitrogen fixation, cytokinin and exopolysaccharide production: After 5 days of enrichment in N-free Ashby's liquid medium, the biofilm appeared in surface of liquid medium (Fig. 1 Above). On N.free Ashby's Mannitol Agar medium the colonies were grows up after three days of incubation, and showed dark brown pigmentation (Fig. 1 Below).

Figure 1. Biofilm appearance in the surface of liquid medium 5 days after incubation (a) from which six pure culture of *Azotobacter* has been isolated

The shape of the colonies was round, margins were entire, the surface of the colonies were glistening, the density of colonies was transparent. Most of colony was watery and mucilaginous. We collected six colonies which grown up to 1-3 mm in diameter and passed to cell morphological test, They might represented the different types of *Azotobater* in Alas Selatan village. Six colonies showed charactersitics of Gram negative bacteri, non spore forming and showed capsular characteristics in negative staining, were purified and maintained in slants with the same medium (Fig. 1). Microscopic cell shape of all isolate (AS1, AS2, AS3, AS4, AS5 and AS6) was diplococcus, suggesting that all isolates belong to *Azotobacter chroococcum*. When six isolates of *Azotobacter* grown in N-free liquid medium, nitrogen content gains ranged from 30 mg/L to 140 mg/L with nitrogenase activity equal to 0.01-0.39 $\mu MC_2H_4/g/h$ (Table 1). Cytokinin were also detected in N-free liquid medium of 0.11-43.14 mg/L (Table 2). By using Kjeldahl Method, nitrogen was not detected in liquid culture of isolate AS5, but ethylene was detected in slant after acetylene injection which verified that this isolate was able to fix N_2. Cytokinin concentration in liquid culture was 0.11-43.14 mg/L depend on the isolate. AS4 isolate showed te highest capacity to produce this phytohormone (Table 2).

Table 1. Nitrogen recovery and nitrogenase activity of six *Azotobacter*

*Azotobacter*Isolates	Total N (mg/L)	Nitrogenase activity ($\mu M\ C_2H_4$/g/h)
AS1	82	0.01
AS2	139	0.14
AS3	58	0.26
AS4	131	0.39
AS5	nd*	0.25
AS6	41	0.11

*nd: not detected.

To induce EPS production, each *Azotobacter* isolates was grown on Vermani plate agar. After three days incubation, colonies were grows up with intensive mucilage and brownish black pigmentation (Fig. 2 left). The EPS was successfully extracted by using acetone and collected on Whatman no 1 (Fig. 2 center and right). EPS concentration was varied between 0,4-27.3 g/L depend on isolates (Table 2).

Table 2. Cytokinin and Exopolysachharide production by six *Azotobacter* isolates

*Azotobacter*Isolates	Cytokinin (mg/L)	EPS content (g/L)
AS1	0.11	0.4
AS2	6.46	2.5
AS3	16.21	4.0
AS4	43.14	3.8
AS5	40.04	27.3
AS6	32.00	13.5

Azotobacter was reported produce phytohormone of Gibberellin, Cytokinin and auxin (Wani et al., 2013). An isolate of *Azotobacter vinelandii* excreted 1.3 mg/L cytokinin of trans-zeatin riboside group and into culture supernatant (Naz et al., 2012). The capacity of all isolates to produce cytokinin will be an important factor when the bacteria will be used as biofertilizer. EPS production in the cell surface of *Azotobacter* was well documented. Emtiazi *et al.* (2004) concluded that EPS production by *Azotobacter* was 1.6 – 7.6 mg/mL depend on bacterial strain and sucrose content in N-free medium. Agro-ecological importance of EPS was related with fitness factors, stress tolerance and nutritional benefits (Gauri et al., 2012). Nine *Azotobacter* isolate generates EPS in liquid medium with and without $CdCl_2$ up to 1.1-1.7 g L^{-1} (Hindersah et al., 2006).

Figure 2. Exopolysachharides at the bottom of tube after 7,000 rpm centrifugation at 4^0C (above), EPS on the Whatman no 1 after heating at 35^0C (below)

At the end of research, six isolates of *Azotobacter* were sucessfully isolated from Entisol from Alas Selatan Village. *Azotobacter* AS4 showed higher nitrogen fixation, nitrogenase activity and cytokinin production in compared to other isolate although their capacity to produce EPS was lower in compared to isolate AS5 and AS4. Based on those results, role of *Azotobacter* AS4 on plant growth had been evaluated in maize's bioassay.

Effect of *Azotobacter* on Maize Seedling Growth

Analysis of variance showed that the effect of microbial treatment was significant on nitrogen uptake and plant height. Total nitrogen content in soil with bacterial inoculation was similar to un-inoculated one (Table 3). *Azotobacter* AS4 of 10^6 CFU/mL increased N uptake in compared with un-inoculated and higher concentration of liquid inoculant; but no change in *Azotobacter* count following inoculation. This bioassay was in line with the results of *A.chroococcum* at concentration of 10^8CFU/mL inoculation on germination of maize seeds in the N-free Hoagland's medium; seedlings harvested in 25-35 days had significant increase in root length

and plant height and also in dry weight of root and shoot (Dhamangaonkar Sachin and Pragati, 2009).

Table 3. Effect of *Azotobacter* AS4 cell density on soil total nitrogen and *Azotobacter* population; and N- uptake and plant height of 3-weeks old maize seedling in N-free liquid culture

Cell density	Soil total N* (%)	N Uptake* (mg/plant)	*Azotobacter* Count* (x 10^6 CFU/g)	Plant height* (cm)
Without inoculation	1.23 a	1.38 a	1.59 a	24.1 a
10^6 CFU/mL	1.15 a	1.48 b	1.38 a	27,9 b
10^8 CFU/mL	1.05 a	1.38 a	1.47 a	26,2 ab

*Numbers followed by the same letter were not significantly different base on 5% Duncan's Multiple Range Test %

Beneficial effect of *Azotobacter* on nitrogen uptake is due to increased N content in soil and root system. Our results were in consistency with those reported in other bioassay with agricultural crops; *A. vinelandii* enables to enhance growth of maize seedlings; shoot and root length as well as shoot and root length (Naz et al., 2012). Our experiment was terminated at three weeks after planting; but in this present study positive role of indigenous *Azotobacter* on nitrogen level in soil and N uptake has been demonstrated. Extracellular polysaccharideexcreted by *Azotobacter* may take part of plant development due to their participation in plant-bacteria interaction (Mandal et al., 2008). This results verified that local *Azotobacter* strain might be used as biofertilizer since they demonstrated plant growth promoting ability on maize seedlings.

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