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Properties of Azotobacter as Biofertilizer and Biocontrol of Fusarium

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ABSTRACT

The Nitrogen-fixer bacteria, Azotobacters, are the potential Plant Growth Promotion Rhizobacteria for increasing plant growth and productivity due to their prominent role as biofertilizer dan bioprotectant. The objective of the experiment was to identify the plant-growth related characteristic of various Azotobacter isolates and to test their antagonism to soil-borne pathogen Fusarium. The five Azotobacter isolates were grown in Nitrogen-free slant for determining their ability to fix the Nitrogen, producing phytohormones and exopolysaccharides, and to inhibit the growth of *Fusarium oxysforum* f.sp. batatas. The results verified that all isolates synthesize various concentration of Indole acetic acid, Gibberellin, Zeatin and Kinetin as well exopolysaccharide in the N-free media. However, this study found that Azotobacter AzV1 did not detected to fix the N. This study suggests that Azotobacter has a dual function as biofertilizer and bioprotectant.

Keywords: Antagonism, Exopolysaccharides, Nitrogen Fixation, Phytohormones

Karakteristik Azotobacter Sebagai Pupuk Hayati dan Agen Pengendali Fusarium

ABSTRAK

Bakteri pemfiksasi Nitrogen, Azotobacters adalah Rhizobakteri Pemacu Pertumbuhan Tanaman yang potensial untuk meningkatkan pertumbuhan dan produktivitas tanaman karena berperan ganda sebagai pupuk hayati dan pengendali penyakit tular tanah . Tujuan penelitian ini adalah untuk mengidentifikasi karakteristik pertumbuhan tanaman berbagai isolat Azotobacter dan menguji antagonismenya terhadap patogen tular tanah Fusarium. Kelima isolat Azotobacter ditumbuhkan pada agar miring bebas Nitrogen untuk mengetahui kemampuannya dalam memfiksasi Nitrogen, menghasilkan fitohormon dan eksopolisakarida, serta menghambat pertumbuhan Fusarium oxysporum f.sp. batatas. Hasil penelitian membuktikan bahwa seluruh isolat mensintesis berbagai konsentrasi asam Indole asetat, Giberelin, Zeatin dan Kinetin serta eksopolisakarida dalam media bebas Nitrogen; tetapi kemampuan Azotobacter AzV1 untuk memfiksasi Nitrogen tidak terdeteksi. Penelitian ini menunjukkan bahwa Azotobacter memiliki fungsi ganda sebagai pupuk hayati dan secara in vitro dapat mengendalikan pertumbuhan Fusarium.

Kata Kunci: Antagonisme, Eksopolisakarida, Fiksasi Nitrogen, Fitohormon

INTRODUCTION

Nowadays, the Plant Growth Promoting Rhizobacteria (PGPR) is suggested as a source of plant nutrients in agriculture. The PGPR that provide N for plants had been explored and studied due to their ability to fix the dinitrogen gas (N_2) to ammonia (NH_3) that is not yet available for plants. The gaseous NH_3^- are hydrolyzed to ammonium (NH_4^+) by chemical reaction, and further NH_4^+ are enzymatically converted to NO_3^- by nitrification bacteria [1]; both NH_3^- and NH_4^+ are the available for root uptake. Despite the essential role of Nitrogen (N) in food crop cultivation, soil in tropical area in general contain low N as well as carbon [2].

Farmers rely on inorganic N fertilizer to improve plant growth and productivity effectively. In order to support the sustainability of agriculture, other source of N is needed since in fact the effectivity of N fertilizer is only 20%, and the price is always increased. The genus of Azotobacter is a nonsymbiotic N_2-fixer rhizobacteria has widely used a bioactive compound of biofertilizer. The bacteria enable to fix the nitrogen up to up to 73.8 kg ha-1 year-1 in soil [3]. Other study revealed that Azotobacter enable to fix 8.14-8.46 mg N g^{-1} glucose [4]. Nitrogenase that catalyze the N_2 fixation reaction is sensitive to available N; the nifA genes are become non active in the presence of N [5].

The other mechanisms by which Azotobacter enhance plant growth are phytohormone and exopolysaccharides (EPS) production. The Azotobacter produced phytohormones to support roots formation and stimulate plant growth [6]. The saline resistant strain of *A. salinestris* AT19, and *A. chroococcum* produce different concentration of Indole Acetic Acid (IAA), Gibberellin (GA3), and Cytokinin (Zeatin) in N-free broth [7]. Nevertheless, Azotobacter has outer-cell structure namely capsules [8] that also well known as EPS.

Azotobacter species naturally form EPS that composed of simple sugars and organic acids [9]. In contrast with N fixation, the Azotobacter produces more EPS in the presence of available N [10]. The functions of EPS are diverse. In *A. vinelandii*, EPS has a prominent role to protect nitrogenase for high concentration of Oxygen that cease nitrogenase activity [11]. The EPS is reported to improve soil aggregation and pore to facilitate better water and nutrient uptake [12].

Biocontrol or bioprotectant is prominent mechanisms by which the Azotobacter promote plant growth. Azotobacter is able to control the soil pathogen including Fusarium that attack important food crops. Antifungal activity of Azotobacter against *Fusarium* spp. in banana as well as maize, sorghum, and wheat [13][14]. In vitro experiment shows the ability of *A. chroococcum* to inhibit growth of pathogen *Rhizoctonia solani, Sclerotia rolfsii*, and *F. oxysprorum* [15].

Researchers agree that Azotobacter is considered as an important biofertilizer to provide available N and lower chemical fertilizer dose [16][17]. Since the Azotobacter is easily isolated from the rhizosphere, researcher need to find the isolate that have multiple role as the PGPR through the characterization. The objective of this laboratory study was to verify the plant-growth related characteristic of five Azotobacter isolates and to identify their antagonism to Fusarium fungi, the important soil-borne pathogen.

MATERIAL AND METHOD

The laboratory research has been conducted in the Soil Biology Laboratory, Faculty of Agriculture, Universitas Padjadjaran on Oktober - November 2019. The Azotobacter belong to the Faculty of Agriculture, Universitas Karawang that isolated from various isolate sources (Table 1). All isolates were maintained on N-free Ashby slant at 4^0C; the pure cultures were transferred to similar slants and stored at 30^0C three days before characterization. All the isolates were Gram negative and cocci in shapes (Figure 1).

Table 1. Azotobacter isolates used in the characterization

Isolates	Source
AzV1	Rhizosphere of sweet potatoes
AzV2	Rhizosphere of paddy
AzV3	Hydrocarbon-contaminated soil
AzV4	Rhizosphere of paddy
AzV5	Hydrocarbon-contaminated soil

Determination of Nitrogen-fixing Capacity

The N_2 fixation capacity test was carried out on Ashby's broth by using the Acetylene Reduction Assay (ARA) method.

Each isolate was grown separately into 50 ml of Ashby broth media in the 100-ml Erlenmeyer flask by adding 10% of liquid pure culture. The cultures were incubated at room temperature (25°C-27°C) for 72 h on the 110-rpm gyratory shaker. A total of 10 mL acetylene gas was injected to each culture prior to incubating at room temperature for 1h. The N_2-fixation capacity of all isolated were follow the Acetylene Reduction Assay [18]. The tests were performed in duplicate.

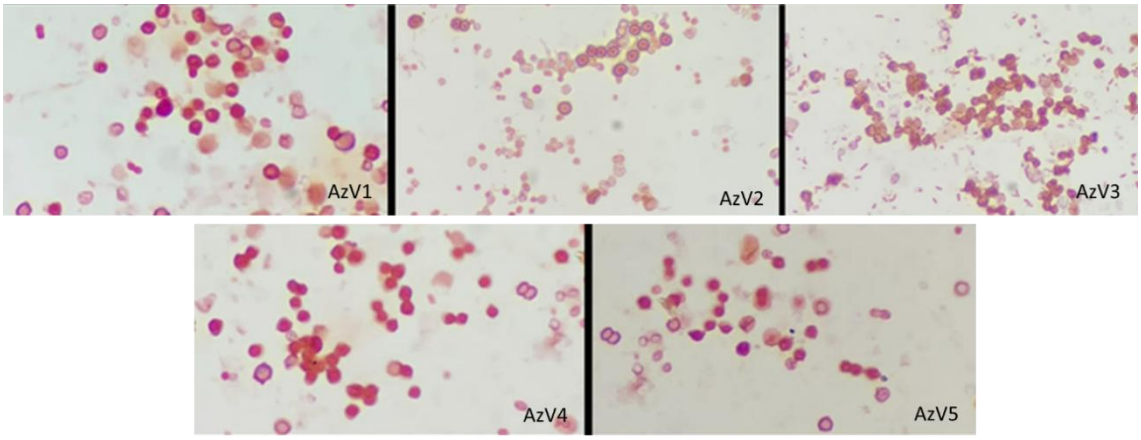

Figure 1. The cell morphology of five Azotobacter's isolates used in the characterization

Metabolites characterization

The determination of EPS concentration in each Azotobacter liquid culture was carried out by gravimetric method. The bacteria was grown in N-free Ashby broth by inoculating 10% of Azotobacter pure culture with the cell density of 106 CFU mL-1 at room temperature on the gyratory shaker for 96 h. The cultures were further centrifugated at 10,000 rpm at 40C for 15 minutes. The supernatants were collected and extracted with cold acetone [9]. The EPS determination was performed in triplicate from each culture.

The ability of Azotobacter to synthesize the Gibberellin (GA3) and Cytokinin (Zeatin and Kinetin) in the liquid culture was determined by using High Performance Liquid Chromatography (HPLC) [19]. A total of 10% pure liquid culture of each isolate were grown for 72 h at room temperature; the culture then passed the centrifugation at 10,000 rpm at 40C for 15 minutes. The supernatants were extracted with acetonitrile for gibberellin and methanol for Cytokinin prior to be injected to HPLC. The ability of bacteria to produce IAA was conducted by using spectrophotometry with Salkowski's solution.

Antagonistic Test on Fusarium

The antagonistic test was done by dual culture method [20]. A 0.5-cm culture disk of F. oxysporum f.sp. batatas (Fob) colony was put 3 cm away from the edge of Potato Dextrose Agar (PDA) plate. A single culture disk of Azotobacter with the cell density of 10^4 or 10^5 CFU mL^{-1} was put separately on the PDA plate 3 cm away from the Fob culture. Control PDA plate contained only a single Fusarium culture disk without Azotobacter. The test was conducted in duplicate; all cultures were incubated at 30°C for 72 h. The diameter of inhabitation zone was measured at 24h and 27h after incubation; the ability of Azotobacter to inhibit Fusarium growth was calculated by using this equation:

$$GI = [(r1 - r2)/r1] \times 100\%$$

where:
GI: growth inhibition of Fusarium (%)
r1: the radius of radial growth to the opposite direction in the control Petri dish
r2: the radius of radial growth in the treated petri dish.

The average of all data was calculated and presented in the table or figure.

RESULTS AND DISCUSSION

Five Azotobacter isolates produced significant amounts of exopolysaccharides in N-free media but only three isolates fixed N (Table 2). AzV1 isolate isolated from sweet potato rhizosphere showed the highest EPS production compared to other isolates. N fixation activity determined based on acetylene reduction was only shown by isolates AzV2, AzV3 and AzV5.

All Azotobacter isolates produce phytohormones IAA, gibberellin, zeatin, and kinetin with various concentration (Figure 2). In general, isolat AzV1 (isolated from the rhizosphere of sweet potatoes) produced less phytohormones compared to the other isolates. In this study, the higher phytohormones content was found in the culture of AzV5; included 2.99 mg/L IAA, b2.8 mg/L GA3, 0.45 mg/L Zeatin, but Kinetin production was just 0.62 mg/L Kinetin, and it was lower than AzV4.

Table 2. Nitrogen fixation capacity and exopolysaccharides production of various Azotobacter's isolates in N-free Ashby broth

Isolates	EPS* (g/L)	Nitrogen Fixation (µmol/ h)
AzV1	68.67	0.005
AzV2	50.23	0.056
AzV3	41.31	0.002
AzV4	51.33	0.001
AzV5	52.36	0.015

* Exopolysaccharides

Table 3 and 4 revealed the potential of Azotobacter isolates to inhibit the growth of Fusarium. The ability of Azotobacter to inhibit Fob growth was vary and depend on the cell density of Azotobacter and incubation time (Table 3 and Table 4). At the cell density of 10^4 CFU/mL, the AzV1, AzV2, and AzV4 showed similar inhibition on Fusarium at 24 h and 72 h (Table 3). However, the ability of Azotobacter AzV3 and AzV5 were reduced at 72h compared to 24 h.

The AzV5 exhibited the highest inhibition ability at a cell density of 10^4 CFU/mL, but this inhibitory effect was sustained only for 24 hours before significantly decreasing. Conversely, AzV4 demonstrated the highest inhibition effectiveness at a cell density of 10^5 CFU/mL (see Table 4). Our study reveals that the cell density of each species and the duration of incubation for inhibiting Fusarium growth did not follow distinct patterns, which warrant further investigation. Particularly, AzV3 emerged as the most sensitive isolate to incubation duration. Hypothetically, this sensitivity of antifungal produced by AzV3 may be attributed to this oxic substances is degraded easily into compounds with reduced or less-toxicity towards Fusarium.

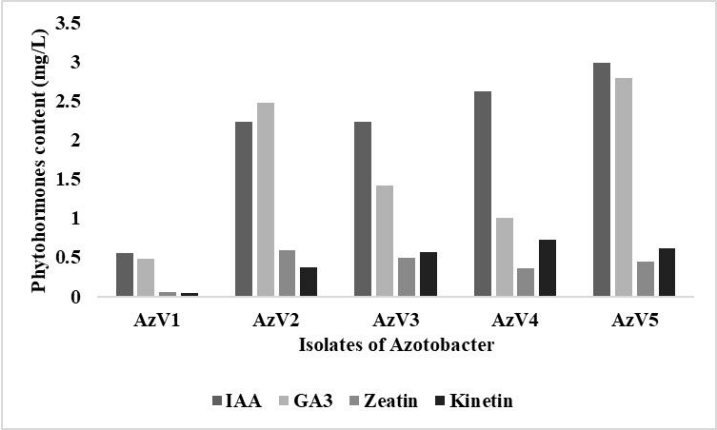

Figure 2. Phytohormone production by various isolates of Azotobacter in N-free Ashby broth

Table 3. Growth inhibition rate of *Azotobacter* with the cell density of 10^4 CFU/mL on Fusarium growth

Isolat	GI* (%) at two incubation time	
	24h	72h
Azv1	5.1	5.8
Azv2	2.4	2.8
Azv3	7.7	3.0
Azv4	4.8	4.8
Azv5	2.2	2.6

*Growth Inhibition

Table 4. Growth inhibition rate of *Azotobacter* with the cell density of 10^5 CFU/mL on Fusarium growth

Isolat	GI* (%) at two incubation time	
	24h	72h
Azv1	7.9	8.3
Azv2	2.6	3.0
Azv3	2.6	0.0
Azv4	12.3	5.6
Azv5	5.1	5.6

*Growth Inhibition.

The growth inhibition rate of Azotobacter isolates were slightly changed in the higher cell density (10^5 CFU/mL). Despite constant inhibition rate at low cell density (Table 3), the ability of AzV4 to inhibit Fusarium growth at higher cell density, 10^5 CFU/mL, at 24h (Table 4) was clearly 58 % lower compared to lower concentration (Table 3). Nonetheless, the inhabitation capacity of higher inoculant concentration ofAzV4 at 72h was 56% higher than lower concentration.

This investigation found that the ability of five isolates Azotobacters in inhibiting Fusarium growth was lower than Azotobacter salinestris Strain Azt 31 that enable to reduce the growth of Fusarium up to 50% (Nagaraja et al., 2022). In general, in this study Azotobacter isolates had a dual role as biofertilizer and bioprotectant. They enable for producing the phytohormone and exopolysaccharides, even though the nitrogen fixation capacity of AZV1 and AzV4 were less than 0.005 ug/hour (Table 2). The nif genes of N-fixing bacteria conservative genes and is an operon composed of several *nif* gene including nif D, nifH and *nifK*; as well as *nifA* that initiate the nitrogenase activity in the low-N environment [21].

Azotobacter are the PGPR (Plant Growth Promoting Rhizobacteria) bacteria which are capable of producing phytohormones and increasing the uptake of nitrogen, phosphorus and other essential minerals [7][22]. Nonetheless, the study verified that all Azotobacter were able to inhibit the growth of

F. oxysforum based on in vitro antagonist test (Table 3 and Table 4) in various inhibition effectively pattern; which is in accordance with the other experimental results [13][14].

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