Effect of urea-N on growth and indoleacetic acid production of Stenotrophomonas maltophilia (Sb16) isolated from rice growing soils in Malaysia

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INTRODUCTION

Rice (Oryza sativa L.) is one of the major staple food crops in the world (Wang et al., 2004), and N is the most important input for high yield. It is known that rice requires 1 kg N to produce 15-20 kg of grain. Yields per hectare are critically dependent on the nature, and amount and timing of N supply (George et al., 1992). Urea is the most commonly used N fertilizer in rice cultivation, produced in granular or pellet forms, and is coated with a non-hygroscopic inert material. After applied to the soil, its N is rapidly changed into ammonia.

Wetland rice ecosystem is unique where N can be supplied to plant through biological N fixation (BNF). Wetland rice ecosystem is unique where N can be supplied to plant through biological N fixation (BNF). The rice ecosystem harbors diverse groups of diazotrophs and BNF is a spontaneous process of diazotrophic strain Sb16 but significantly reduced indoleacetic acid production.

Key words: Incubation study, paddy soil, diazotroph.

Materials and Methods

Inoculum preparation

The bacterial strain Stenotrophomonas maltophilia Sb16 (accession number JQ820255) was previously isolated from Tanjong Karang Rice growing area in Malaysia (Naher et al., 2008). The bacterial strain (Sb16) used was Gram negative rod, with cellulolytic enzyme activity, high IAA (60 mg L⁻¹), nitrogenase activity of 1.4 × 10⁻⁷ µmol C₂H₄mol⁻¹ h⁻¹ and 43% Nfda (Naher et al., 2009; 2011). Starter
culture was prepared by growing pure culture of Sb16 in Jensen’s N-free broth for 36 h. Composition of broth (L⁻¹): 20.0 g sucrose, 1.0 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.5 g NaCl, 0.1 g FeSO₄, 0.005 g Na₂MoO₄, 2.0 g CaCO₃. pH was adjusted to 6.8-7.0. One milliliter of the starter culture containing approximately 2 × 10⁷ cfu mL⁻¹ was transferred to a 100 mL flask containing 50 mL of Jensen’s N-free broth medium and allowed to grow to exponential growth phase.

**Soil inoculation and incubation**

Bacteria cells were harvested, washed with phosphate buffer solution (0.85% PBS), and immediately suspended into PBS solution. Before applied inoculum optical density (OD₆₀₀) of washed cells were checked and adjusted to 0.1 and the population was confirmed by cell enumeration using drop plate method on N-free media (Somasegaran and Hoben, 1985). Composition of the medium is (L⁻¹): 20.0 g sucrose, 1.0 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.5 g NaCl, 0.1 g FeSO₄, 0.005 g Na₂MoO₄, 2.0 g CaCO₃. pH was adjusted to 6.8-7.0. One milliliter of the starter culture containing approximately 2 × 10⁷ cfu mL⁻¹ was transferred to a 100 mL flask containing 50 mL of Jensen’s N-free broth medium and allowed to grow to exponential growth phase.

**Extraction of IAA from soil and water**

The IAA concentration of soil sample was determined using modified method of Sarwar et al. (1992). Three grams of soil were placed into a 50 mL Erlenmeyer flask and treated with 6 mL of phosphate buffer (0.2 M, pH 7.0) and 4 mL of L-tryptophan solution (5.3 g L-tryptophan kg⁻¹ soil). The flask was covered with parafilm and incubated in darkness at room temperature (± 28 °C) for 12 h on a shaker (~ 150 rpm). After incubation, flask contents were treated with 2 mL trichloroacetic acid (5 g 100 mL⁻¹ H₂O₂) to terminate the reaction and 1 mL calcium chloride (0.5 M) to facilitate filtration. The soil standing water was filtered through Whatman Filter paper nr 2. A buffer solution without incubation of soil was also prepared as a standard solution. For soil and soil standing water sample, approximately 2 mL water supernatant were mixed with Salkowski reagent (Gordon and Weber, 1951) and the mixture was allowed to stand 30 min for color development. The intensity of the color development was measured at 535 nm by using a spectrophotometer (Milton Roy, Rochester, New York, USA). The amount of L-tryptophan-derived auxins content in soil and soil standing water was determined as IAA-equivalents (mg kg⁻¹ soil) using standard IAA solution.

**Statistical analysis**

The experiment was conducted in factorial completely randomized design with three replicates. All experimental data were statistically analyzed by ANOVA using SAS (9.1 version) statistical software. Treatment means were compared using Tukey’s test (p ≤ 0.05).

**RESULTS AND DISCUSSION**

**Population of diazotrophs Sb16**

Application of different levels of urea-N increased population of Sb16. However, population differed significantly with time (Figure 1). In general bacterial population in the soil-standing water was higher than in soil. Significantly high population growth was observed in soil treated with 200 kg N ha⁻¹. Soil applied with urea-N showed higher population growth at first week and then decreased with increasing time. On the other hand, population in soil standing water was found high at second week of incubation. It is known that population of the diazotrophs can be affected by several factors including pH, temperature, nutrients, water, oxygen, and metabolic compounds (Döbereiner and Pedrosa, 1987). This study showed that population of Sb16 was indeed affected by the use of urea-N in the soil. The bacterial population decreased with increasing incubation time probably due to urea-N reduction over time and it was an important nutrient source for bacteria growth. Compared to soil, soil standing water maintained higher population as the applied bacteria contributed some of NH₄-N to the water. Previous study also showed that application of Sb16 increased NH₄-N level in soil water (Othman et al., 2012).

There was a positive significant relationship found between N rates and population of bacteria (Figure 2).
There were significant differences in population at first, third, and sixth week with increasing rates of urea-N. This could be due to the utilization of available N for cell growth. During the high bacterial growth in urea-N treatments, gelatinous material was observed to form in the soil layer of the incubation flask. The material could be the extra cellular polysaccharide produced by bacterial cells. Polysaccharide is a polymer that plays an essential role for bacterial growth and survival (Castro et al., 2008), it protects cell from desiccation and help in N\textsubscript{2} fixation by preventing high oxygen (O\textsubscript{2}) tension (Kumari et al., 2009).

**Soil chemical properties**

The pH was significantly affected by different levels of urea-N (Figure 3). Soil pH increased after first week of incubation and stabilized thereafter. After the first

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Figure 1. Effect of urea-N on population of Sb16 in soil (a) and soil standing water (b). Bars indicate standard error n = 5.

Figure 2. Bacterial population at different levels of urea-N in soil at first week (a), third week (b), sixth week (c), and in soil standing water at first week (d), third week (e), and sixth week (f).

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y = 2E-06x\^2 + 0.000x + 6.681
R\(^2\) = 0.887

y = 4E-06x\^2 - 0.000x + 6.614
R\(^2\) = 0.966

y = 3E-06x\^2 + 0.001x + 5.843
R\(^2\) = 0.965

y = 1E-06x\^2 - 0.001x + 5.68
R\(^2\) = 0.974

y = 2E-06x\^2 + 0.001x + 5.711
R\(^2\) = 0.980
week of incubation, soil pH increased from 6.7 to 7 and pH of control soil was lower than the other treatments. Soil treated with urea-N had higher pH than control as presence of high NH₄⁺ concentration in the soil increased pH. Optimal pH for N₂ fixation is 5-8 (Leigh, 2002). The consistencies of soil pH provide a stable environment for the growth of Sb16 that increases the survival and activity of Sb16 in soil and water.

The pH of soil-standing water was higher compared to pH of soil which ranged from 7.4 to 8.4 (Figure 3b). The pH change was probably due to the formation of ammonium ion in the soil standing water. Ammonium ion can also be formed through N₂ fixation by Sb16. The pH of soil and soil water in the first, third, and sixth weeks of incubation increased significantly with increasing rate of urea-N (Figure 4).

![Figure 3. Effect of urea-N on soil pH (a) and soil standing water pH (b). Bar indicates standard error n = 3.](image)

![Figure 4. pH changes at different levels of urea-N in soil at first week (a), third week (b), sixth week (c), and in soil water at first week (d), third week (e), and sixth week (f).](image)
There were significant effects of different levels of urea-N on total soil N content. Total N in soil decreased with increasing time of incubation (Figure 5). Total N content in soil and soil water decreased with increasing incubation time as it was used up by the applied bacteria. Other important factors may regulate NH$_3$ loss as the pH of the soil solution was high. However, an increasing trend of total N in the soil standing water observed at the 5th and 6th week of incubation which showed the biological N fixation activity.

**Concentration of IAA**

The IAA production by bacteria was significantly affected by urea-N levels. Significantly high amount of IAA was produced in the control treatment and lowest amount produced in the 200 kg ha$^{-1}$ N applied treatment. The IAA concentration in the soil standing water was higher than in the soil fraction. The initial IAA concentration in soil ranged from 1.5 to 2 mg g$^{-1}$ and it was observed to decrease with increasing time of incubation (Figure 6). The amount of IAA produced in this study was comparatively low. Previously the Sb16 was shown to produce high amount of IAA in the presence of tryptophan (Naher et al., 2009). The low concentration of IAA could be due to the presence of low amount of precursor, L-tryptophan in the soil and water. The presence of IAA stimulating amino acid such as L-alanine, L-asparagine, and L-lysine have been reported to be present in root exudates, which stimulate formation of IAA in soils (Naher et al., 2008).

A significant decrease in IAA concentration in the soil and soil standing water found with increasing rates of urea-N (Figure 7) which might be due to less activity of the added microbes.

**CONCLUSIONS**

Application of different rates of urea-N significantly increased population of diazotrophic strain Sb16. The total N decreased until sixth week either it was used by the bacteria as a nutrient or NH$_3$ lost during incubation time. Application of urea-N significantly reduced the IAA production. The higher IAA value in control N treatments proved that higher doses of nitrogen reduced bacterial activities but did not hamper its growth.

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Figure 7. Effect of urea-N on indoleacetic acid (IAA) production of Sb16 in soil at a) first week, b) second week, c) third week, and in soil water at e) first week, d) second week, and f) third week.

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