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EFFECT OF NITROGEN ON NITROGENASE ACTIVITY OF DIAZOTROPHS AND TOTAL BACTERIAL POPULATION IN RICE SOIL

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ABSTRACT

Nitrogen (N) is the most limiting nutrient for rice and thisinput is required in the largest quantity for rice production. Laboratory and glasshouse studies were conducted at Universiti Putra Malaysiato determine the effect of urea-N on diazotrophs (*Stenotrophomonas maltophila*) growth and colonization on the rice roots and the effect of inoculation on bacterial population. *Stenotrophomonas maltophila* was grown under laboratory condition and applied with five levels of nitrogen in the form of urea (urea-N) (0, 50, 100, 200 and 300 mg L^{-1}). The same treatments were given to rice plants under glasshouse condition for growth performance effect. Results showed that application of urea-N significantly influenced the population and nitrogenase activity of *Stenotrophomonas maltophila*. Nitrogenase activity was reduced with increased of urea-N application. The bacteria showed highest acetylene reduction assay (ARA) value of 0.042 µmol C_2H_4 h^{-1} mL^{-1} at 0 mg L^{-1} urea-N and the ARA was totally inhibited at 300 mg L^{-1} urea-N. In glasshouse study, the rhizosphere population was reduced by 7.6% with addition of 50 $kgha^{-1}$, and endosphere population was reduced by 8% with addition of 200 $kgha^{-1}$ of urea-N. However, photosynthesis and plant biomass were significantly increased by inoculation without affecting the non-rhizosphere population. In general high application of N negatively affected the nitrogenase activity, diazotrophs colonization on rice roots, photosynthesis and plant growth.

Keywords: Acetylene reduction assay, diazotrophs, *g*rowth, photosynthesis, plant biomass, *Stenotrophomonas maltophila*.

INTRODUCTION

Urea is the most common nitrogen fertilizer used worldwide for wetland rice cultivation. High rates of N fertilizer used in rice have led to serious environmental problems including soil quality deterioration, increased greenhouse gas emissions and surface water eutrophication (Fan *et al.,* 2011).Mineral nitrogen can influence the diazotrophs colonization in the plant roots and cause inhibition of the nitrogen (N_2) fixation process. To reduce this excessive use of N fertilizer, biological nitrogen fixation (BNF) is the most promising approach in plant N uptake efficiency. It is proven that BNF association with rice can potentially supply N to rice plants (Naher *et al.,* 2011; Ladha *et al.,* 1997). It is the process in which N_2 is reduced to ammonium (NH_4^+) by nitrogenase enzyme.The isolation and identification of N_2 fixing bacteria in rice fields has been well documented over the past two decades (Xie *et al.,* 2003). Other than fixing N_2, BNF can also produce phytohormones which can stimulate plant growth (Naher *et al.,* 2009) and it functions as an antagonist to plant pathogens (Piao *et al*., 2005). A low concentration of available N and O_2 in substrate is important for biological N_2 fixation, even though addition of nutrient may stimulate bacterial growth and increase the number of bacteria in the rhizosphere. However, not all bacterial groups are stimulated by N fertilization (Marschner *et al*., 1999). Previous studies of Laane *et al*. (1980) showed that the process of N_2 fixation by free-living bacteria as well as by symbiotic associations is inhibited in the presence of N especially ammonium (NH_4^+)and the amount of N fertilizer application influenced the presence of N_2-fixing bacteria in plant's rhizosphere (Coelho *et al*., 2009). Previous study conducted by Yoshida *et al.* (1973) concluded that N_2 fixation was completely inhibited when 400 kg ha^{-1}fertilizer N was applied to a paddy soil. In addition, laboratory experiment conducted by Rao (1976) reported that N application of 100 to 150 $kgha^{-1}$ inhibited the N_2 fixation to approximately 60% and completely suppressed when more than 300 $kgha^{-1}$ N was applied (Tanaka *et al*., 2006).

Besides chemical fertilizer application free living associative bacteria and endophytes can supplement nitrogen to the plant and improve soil N. For sustainable rice production there is a need of genotypes with better N_2 fixation stimulation traits. Genetic variability for N_2 fixation feature exists in rice. The trait is heritable selected and can be used in breeding of rice genotypes with high biological N_2 fixation (Reddy *et al*., 2002). Diazotroph are N_2 fixing bacteria that colonize and contribute biological nitrogen to the crops (Kundu and Ladha, 1995). Rice plant can form natural associations

with various N_2-fixing bacteria, both phototrophs and heterotrophs. These diazotrophs can improve growth and development of rice plants by transferring fixed N_2 or by producing phytohormone. The N_2 fixed by asymbiotic diazotroph may not be immediately available for plant growth. The plant may benefit from asymbiotic N_2 fixation in the long term, as nitrogen gets released through biomass turnover (Dobbelaere *et al.,* 2003). Biological nitrogen fixation by the diazotrophs is an energy involving process and about 64-86 % of the carbon released into the rhizosphere is respired by microorganisms (Hutsch *et al.,* 2002). Diazotroph utilized rhizosphere carbon substrates as their energy and fix N_2 from the atmosphere and form natural association with plants.

Nitrogen is obligatory for all living cells and the N content in soil has influence on total microbial population. Application of high N may benefit total bacterial population. Soil microorganisms would use this excessive N for their growth and metabolism which will reduce the N concentration in the soil and indirectly reduce environmental pollution. Hence, the present studies were conducted with the aim to assess the influence of high N fertilizer on *Stenotrophomonas maltophila* nitrogenase activity, colonization on rice rootsandtheir effects on bacterial population and growth of wetland rice.

MATERIALS AND METHODS

Substrate preparation and inoculation: The strain used in this study was *Stenotrophomonas maltophila*, a N_2 fixing bacteria previously isolated from rice field in Tanjung Karang, Selangor (Naher *et al*., 2008). The bacterial strain was Gram negative rod, with cellulolytic enzyme activity, high IAA (60 mg L^{-1}), nitrogenase activity of 1.4×10^{-7} µmol $C_2H_4^{-1}$ cfu^{-1} h^{-1} and 43% Nfda (Naher *et al.,* 2011; Naher *et al.,* 2009). Cells were grown in N-free liquid medium and shaken for 48 hours at 130 rpm. Composition of broth (L^{-1}): 20.0 g sucrose, 1.0 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g NaCl, 0.1 g $FeSO_4$, 0.005 g Na_2MoO_4, 2.0 g $CaCO_3$. pH adjusted to 6.8 – 7.0. Approximately, 1×10^6 live washed cells were used to inoculate fresh N-free medium with different concentrations of N (0, 50, 100, 200 and 300 mgL^{-1}) in the form of urea. The cultures were shaken at 100 rpm for 48 hours. Bacterial population in each concentration was determined using drop plate technique and nitrogenase activity was measured by acetylene reduction assay (ARA).

Acetylene reduction assay: Acetylene reduction assay (ARA) of the bacteria was performed immediately after 48 hours of growth. The cultures were aseptically transferred to sterile vacuum tube (Vacutainer). By using an airtight syringe, 10% (v/v) of the air from the head-phase of each bottle was removed and replaced with purified acetylene gas (99.8%). The cultures were incubated for 30 min for the reduction of acetylene (C_2H_2) to ethylene (C_2H_4) to take place. Gas samples (0.5 mL) were removed after 30 min and assayed for C_2H_4 production using a gas chromatograph (Perkin Elmer Auto system GC) with flame ionization detector (FID). Nitrogen was used as the carrier gas and the temperature for detector and injector was maintained at 200ºC and 60ºC, respectively. Values were expressed as µmol C_2H_4 h^{-1} mL^{-1}.

Inoculums preparation and inoculation: Nitrogen-fixing bacteria (*Stenotrophomonas maltophila*) was grown in N-free broth and shaken for 48 hours. At exponential growth phase the broth culture was transferred into Eppendorf tube and centrifuged at 40000 rpm for 40 minutes. The supernatant was decanted and cells was washed with 0.85% sterilize phosphate buffer solution. Approximately, $1x10^9$cfu mL^{-1} of washed cells were applied to each treatment. The bacterial populations were determined by using drop plate technique (Somasegaran and Hoben, 1985).

Seeds germination and surface sterilization: Rice seeds of MR219 were surface sterilized according to the method modified from Amin *et al*. (2004). Rice seeds were immersed in 70% alcohol for 4 minutes and shaken in 10% (w/v) NaOCl solution. The seeds were then washed with sterilized distilled water for 5 seconds and were soaked in fungicide for 3 hours. Rice seeds were blotted dry on sterilized moist filter paper and left to germinate before they were transplanted into pots.

Transplanting: Four uniformedsize 7 day old rice seedlings were transplanted into pots containing 2 kg of unsterilized soil mix containing75% mineral soil and 25% sand. The different rates of urea-N used were 0, 50, 100, 200 and 300 $kgha^{-1}$. Rice seedlings were inoculated with 100 mL of inoculums (approximately 1×10^9cfu mL^{-1}) one week after transplanting and the same amount of autoclaved culture (dead cells) was applied to the control treatment. Plants were harvested at 45 days after transplanting (DAT). At harvest, shoots were separated from roots and the roots were thoroughly washed free of soil with tap water. Bacterial colonies of rhizosphere, endosphere and non-rhizosphere were determined.

Determination of bacterial population

Rhizosphere population: Roots were gently washed with sterilized distilled water and 2-4 g of root was placed into conical flask containing 99 mL of sterilized distilled water. Conical flasks were shaken for 15 minutes at 100 rpm. A series of 10 fold dilutions were prepared. Bacterial population was determined in N-free medium. Composition of the N-free medium was: 5g malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$ 7 H_2O, 0.1 g NaCl, 0.02 g

$CaCl_2$ and 0.5% bromothymol blue in 0.2 N KOH (2 ml), 1.64% Fe-EDTA solution (4 ml), 20 g agar. Other bacterial populations were determined in Nutrient Agar (NA)medium.

Endosphere population: Roots were surface sterilized with 70% ethanol for 3 to 5 min and were treated with 3% sodium hypochlorite for 3 seconds. Stem of the roots were cut into small pieces approximately 5 cm and were surface sterilized by dipping into 95% ethanol. To validate the sterilization procedure 1 cm of each ends were removed and rolled onto nutrient agar. Roots were then homogenized using sterilized mortar and pestle in sterilize 0.85% phosphate buffer solution. A series of 10 fold dilutions were made. Populations of diazotrophs and endophytes were determined on N-free (Nfb) medium using total plate count method.

Non-rhizosphere population: Ten grams of non-rhizosphere soil was placed in to 250 ml Erlenmeyer flask containing 95 ml sterilized water, and content was shaken for 15 to 20 minutes. A series of 10-fold dilutions were prepared up to 10^{-10}. Diazotrophs populations were determined using N-free (Nfb) medium. Other bacterial populations were determined using NA media.

Photosynthesis measurement: The single-leaf net photosynthesis rate (A_{max}) was determined (45 days of transplanting) from youngest expanded leaf (YEL) of each treatment using LI-6200 Portable photosynthesis system, LI-COR Inc. Lincoln, Nebraska, USA. Measurements were done under full sunlight and constant CO_2 of 380 µmol CO_2 mol^{-1} in the chamber.

Statistical analysis: Laboratory study was laid out in a complete randomized design (CRD)while the glasshouse study was a factorial experiment with 2 factors (bacterial inoculation and 5 levels of N).Data were analyzed by ANOVA using SAS statistical program version 9.2. The treatments means were separated using Tukey's test at the 5% level of probability.

RESULTS AND DISCUSSION

Effect of nitrogen on *Stenotrophomonas maltophila* population: Result of the laboratorystudy showed that application of urea-N significantly influenced the population of *Stenotrophomonas maltophila*. Application of 50 mg L^{-1} urea-N significantly increased the bacterial population, however, further increase in the level did not affect the population (Figure1a). The lowest population was found in control treatment. However, the bacteria was able to grow even at 0 mg L^{-1} urea-N indicating that it can fix N_2 as the media used was N-free. Addition of 50 mg L^{-1} urea-N increased 20% bacterial population compared to control. This might be due to the utilization of available N for cell growth. High gelatinous material was found during growth of bacteria in urea-N which could be due to production of extracellular polysaccharide. Castro *et al.* (2008) explained that extracellular polysaccharide is a polymer which plays an essential role for bacterial growth and survival. It protects cell from desiccation and helps in N_2 fixation by preventing high oxygen (O_2) tension (Kumari *et al.*, 2009). Furthermore, it demonstrated the capability of N_2-fixing bacteria to survive on the sources of ammonia and carbon (Kavadia *et al*., 2011).

Effect of N on *Stenotrophomonas maltophila* nitrogenase activity: Application of urea-N significantly affected nitrogenase enzyme activity of *Stenotrophomonas maltophila*. Nitrogenase enzyme activity was reduced with increased rate of urea-N application (Figure 1b). The bacteria showed highest ARA of 0.042 µmol C_2H_4 h^{-1} mL^{-1} at 0 mg L^{-1} urea-N. The application of 50 mg L^{-1}N decreased the nitrogenase activity by 25% and was totally inhibited when 300 mgL^{-1}urea-Nwas added. This can be supported by the previous study of Veronica and Dobereiner (1989) who stated that microorganisms developed a mechanism to turn off nitrogenase enzyme when fixed N is available to supply the organism's need. Addition of NH_4^+ inhibited nitrogenase enzyme activity and probably followed the "NH_4^+ switched off" mechanism. Excess amount of nitrogen in the solution may be utilized for cell metabolism of the bacteria rather than nitrogen fixation process. Brooks *et al*. (2004)stated that at high concentration of NH_4^+, cells assimilate the compound via the glutamate dehydrogenase reaction for cell growth.

Effect of inoculation and N on diazotrophs colonizationon rice plant: Inoculation increased rhizosphere and endosphere population compared to non-inoculated treatment. This proved that inoculation increased diazotrophs colonization at the rhizosphere and endosphere and the bacteria form close association with the rice plants. Previous study of Naher *et al.*(2011) also found that MR219 rice root exuded favored diazotrophs association and root colonization. The rhizosphere populations were significantly higher at 0 $kgha^{-1}$ N treatment and were reduced by 7.6% with addition of 50 $kgha^{-1}$. Further increased of N concentration did not affect the rhizosphere population (Figure 2a). High supply of urea-N (200 $kgha^{-1}$) decreased the endosphere population by 8% (Figure 2b). It is observed that the population of diazotrophs was higher in endosphere than in the rhizosphere. This may be due to the lesser competition for nutrients and low concentration of oxygen inside the root tissue which favored the growth of the diazotrophs (Boddey and Dobereiner, 1995). In addition, the N fertilization had a stronger effect on *nifH* community structure that can suppress the diversity and abundance of N_2-fixing bacteria (Berthrong *et al.,* 2014).

Effect of inoculation and N fertilizer on total bacterial population: There were significant effects of diazotrophs inoculation and N fertilizer application on the non-nitrogen fixing soil bacteria. Nitrogen-fixing bacterial population was found higher in the non-rhizosphere soil compared to plant rhizosphere and endosphere. The addition of 50 $kgha^{-1}$or moreof N fertilizer significantly increased the bacterial population in both inoculated and innon-inoculated treatments(Figure 2c). This might be due to the less NH_4 presence in the soil at lower rate and with increase of N application, population was slightly decreased and remain constant at higher rates of N. In general, this result suggests that soil indigenous microorganisms responded rapidly to increased N application. The decrease in the proportion of diazotrophs suggests a competitive suppression by non-diazotrophs in the presence of high N (Kolb and Martin, 1988). Similar findings were observed by Mihir Lal and Srivastava Ramesh (2014). The diazotrophs population in paddy soil was enhanced with N_2-fixing bacteria inoculation. The population was severely affected at 75 and 100% of the recommended N fertilizer rate. The diazotrophs number at any plant development stage significantly increased compared to plots that did not receive urea-N application (Hai*et al.,* 2009).

Effect of N on plant growth: Inoculation with *Stenotrophomonasmaltophila*and application of different rates of N significantly affected the photosynthesisand plant biomass compared to non-inoculated plants(Figure 3a). Regardless of different N treatments, photosynthesis was higher in the inoculated than the non-inoculated plants. Photosynthesis was highest at 50 $kgha^{-1}$Nand decreased 56% after addition of 300 $kgha^{-1}$.The highest photosynthesis at the lower rates of N might be due to the initial low requirement of N application. Addition of N increased shoot biomass in both inoculated and non-inoculated treatments and the highest biomass was obtained in inoculated plants applied with 200 kg N ha^{-1}.Higher N application reduced the plant biomass. The study is in agreement with Asilah *et al*., (2012) who found that application of more than 60 kg N ha^{-1}in the presence of diazotrophs significantly reduced MR219 rice growth. On the other hand plant growth in inoculated treatment at low N concentration (0 $kgha^{-1}$N) was low and this could probably be due to competition for N. It is also known that soil bacteria are better competitors than plants as their growth rate is faster than plants. Meanwhile, plant growth was retarded by high N rate of 300 kg ha^{-1} which can be toxic to plants (Li *et al*., 2011). In soil with high N concentration, microorganism would use the available N for their rapid growth and metabolism rather than fixing the N_2. Shrestha and Maskey (2005) also reported that excessive use of N fertilizer to lowland rice significantly improved plant growth but inhibited N_2 fixation by diazotrophic bacteria. On the other hand inoculation of the diazotrophs was most effective to increase the nitrogen status of the rhizosphere soils that was similar effect under the non-inoculated soils with application of 100 kg ha^{-1} as urea-N (Das and Saha, 2007).

Plant growth in the non-inoculated treatment was also found to be high. This might be due to the initial soil N content. In general, higher plant photosynthesis and biomass were found in inoculated compared to non-inoculated treatments. This might be the resultant effect of phytohormones production by the inoculated bacteria. Similar finding were reported by the Laskar and Sharma (2013) that the nitrogen fixing bacteria KR-6 (*Stenotrophomonas maltophila*) and KR-7 (*Herbispirillumrubrisubalbicans*) have great potential for nitrogen fixing and produced phytohormones that may lead to improved plant growth.

In conclusion the studyshowed that high N supply had negative effect on the diazotrophs population and nitrogenase activity as well as plant growth. Inoculation of *Stenotrophomonas maltophila* with lower N (50 $kgha^{-1}$) increased colonization in the rhizosphere and endosphere and enhanced rice plant photosynthesis, and shoot and root biomass.

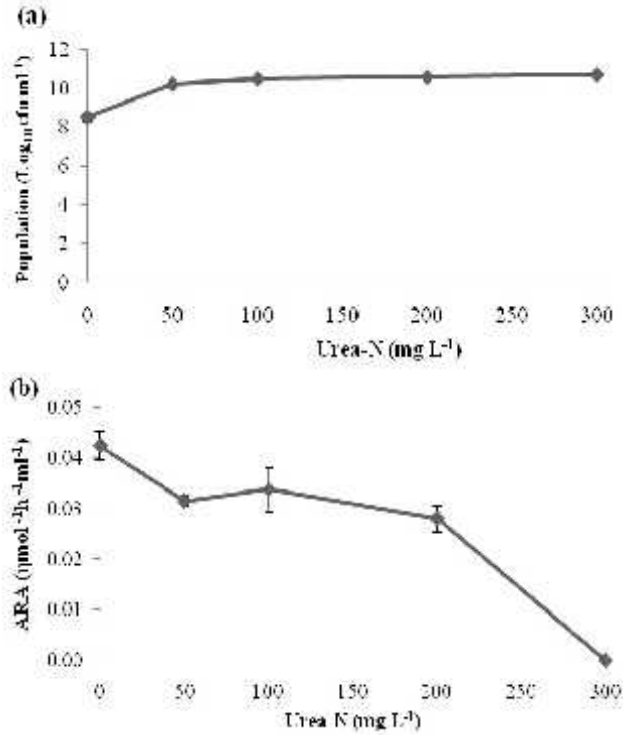

Figure 1. (a)Effect of different levels of urea-N on population of *Stenotrophomonas maltophila* (b) Effect of different levels of N on nitrogense activity of *Stenotrophomonas maltophila*. Bars indicate SE (n=4)

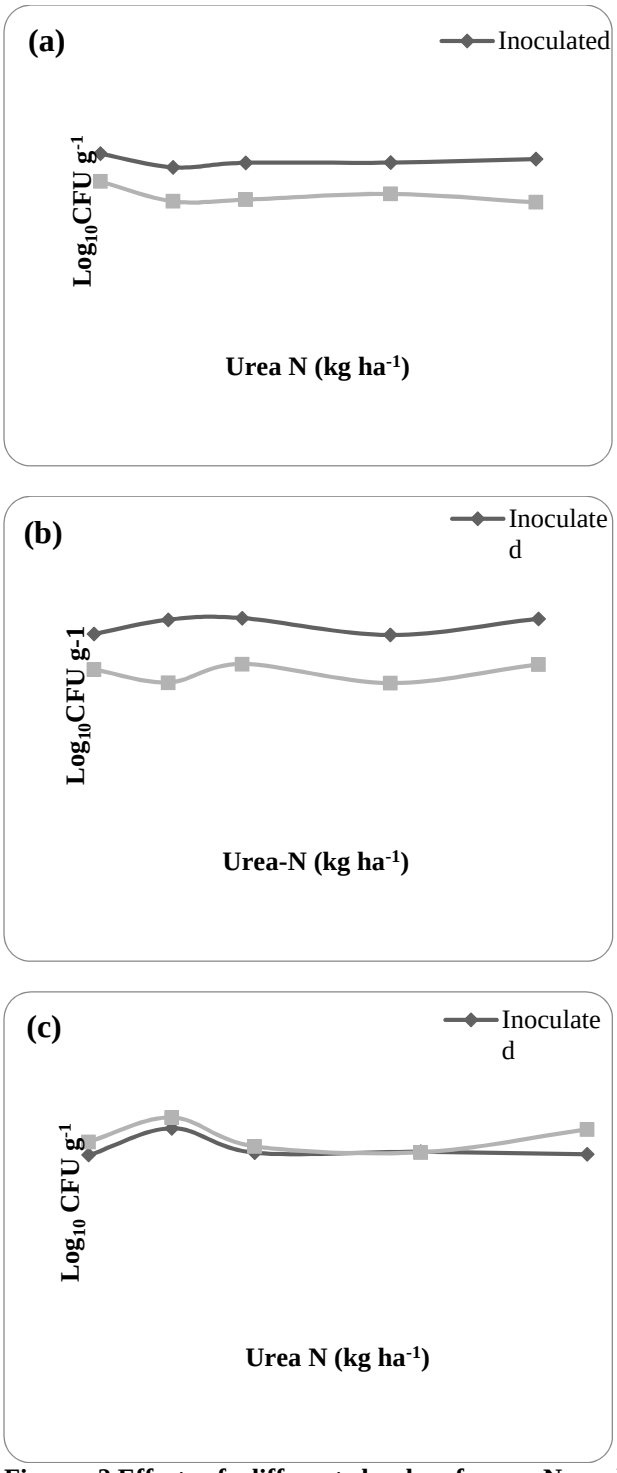

Figure 2.Effect of different levels of urea-N and *Stenotrophomonasmaltophila*inoculation on MR219 rice colonization and total bacterial population. (a) rhizosphere population. (b) endosphere population (c) non-rhizosphere. Bars indicate SE (n=5)

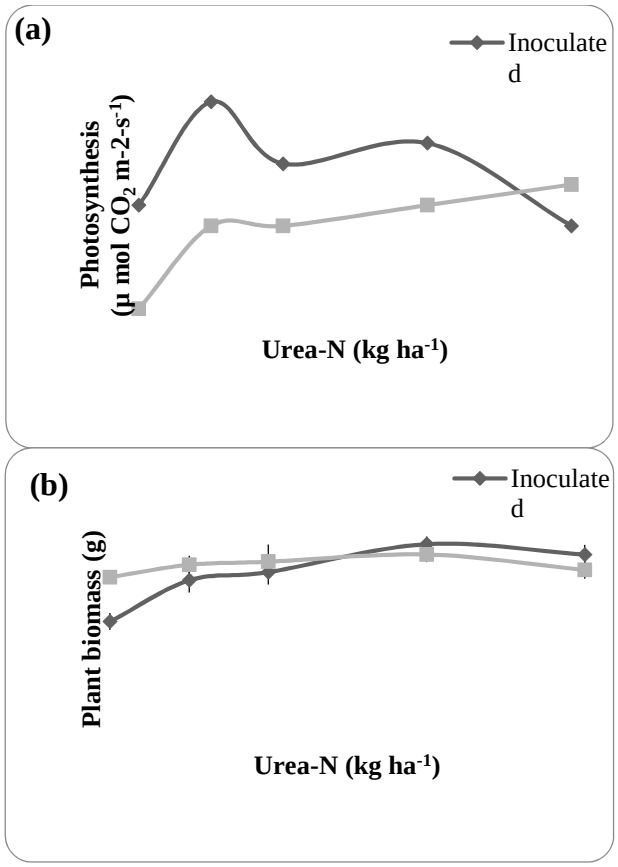

Figure 3.Effect of different levels of urea-N and *Stenotrophomonas maltophila* inoculation on MR219 rice (a) photosynthesis, (b) plant biomass production. Bars indicate SE (n=5).

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