Polish Journal of Microbiology
2016, Vol. 65, No 3, 377–382

SHORT COMMUNICATION

Effects of Selected Herbicides on Growth and Nitrogen Fixing Activity of *Stenotrophomonas maltophilia* (Sb16)

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Submitted 5 July 2015, revised 15 January 2016, accepted 11 February 2016

Abstract

A study was carried out to determine the effects of paraquat, pretilachlor and 2, 4-D on growth and nitrogen fixing activity of *Stenotrophomonas maltophilia* (Sb16) and pH of Jensen's N-free medium. The growth of Sb16 and pH of medium were significantly reduced with full (X) and double (2X) doses of tested herbicides, but nitrogen fixing activity was decreased by 2X doses. The nitrogenase activity had the highest value in samples treated with 1/2X of 2, 4-D on fifth incubation day, but 2X of 2, 4-D had the most adverse effect. An inhibition in the growth and nitrogenase activity was recovered on the last days of incubation.

Key words: *Stenotrophomonas maltophilia* (Sb16), nitrogenase activity, paraquat, pretilachlor, 2, 4-D

Microorganisms employed to enhance the availability of nitrogen by fixing atmospheric N_2 to the crops are called N_2 fixing bacteria. There is a natural association between rice plant and N_2 fixing bacteria. *Stenotrophomonas maltophilia* is broadly found on or in plants and has a worldwide distribution (Denton and Kerr, 1998). Diazotrophic *S. Maltophilia* strain Sb16 isolated from Tanjong Karang, a rice (Oryza sativa L.) growing area in Selangor, Malaysia, has been proven to improve rice production (Naher *et al*., 2009). The Sb16 strain used in this study was obtained from the Soil Microbiology Laboratory, Department of Land Management, Universiti Putra Malaysia (UPM), Serdang, Selangor. Herbicides have been the most efficient chemical weed management approach since they were introduced to agriculture. They may cause undesirable effects when applied at high concentrations. Various herbicides act on various types of plant species, on various processes of plant metabolism, and at various periods in plant growth cycles. Translocated herbicides are taken up into the plant's vascular system, while contact herbicides only affect the part of the plant contacted by the spray. Some herbicides represent a risk of vapourising to other sites, while others stay effective for a long term in the soil, harming planted crops at a later time. If they come into contact, herbicides applied to soil or plants might interfere with the microbial biofertiliser inoculated to crop plants. The herbicides commonly used for rice production are paraquat, glyphosate, oxadiazon, propanil, pretilachlor, 2, 4-D, *etc*. Paraquat, pretilachlor and 2, 4-D have been chosen for the present study. The effect of herbicides on N_2 fixing activity of diazotrophs is a major concern among researchers as it is vital to the soil fertility of rice fields. Meanwhile, studies on the effects of herbicides on symbiotic N_2 fixation have concentrated on *Rhizobium* sp. under *in vitro* conditions (Moorman, 1986). However, there are insufficient studies on the effects of herbicides on other species of N_2 fixing bacteria. Besides, as attributing of N_2 fixation to the specific bacterium in the plant system is impossible, the effects of herbicides on N_2 fixation ability of the specific bacterium need to be determined in laboratory conditions to predict the role of strain in N_2 fixation process in the plant system under natural soil condition. The objective of the present investigation was to determine the effect of paraquat, pretilachlor and 2, 4-D at concentrations corresponding to 1/2, 1 and 2 times of their recommended field application rate (X) on the growth and N_2 fixing

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activity of Sb16 and pH of Jensen's N-free medium (Jensen, 1951) within a determined incubation time.

Medium was inoculated with a colony of Sb16 strain and incubated at 35°C on a rotary shaker at 150 rpm for 3 days. Aliquots of 1 ml of approximately 10^8 cfu/ml of live cells, with adjusted optical density $(OD)_{600}$ of ≈ 0.1, was inoculated to each culture flask.

The herbicides used in this study were paraquat dichloride (13% w/w) Syngenta Capayam, pretilachlor (28.7% w/w) Syngenta Sofit N300 EC and 2, 4-D isopropylamine (28% equivalent) (35.5 % w/w) Kompressor Ancom Cropcare. The herbicides solutions were prepared by mixing the required amount of active ingredient in sterilised distilled water to obtain concentrations corresponding to 0, 1/2, 1 and 2 times of the recommended field application rate. Therefore, four rates of 0, 0.78, 1.56 and 3.12 mg/ml of paraquat at the rate of 12 g/l; 0, 0.72, 1.44 and 2.87 mg/ml of pretilachlor at 5 g/l; 0, 1.42, 2.84 and 5.68 mg/ml of 2, 4-D at 8 g/l were prepared to get the concentrations of 0, 1/2, 1 and 2 times of the recommended field application rate, respectively. Thereafter, herbicides were sterilised by filtration (Millipore filter, 0.22 µm) aseptically in a laminar flow cabinet.

The prepared herbicides solutions were added to each sterilised flask (250 ml) containing 75 ml Jensen's N-free broth medium. Control flasks were without herbicides. Before inoculum application, optical density (OD_{600}) of inoculum was checked and regulated to approximately 0.1 and the drop plate method for cell count on N-free agar was employed to confirm the population (Somasegaran and Hoben, 1985). Aliquots of 1 ml of the desired inoculum (approximately 10^8 cfu/ml) of live cells was transferred to each culture flask using sterilised pipette. The flasks were incubated at 28°C on a rotary shaker for 7 days till the end of the experiment.

At each sampling period, 1 ml of the culture was sampled and 10-fold serial dilutions were made up to 10^{-8}. The mixture in each test tube was shaken vigorously to suspend bacterial cells. Aliquots of 0.1 ml of appropriate dilutions was placed on each Jensen's agar plate. The plates were then incubated at 32°C. The population was determined using the drop plate method at 24 h intervals within 7 days.

The N_2 fixing activity of Sb16 strain in Jensen's N-free broth, amended with paraquat, pretilachlor and 2, 4-D, was determined using acetylene reduction assay (ARA) based on the methods by Hardy *et al.* (1968) and Somasegaran and Hoben (1985). At every 24 h interval, 1 ml of the suspension was taken from each flask and transferred to a 10 ml air-tight Syringe. A sample of 10% of air was extracted from each syringe and pure acetylene gas (99.8%) was injected with a gas-tight syringe. The syringes containing bacterial suspensions were allowed to incubate on incubatory shaker for 1 h. Thereafter, 1 ml of the air sample from each incubated syringe was injected into a Gas Chromatography (HP 6890) equipped with Hydrogen Flame Ionisation Detector (FID) with a temperature of 120°C, injector temperature 150°C with Column (Agilent J&W GC Column, HP-PLOT/Q, 30 M, ID 0.53, Film Thickness 40 µm) and carrier gas (nitrogen) 70–80 kPa for lighting the FID, Hydrogen 100 kPa and air 10 kPa. The produced ethylene was determined based on the transformation rate of acetylene to ethylene (% v/v).

Changes in the pH of Jensen's N-free broth, inoculated with Sb16 strain and amended with herbicides, were determined using a standard pH meter (pHM 210, MeterLab®) equipped with a glass electrode at every 24 h interval within 7 days.

The study was conducted as factorial complete randomised design (CRD) with four replications. The factors were 3 types of herbicides with 4 different concentrations and 7 incubation periods. The data were subjected to analysis of variance (ANOVA) and analysed using SAS (version 9.3). The treatment means were compared by using Duncan's multiple range test (DMRT). The number of bacteria was $\log_{10}$ transformed before statistical analysis.

Results of our study shown that population of bacteria in the presence of each of the herbicides exhibited a similar trend. The population significantly increased from day 1 – day 3 of the incubation period. However, it decreased on the fourth day, followed by a significant increase on the fifth day. The population significantly declined from the sixth-seventh day. Meanwhile, the population in the samples treated with double dose of paraquat reached that of the control sample on the last day of incubation (Fig. 1A). In particular, Sb16 had the lowest population (6.25 $\log_{10}$ cfu/ml) in the samples amended with double dose of pretilachlor on the seventh day (Fig. 1B). However, the highest population (7.93 $\log_{10}$ cfu/ml) was obtained in the samples

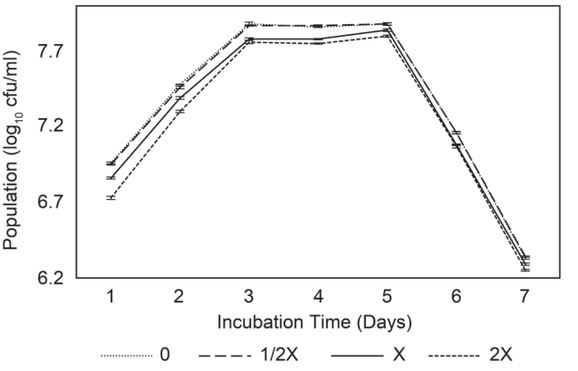

Fig. 1A. Effect of different concentrations of paraquat on population of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

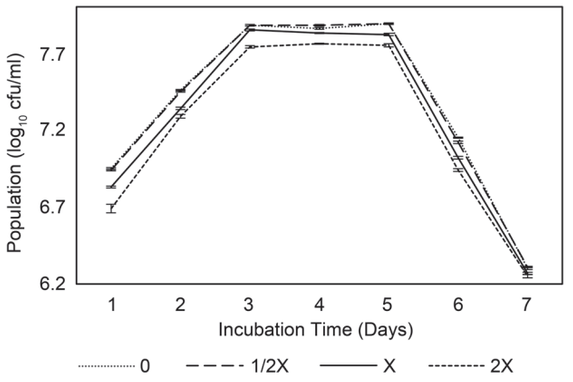

Fig. 1B. Effect of different concentrations of pretilachlor on population of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

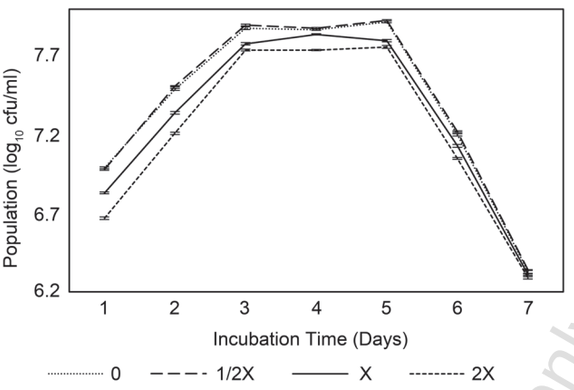

Fig. 1C. Effect of different concentrations of 2, 4-D on population of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

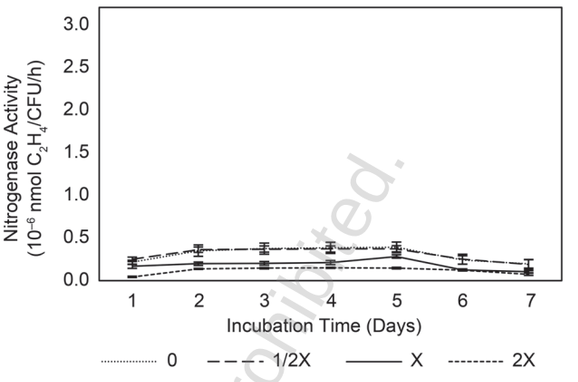

Fig. 2A. Effect of different concentrations of paraquat on nitrogenase activity of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

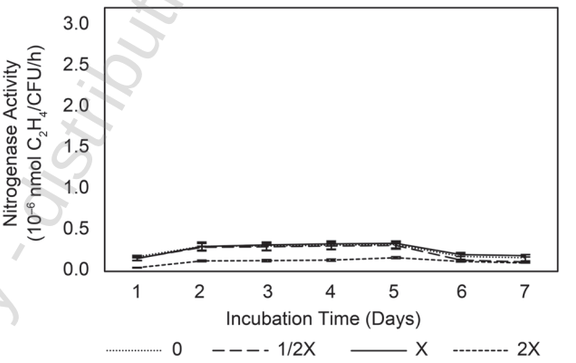

Fig. 2B. Effect of different concentrations of pretilachlor on nitrogenase activity of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

amended with half dose of 2, 4-D on the fifth day (Fig. 1C). Based on the results of statistical analysis, the population was significantly influenced by the type of herbicides. The population decreased significantly upon treatment with herbicides at full and double doses when compared with the control. There was a significant three-way interaction effect between the herbicides, concentrations and incubation time on the Sb16 population.

The nitrogenase activity in the presence of each of the herbicides increased from the first to fifth incubation day, followed by a decline up to day 7. The nitrogenase activity in the samples amended with double dose of paraquat started to decline from the fifth incubation day, but those with the half and full doses decreased from the sixth day (Fig. 2A). The nitrogenase activity in the samples amended with full dose of pretilachlor reached to that treated with half dose on the second day (Fig. 2B.). The highest nitrogenase activity (3.1×10^{-6} nmol C_2H_4/cfu/h) was obtained in the samples amended with half dose of 2, 4-D on

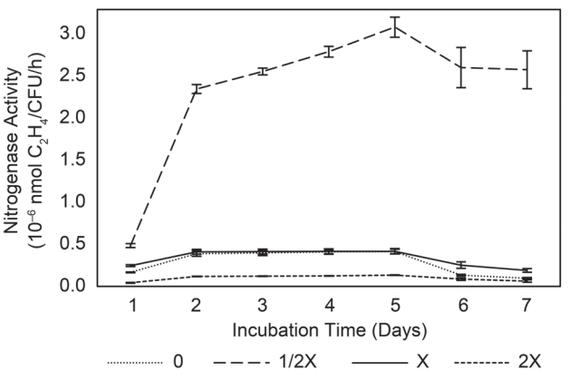

Fig. 2C. Effect of different concentrations of 2, 4-D on nitrogenase activity of diazotrophic Sb16 in Jensen's N-free medium during 7 days of incubation period; Bars indicate standard error (n = 4).

the fifth day. However, the lowest nitrogenase activity (4.2×10^{-8} nmol C_2H_4/cfu/h) was recorded in the samples amended with double dose of 2, 4-D on the first day (Fig. 2C). Results of the statistical analysis

showed no significant differences in nitrogenase activity between samples amended with paraquat and pretilachlor. Addition of half dose of herbicides resulted in a significant increase in nitrogenase activity compared to the control, while nitrogenase activity decreased significantly in the presence of double dose of herbicides. Herbicides, concentrations and incubation time showed a significant three-way interaction effect on the nitrogenase activity of Sb16.

Acidity (pH) in the presence of each of the herbicides significantly increased from days 1–5 of incubation, followed by a significant decline till day 7. Acidity in the samples amended with half, full and double doses of paraquat reached the value of the control sample on the seventh day (Fig. 3A). The highest pH in herbicide amended samples was recorded by 7.46 with half dose of pretilachlor on the fifth day (Fig. 3B). However, the lowest pH (6.8) was obtained in the samples amended with double dose of 2, 4-D on the first day (Fig. 3C). The statistical study of the results showed that pH did not differ significantly between samples amended with paraquat and pretilachlor. Similarly, pH of Jensen's medium was not affected significantly by half dose of herbicides but significantly decreased in the presence of full and double doses of herbicides. There was a significant three-way interaction effect between herbicides, concentrations and incubation time on the pH of Jensen's medium.

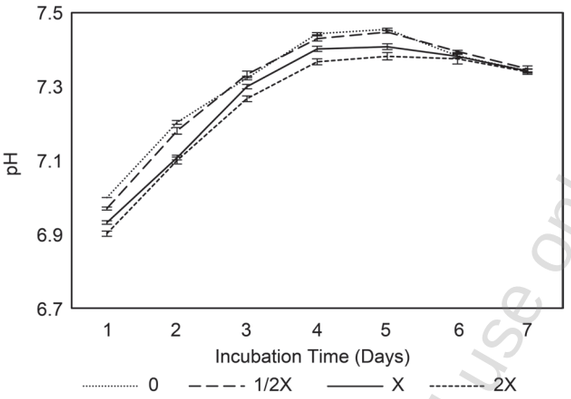

Fig. 3A. Effect of different concentrations of paraquat on pH of Jensen's N-free medium inoculated with diazotrophic Sb16 during 7 days of incubation period; Bars indicate standard error (n = 4).

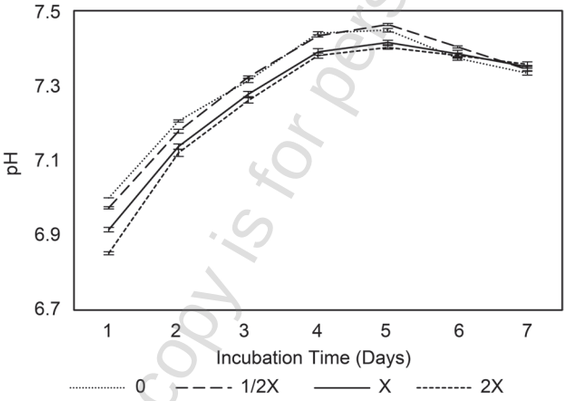

Fig. 3B. Effect of different concentrations of pretilachlor on pH of Jensen's N-free medium inoculated with diazotrophic Sb16 during 7 days of incubation period; Bars indicate standard error (n = 4).

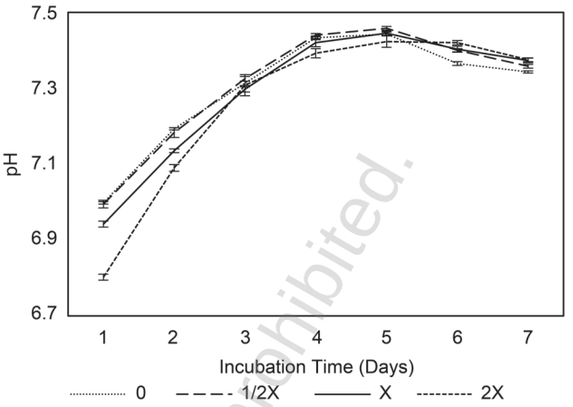

Fig. 3C. Effect of different concentrations of 2, 4-D on pH of Jensen's N-free medium inoculated with diazotrophic Sb16 during 7 days of incubation period; Bars indicate standard error (n = 4).

An inhibition in the growth of Sb16 resulting from the addition of higher concentrations of herbicides was recovered on the last incubation days. This finding concurs with the study of Latha and Gopal (2010) which revealed an initial decrease in the growth of *Azospirillum* lipoferum with herbicides 2, 4-D, butachlor, pretilachlor and pyrazosulfuron compared to the control treatment after 24 h of incubation, followed by an increase over time. The increase in bacterial population over time can be due to the mineralisation of the herbicides by the bacteria as energy and carbon sources. Soil microorganisms use many herbicides as good carbon and/or nitrogen sources (Qiu *et al.*, 2009). The insignificant effect of half dose of herbicides on growth of Sb16 and pH of medium can be explained by alterations in the medium nutrient, chemical structure and herbicide degradation by the bacteria. Ayansina and Oso (2006) found lower microbial counts in higher concentrations of herbicides compared to the recommended doses.

Adeleye *et al.* (2004) studied the toxicity of 2, 4-D amine to *Bacillus subtilis* and found that a decrease in the survival percentage occurred at higher concentrations. Smith and Beadle (2008) also reported the toxicity of 2, 4-D and its metabolic intermediates on *Burkholderia cepacia.* The significant stimulation in the nitrogenase activity of Sb16 in lower concentrations of 2, 4-D in the present study corresponds to the findings of Saikia *et al.* (2014) who reported a higher rate of acetylene reduction in seedling roots of citronella ino-

culated with *Azospirillum brasilense* and treated with 2, 4-D than with *A. brasilense* alone. 2, 4-D does not only seem to affect several distinct metabolic pathways in a variety of organisms, but shows a direct biphasic effect depending on its initial concentration (Toyoshiba *et al.*, 2006). At low concentrations, 2, 4-D might stimulate growth of the organism by cell division and elongation; however, it may induce abnormalities at high concentrations. Non-targeted organisms exposed to 2, 4-D might respond by starting a metabolic chain-reaction that is commonly associated with changes in the cellular membrane integrity and fluidity.

Based on the data obtained on growth and N_2 fixing activity in the present study, paraquat showed moderate toxicity on Sb16 strain. Drouin *et al.* (2010) found that paraquat applied in a range between 0.367–367 kg/ha had no bactericidal effect on *Sinorhizobium* strains, but it inhibited three strains of *Bradyrhizobium* at 36.7 kg/ha. Paraquat toxicity is thought to be mediated by the superoxide anion (O_2^-), a reactive species generated by the reoxidation of reduced paraquat by molecular oxygen (Bus *et al.*, 1974).

According to the results of the present study, pretilachlor had the highest adverse effect on the growth and nitrogenase activity of Sb16. However, as research on the effect of pretilachlor on pure cultures of bacterial strains *in vitro* condition is scarce, the exact mechanism of the action of this chemical in microorganisms remains unknown. In general, the microbial degradation of chloroacetanilide herbicides can be initiated by two reactions; formation of glutathione conjugate (Stamper and Tuovinen, 1998) or N-dealkylation (Li *et al.,* 2013).

Meanwhile, during the incubation period, a variety of factors can influence the growth and nitrogenase activity of bacteria. These factors include O_2, CO_2, nutrients and inorganic salts of the medium, as well as some environmental factors such as light, pH and temperature. Thus, changes in any one of these factors, within the seven-day culture, affect the growth and nitrogenase activity of Sb16.

In the present study, the decrease of pH in the Jensen's medium amended with herbicides could be due to the decrease in the growth and nitrogenase activity of Sb16 following the contact with herbicides, leading to a more acidic medium. Optimal pH for N_2 fixation is 5–8 (Leigh, 2002). Through N_2 fixation by Sb16, ammonium ion (NH_4^+) can be produced. The presence of high NH_4^+ concentration increases pH of the medium. The increase in the pH of the medium on the last incubation days after the initial inhibition could be related to an increase in the growth and activity of Sb16 and also adaptability to stressful conditions.

The study showed that the tested herbicides at half dose had a positive effect on the growth and nitrogenase activity of Sb16. However, double dose significantly decreased the growth and nitrogenase activity of Sb16 and pH of the Jensen's medium. An increase in the activity and growth of Sb16 and pH of the Jensen's medium amended with double doses was found to occur on the last incubation days. It can be concluded that at their recommended doses, the tested herbicides might have insignificant effects on the growth and nitrogenase activity of Sb16 under natural field conditions. Further studies involving other strains of *S. maltophilia* need to be carried out so as to better clarify the susceptibility of these diazotrophic strains to the herbicides.

Acknowledgements

The authors are grateful to the Department of Land Management, Faculty of Agriculture and Department of Preclinical Sciences, Faculty of Veterinary, UPM.

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