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EFFECTS OF SELECTED HERBICIDES ON ACTIVITY OF Stenotrophomonas maltophilia AND GROWTH OF AEROBIC RICE, AND THEIR PERSISTENCE IN SOIL

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EFFECTS OF SELECTED HERBICIDES ON ACTIVITY OF *Stenotrophomonas maltophilia* AND GROWTH OF AEROBIC RICE, AND THEIR PERSISTENCE IN SOIL

By

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EFFECTS OF SELECTED HERBICIDES ON ACTIVITY OF *Stenotrophomonas maltophilia* AND GROWTH OF AEROBIC RICE, AND THEIR PERSISTENCE IN SOIL

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April 2015

Chairman: Associate Professor Radziah Othman

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Aerobic rice cultivation has come up as a promising alternative for flood-irrigated rice system in terms of water use recently. Bio-fertilizer, consisting of beneficial N_2 fixing bacteria can promote crop production and nutrient uptake efficiency. Herbicides, the most succesfull chemical weed management approach in direct seeded rice may cause hazardous effects on plant and soil microbes when they come into contact. This study was conducted in laboratory and glasshouse conditions with the following objectives; (i) to determine the effects of three selected rice herbicides on growth and N_2 fixing activity of diazotrophic *Stenotrophomonas maltophilia* (Sb16) (ii) to determine the effects of Sb16 bacterial inoculation and application of three selected rice herbicides on growth of aerobic rice and the soil microbial and chemical properties (iii) to determine the effect of Sb16 on persistence of three selected rice herbicides in soil. The effects of paraquat, pretilachlor and 2, 4-D on growth and N_2 fixing activity of Sb16 and pH of Jensen N-free medium were determined *in-vitro* at every 24-h interval within 7 days of incubation period. The effects of Sb16 bacterial inoculation and application of paraquat, pretilachlor and 2, 4-D on growth of aerobic rice, soil microbial population and chemical properties and their persistence in sterilized and non-sterilized soil were determined under glasshouse condition. Results from *in-vitro* experiment showed the significant ($P \leq 0.05$) decrease of growth of Sb16 by 7.29 and 7.22 $\log_{10}$ cfu.mL^{-1} in samples amended with full and double doses of herbicides compared to control and half dose (7.37 $\log_{10}$ cfu.mL^{-1}). N_2 fixing activity of Sb16 significantly ($P \leq 0.05$) increased by 1.66 Nmol C_2H_4/mL/h with half dose of 2, 4-D compared to control (0.58 Nmol C_2H_4/mL/h). The growth parameters of aerobic rice, population of total bacteria and diazotrophs and soil chemical properties showed higher values in inoculated samples treated with herbicides compared to non-inoculated samples. The longest half lives of paraquat in sterilized and non-sterilized soil were

recorded by 866.38 and 198.3 days in non-inoculated samples treated with double dose. Half lives of 58.74 and 99.01 days were obtained in inoculated and non-inoculated sterilized soil samples, respectively treated with double dose of pretilachlor. Sb16 is recommended as a beneficial biofertilizer in aerobic rice cultivation. Sb16 can promote the aerobic rice production and nutrient uptake in soil applied with paraquat, pretilachlor and 2, 4-D at their recommended dose. Sb16 might be useful in decontamination of aerobic rice field soil applied with pretilachlor and 2, 4-D at their recommended dose under natural soil conditions.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia Sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN-KESAN RACUN RUMPAI TERPILIH PADA AKTIVITI *Stenotrophomonas maltophilia* DAN PERTUMBUHAN PADI AEROB, DAN KETAHANAN DALAM TANAH

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Penanaman padi aerob telah menjadi alternatif yang baik terhadap sistem penanaman anaerob dalam kecekapan penggunaan air. Bio-fertilizer, mengandungi bakteria pengikat N_2 yang meningkatkan hasil tanaman dan kecekapan pengambilan nutrien. Racun rumpai, bahan kimia yang paling Berjaya dalam pengawalan rumpai dalam kaedah tabor terus padi boleh mendatangkan kesan yang berbahaya kepada tumbuhan dan mikrob tanah apabila berlaku sentuhan. Kajian ini teluh dilakukan di lab dan rumah kaca dengan objektif berikut: i) Untuk mengkaji kesan tiga jenis racun rumpai yang terpilih terhadap pertumbuhan dan aktiviti N_2 bakteria *Stenotrophomonas maltophilia* (Sb16) ii) Untuk mengkaji kesan inokulasi Sb16 dan aplikasi tiga jenis racun rumpai terhadap pertumbuhan padi aerob dan mikrob tanah dan sifat-sifat kimia iii) Untuk mengkaji kesan Sb16 terhadap ketahanan racun rumpai dalam tanah. Kesan paraquat, pretilachlor dan 2, 4-D terhadap pertumbuhan dan aktiviti pengikatan N_2 oleh Sb16 dan pH media Jensen bebas N ditentukan secara in-vitro pada setiap 24 jam selamasa dalam masa 7 hari inkubasi. Kesan inokulasi Sb16 dan aplikasi paraquat, pretilachlor serta 2, 4-D terhadap pertumbuhan padi aerob, populasi mikrob tanah dan sifat-sifat kimia dan ketahanan dalam tanah steril dan tak-steril ditentukan dalam keadaan rumah kaca. Keputusan kajian in-vitro mendapati kesan yang signifikan ($P \leq 0.05$) pengurangan pertumbuhan Sb16 sebanyak 7.29 dan 7.22 Log_{10} cfu.mL^{-1} dalam sampel yang mengandongi dos penuh dan dua kali gand dos penuh racun rumpai berbanding kawalan dan dos separon (7.37 Log_{10} cfu.mL^{-1}. Aktiviti pengikatan N_2 oleh Sb16 meningkat ($P \leq 0.05$) sebanyak 1.66 Nmol C_2H_4/mL/h dengan separuh dos 2, 4-D berbanding kawalan (0.58 Nmol C_2H_4/mL/h). Parameter pertumbuhan padi aerobic, populasi keseluruhan bacteria dan sifat-sifat kimia tanah menunjukkan peningkatan terhadap sampel yang diinokulasi oleh bacteria dan racun rumpai berbanding sampel yang tidak diinokulasi. Separuh hayat terpanjang paraquat dalam tanah steril dan

tidak disteril mencatatkan sebanyak 866.38 dan 198.3 hari. Sampel yang tidak di steril dan di rawat dengan dos dua kali ganda. Separuh hayat bagi sampel yang diinokulasi dan tidak diinokulasi tanah steril mencatatkan separuh hayat selama 58.74 dan 99.01 hari. Keduanya di rawat dengan dos dua kali ganda pretilachlor. Sb16 adalah disyorkan sebagai biobaja bermanfaat dalam penanaman padi aerobik. Sb16 boleh menggalakkan pengeluaran padi aerobik dan pengambilan nutrien dalam tanah digunakan dengan paraquat, pretilachlor dan 2, 4-D pada dos yang mereka cadangkan. Sb16 mungkin berguna dalam dekontaminasi daripada aerobik tanah sawah digunakan dengan pretilachlor dan 2, 4-D pada dos mereka disyorkan di bawah keadaan semula jadi tanah.

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I certify that a Thesis Examination Committee has met on 17 April 2015 to conduct the final examination of Armita Nahi on her thesis entitled "Effects of Selected Herbicides on Activity of *Stenotrophomonas maltophilia* and Growth of Aerobic Rice, and Their Persistence in Soil" in accordance with the Universities and University College Act 1971 and the Constitution of the University Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ARA	Acetylene Reduction Assay
BDL	Below Detectable Level
BNF	Biological Nitrogen Fixation
CEC	Cation Exchange Capacity
Cfu	Colony Forming Unit
CRD	Completely Randomized Design
DAA	Days After Application
DAT	Days After Transplanting
DMRT	Duncan Multiple Range Test
DNA	Deoxyribonucleic acid
HPLC	High Performance Liquid Chromatography
GC	Gas Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
NA	Nutrient Agar
Nfb	Nitrogen Free Broth
OD	Optical Density
OM	Organic Matter
PDA	Potato Dextrose Agar
PGPB	Plant Growth Promoting Bacteria

PGPR	Plant Growth Promoting Rhizobacteia
RCBD	Randomized Complete Block Design
RNA	Ribonucleic acid
SAS	Statistical Analysis System
SD	Standard deviation
SPAD	Soil Plant Analysis Division: Chlorophyll meter
UV- VIS- NIR- Spectrophotometric	Ultraviolet- Visible-Near Infrared Reflection Spectrophotometric
WHC	Water Holding Capacity
2, 4-D	2, 4-dichlorophenoxy acetic acid

CHAPTER 1

INTRODUCTION

Rice (*Oryza sativa* L.) is considered the chief food for majority of the population globally (Chauhan and Johnson., 2011). Water plays a significant role in rice production system. Therefore, water-efficient strategies are required to be applied. Aerobic rice system has emerged as the most promising water-wise approach in rice culture system where the crop is cultivated through direct seeding on non-puddled and non-swamped soils (Anwar *et al.*, 2010). Aerobic rice is more inclined toward sustainable agriculture due to lower water requirement during cultivation. In aerobic rice cultivation, lower yield is anticipated compared to flooded rice due to poor water access and seed germination, unfavorable crop stand, high weed competition and nutrients stress.

The use of beneficial microorganisms would further increase the efficiency of natural resources in rice cultivation. In recent decades, soil-plant-microbe interaction has turned out a significant issue in sustainable agriculture system. Many kinds of microorganism have been known to play significant role in plant growth and development, as they inhabit in soil, especially rhizosphere. Therefore, altering the rhizosphere microflora by seed, soil or root inoculation with specific organism is a possible alternative. The use of diazotrophs (N_2 fixing bacteria) as bio-fertilizer could reduce the need for inorganic fertilizer (Sofi and Wani., 2007). A diazotrophic strain Sb16 (previously isolated from rice rhizosphere) as an inoculant was introduced to soil and formed natural association with rice plant (Naher et al., 2009). Investigations have pointed out that indigenous microflora play a key role in the establishment of the introduced microbes.

On the other hand, aerobic rice, causes higher weed pressure (Balasubramanian and Hill., 2002) compared to flood irrigated rice. Hence, an effective weed management approach in aerobic rice cultivation is required toward sustainable weed management. Chemical control is considered the most efficient, cost-effective and practical weed management approach among various alternatives including physical, cultural, biological and chemical control (Hussain *et al*., 2008). The herbicide has been recommended as a viable alternative to hand weeding in direct seeded rice by many researchers working on weed management (Anwar *et al*., 2012). Herbicides might develop a wide range of toxic side effects to the environment despite of their benefits. Since they can cause a contamination in groundwater due to leaching or become immobile and persist on top soil, their fate in soil is gaining a great importance (Ayansina *et al*., 2003). Most herbicides applied will ultimately reach to soil where they come into contact with various microflora performing biochemical transformations related to the plant mineral nutrition. The soil microbes are the first to be influenced directly or indirectly by the herbicides. As they respond immediately to stressful conditions, they are considered as more suitable indicators than other organisms or chemical parameters (Filip., 2002).

Herbicides applied to soil may be removed by soil indigenous microorganisms as well as inoculated microbe, which would lead to an enhancement in microbial population and root and shoot biomass. Microbes could develop extremely well on herbicide compounds in the soil by utilizing them as nutrients and energy sources. Some strains of microbes are capable of utilizing the chemicals and change them to useful compounds, leading to multiplication of the microbes. This will result in an improvement of crop production through immunizing the plant from hazards of herbicides. High amounts of herbicides are applied to soil, as farmers have perceived their benefit. They may come in contact with non-target organisms and show toxicity to soil microflora and plants. The biofertlizer containing bacteria can protect the plant from herbicidal effect through either the improvement of growth of plant or degradation of herbicides in soil. There is no study on the effects of herbicides and N_2 fixing bacteria on aerobic rice. Moreover, there is very little information on effect of introduced bacteria on persistence of herbicides applied to the soil under natural conditions. Therefore this study was conducted with following objectives:

1. To determine the effects of three selected rice herbicides on growth and N_2 fixing activity of *Stenotrophomonas maltophilia* (Sb16)

2. To determine the effects of Sb16 and three selected rice herbicides on growth of aerobic rice, microbial population and chemical properties of soil

3. To determine the effect of Sb16 on persistence of three selected rice herbicides in soil cropping with aerobic rice

CHAPTER 2

LITERATURE REVIEW

2.1 Rice (*Oryza sativa* L.)

Rice (*Oryza sativa* L.) is one of the essential food crops globally. Over a half of the world's population, predominantly in Asia consume the rice as their staple food. After wheat, rice is placed at the second grade among cultivated cereals. A considerable amount of minerals, vitamins, essential fatty acids and dietary fiber are provided from rice in addition to energy. Between 8000 and 15000 years ago, the South-East Asia, India and China were the initial places where the rice (*Oryza sativa* L.) was cultivated. Asia grows and consumes 90% of all rice. An equivalent to around 10% (144 million ha) of total available crop land is allocated for cultivation areas globally. Both temperate and tropical climates from sea level to 3000 m are suitable for rice cultivation. In rice cultivation, the water regimes deployed are 10% of total flooded upland rice; about 45% of irrigated, 45% or 30% of rain-fed flooded lowland rice and up to six m of water of flooded rice. Broad soil varieties including saline, alkaline and acid-sulfur soils are suitable for rice cultivation (OECD., 1999).

2.2 Rice Importance in Malaysia

In Malaysia, more than 100,000 farmers survive their livelihoods through rice cultivation and there are many rice-related occupations. Malaysia has to increase the rice production in order to fulfill the demand for the growing population. In Malaysia like the other parts of the world, the weather changes and natural disasters greatly influence the paddy production. Although the majority of Malaysia's population consumes the rice as staple food, the rice production is not yet profitable. However, Malaysia has established short and long term policies in food security to improve the rice production.

2.3 Aerobic Rice System

In agriculture, a huge crisis regarding the water availability and high price is expected which has endangered the common rice production approach. Aerobic rice has appeared as a bright water-saving system of rice plantation (Anwar *et al*., 2010). In aerobic rice system, dry rice seeds are seeded directly in non-mudded soil, followed by irrigations to maintain the soil status humid instead of being saturated (Tuong and Bouman., 2003). The Malaysian Agricultural Research and Development Institute (MARDI) is working on aerobic rice research since 2005 and has developed several varieties of aerobic rice.

2.4 Nitrogen Requirement

Nitrogen is considered an essential nutrient element in rice production. In order to produce 15-20 kg of grain, 1 kg N is required. The nature, quantity and timing of nitrogen supply critically affect the yields per hectare (George *et al*., 1992). The accessibility and form of N present in soil are affected with water management alteration from flooded to aerobic status in aerobic rice system, thus a new N management approach is required (Savant and De Datta., 1982). As nitrogen is the main factor, limiting growth under majority of conditions, the nitrogen fertilizer usage is essential in rice production (Dawe *et al*., 2000). In order to develop 1 ton dry mass of rough rice, including straw, around 16-17 kg N is utilized (De Datta., 1981).

There is a concern regarding the environmental influence of nitrogen fertilization due to the high inputs of N fertilizer. Biological nitrogen fixation (BNF) by rice plants is regarded as an approach to reduce N losses and promote the uptake and consumption of natural and applied nitrogen (Ladha and Reddy., 1995). A particular enzyme, namely nitrogenase, converts the atmospheric nitrogen to ammonia in a BNF natural process, ended up in availability of unavailable form of nitrogen to plants.

2.5 N_2 Fixing Microorganisms

N_2 fixing bacteria that utilize carbon combinations of rhizosphere for growth and fix the nitrogen for plant subsequently are called diazotrophs, the mediator of BNF process. Diazotrophs are categorized as symbiotic and non-symbiotic or free-livings. Free-living nitrogen fixers are attributed to those microbes that fix N independently. Free- living microorganisms can fix less than 1 kg N/ha/year (Shuichi., 1995). Plant systems benefit from biological N_2 fixing microbes, free living in soil media and in association with rhizosphere and the tissues (endophytes) of the healthy plant (Bashan and de-Bashan., 2005). Atmospheric N_2 fixation into NH_3 by nitrogenase enzyme is carried out by minimum of 90 specific microorganisms' genera (Unkovich and Baldock., 2008). Specific microorganisms, mostly free-living bacteria and blue green algae perform non-symbiotic nitrogen fixation. However, in temperate agricultural soils the ability of using free-living diazotrophs as a N source for crops is limited due to the incapability of the effective multiplication of the organism and thus has not been tested extensively (Keeling *et al*., 1998).

2.6 N_2 Fixing Activity

The molecular nitrogen (N_2) is enzymatically reduced to ammonia by prokaryotes through biological N_2 fixation. The reduction of molecular nitrogen is catalyzed by the nitrogenase enzyme complex, and has a high energy demand, two ATP molecules are consumed for

each electron transferred to the catalytic site (Rees and Howard, 1999). The molecular oxygen irreversibly inactivates the nitrogenase enzyme complex. In presence of oxygen and nitrogen the synthesis and activity of nitrogenase is modified by N_2 fixing microorganisms to avoid unnecessary energy consumption.

2.7 N_2 Fixing Bacteria and Rice Association

The interaction between non-legume plants and diazotrophic bacteria is well discussed in the literature. In the study by Naher *et al*. (2009) MR219 and Mayang Segumpal rice seedlings were colonized by *Corynebacterium* and *Rhizobium* sp. both surface and endophytically and the seedling root and shoot biomass increased by the association, which could supplement about 35 kg. ha^{-1} of nitrogen requirement in MR219 and Mayang Segumpal. Diazotrophs elevate crop growth in various procedures, including BNF and assisting in phytohormones production, which lead to the development of root system. As they act on the root system, the root surface area gets wider and the nutrient uptake would increase, leading to promotion of biomass production and yield.

2.8 Bio-fertilizer Inoculation

The beneficial microorganisms which are applied to seed, root or soil, mobilize the accessibility of nutrients with their biological activity and assist in development of lost microflora to boost the soil health, are live formulations of bio-fertilizer (Ismail *et al*., 2014). Biofertilizer is used with the aim of being partial or complete substitute for chemical fertilizer. Diazotrophic bio-inoculant utilization as a substitute to nitrogen fertilizer has gained some success in recent years (Welbaum *et al*., 2004). To exert the overall capability of the diazotrophic PGP bacterial strain, isolation of indigenous bacteria that are well compatible to the environmental circumstances as an inoculant strains is of great importance (Soares *et al.*, 2006).

In recent decades, various inoculation experiments have not been successful to contribute to biological nitrogen fixation (BNF) to the plant in most cases. In field and greenhouse experiments, inoculation with various diazotrophic strains did not eliminate N deficit symptoms in unfertilized maize (Riggs *et al*., 2001). The production of high quality inoculants biofertilizers, leading to promotion plant growth promoting ability of diazotrophs has been reviewed by Kennedy *et al*. (2004). The seed cotton yield, plant height and soil microbial population were significantly enhanced with diazotrophic bacterial inoculation (Anjum *et al*., 2007). It has recently been recommended that the inoculants can benefit various grasses including maize, rice, wheat and sugarcane (Hungria *et al*., 2010).

The equilibrium of soil microbial communities would be disturbed as inoculation provides high densities of viable and effective microbes for a rapid colonization of host rhizosphere. However, the diversity rate and plant-soil-biota interactions make the ecosystem adaptable, moderating the modification in the bacterial community structure induced by inoculation (Kennedy., 1999). Generally, plant growth promoting bacterial (PGPB) strains must have the ability to compete with rhizosphere, sustain and colonize the rhizospheric soil (Cattelan *et al*., 1999).

2.9 Weed Management Approaches

Flooded-rice system goes through a lower weed pressure in comparison with aerobic rice system (Balasubramanian and Hill., 2002). Finding an effective weed management approach has been a serious challenge for researchers and farmers. Physical, cultural, and biological weed management were the weed control strategies till 1940s. In the last few decades, herbicides have been a huge participant in agriculture. The herbicides have been the most attainable choice among the weed management approaches due to shortage and high wages of labor in large-scale rice cultivation. Currently, there has been no practicable alternative for chemical weed control in rice, despite of the herbicides side-effects.

2.10 Herbicides

In 21st century, herbicide utilization is still considered an essential way to control weeds all over the world, including Malaysia (Zoschke and Quadranti., 2002). The herbicide efficiency is evaluated by its potential to cause a desired effect on the target pest, although it does not determine the herbicide adaptability. The economic aspect of the herbicide should be considered prior to deciding about its use (Wibawa *et al*., 2010).

A study on the effects of different herbicides for weed management in aerobic rice in Malaysia in dry season 2008 and wet season 2008 to 2009 showed that proprietary mixture or tank mixture of herbicides with different modes of actions were more effective than their single application (Rahman *et al.*, 2012). The results from a field study done in Malaysia during 2010/2011 on the effects of eight commercial herbicide products applied singly or as tank-mix or in sequence in aerobic rice represented that weed control was greatly done by most of the herbicide treatments and much higher net benefit than weedy or weed-free treatments was provided. No significant phytotoxicity to rice plants was observed by the herbicides (Anwar *et al.*, 2012).

2.11 Classification of Herbicides

The classification of the herbicides is usually done according to their chemical structure, mode of action, application time and selectivity. Toxicity or hazard level of the herbicides also can be considered a factor for herbicides classification (Zimdhal., 1993).

2.11.1 Chemical Group

The herbicide mode of action is determined by the chemical group to which an herbicide belongs. The use of a new herbicide can be anticipated by common physiological properties of the same chemical group that the new herbicides belong. This classification method provides the information about the elementary use, formulations, water solubility and critical oral toxicity of each herbicide. Herbicides classification based on chemical structure include inorganic and organic herbicides according to the Weed Science Society of America (WSSA). (i) Aliphatics, (ii) Amides, (iii) Aryloxy phenoxy propionate, (iv) Benzoics, (v) Bipyridiliums, (vi) Carbamates, (vi) Cyclohexanedione, (vii) Dinitroanilines (viii) Diphenyl Ethers, (ix) Imidazolines, (x) Isoxazolidinones, (xi) Nitriles, (xii) Oxadiazoles (xiii) Oxadiazolides, (xiv) Phenols, (xv) Phenoxy acids, (xvi) N-phenylphthalamides (xvii)Phenylpyridazones, (xviii) Phthalamates, (xix) Pyrazoliums, (xx) Picolinic acids (xxi) Pyridines, (xxii) Quinolines, (xxiii) Sulfonylureas, (xxiv) Thiocarbamates (xxv) Triazolopyrimidine sulfonamide, (xxvi) Triazolinones, (xxvii) Triazines, (xxiv) Uracil, (xxv) Ureas, (xxvi) Unclassified; are the sub-groups of organic herbicides (Senseman., 2007).

2.11.2 Mode of Action

The way that a pesticide prohibits the normal act of pest to repress or kill it, is defined as its mode of action. A biochemical process is prevented when herbicide goes through the plant and ends up at a certain place in plant cells. The herbicides chemistry and the plant species are involved in inhibition of biochemical process in some cases. The categorization of herbicides on their mode of actions according to WSSA is as following:

Acetyl CoA Carboxylase (ACCase) Inhibitors, Acetolactate Synthase (ALS) or Acetohydroxy Acid Synthase (AHAS) Inhibitors, Mitosis Inhibitors, Synthetic Auxins, Photosystem I Inhibitors, Photosystem II Inhibitors, Fatty Acid and Lipid Biosynthesis Inhibitors, Enolpyruvyl Shikimate-3-Phosphate (EPSP) Synthase Inhibitors, Glutamine Synthetase Inhibitors, Carotenoid Biosynthesis Inhibitors, Protoporphyrinogen Oxidase (PPG oxidase or Protox) Inhibitors, Potential Nucleic Acid Inhibitors or Non-descript mode of action, Dihydropteroate Synthetase Inhibitors, Auxin Transport Inhibitors, Cellulose Inhibitors, Oxidative Phosphorylation Uncouplers, and Not Classified (Senseman., 2007).

2.11.3 Application Time

The efficiency and the length of weed control period are determined by herbicides application time (Carter *et al*., 2007). Herbicides can be classified according to their time of application. Herbicides can be applied as pre-planting, applied prior to the crop plantation; pre-emergence, applied after the crop plantation (vegetative parts) or sown (seeds), and before emergence and post-emergence, soil or foliage, applied after the crop emergence.

2.11.4 Selectivity

Non-selective and selective herbicides can be sub-categories in herbicides classification according to their mode of actions. All plant species are destroyed with non-selective herbicide as the herbicide posses no selectivity, while some plant species are killed with selective herbicides, with little or no hazards to other species. The selective herbicides have the ability to recognize the plants which they affect and destroy through the interference with their biochemical procedures.

2.12 Application Methods

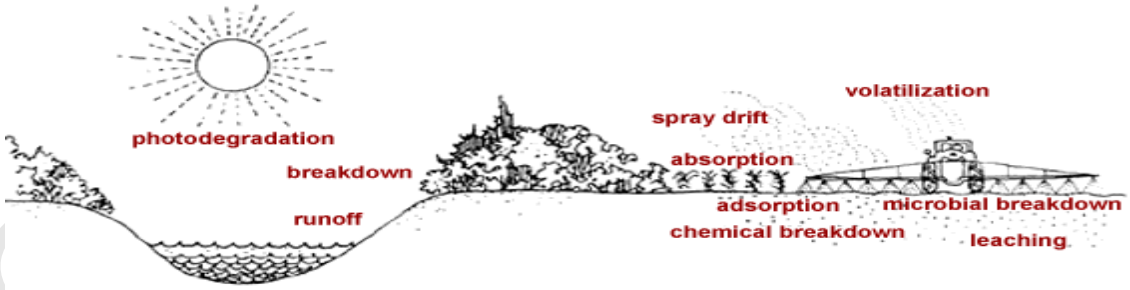
Soil treatment and foliar spray are two methods of herbicides application. Soil-applied pre-emergence herbicides are applied prior to crop emergence and after sowing. Germinated seed or small seedlings are influenced by soil-applied herbicides, which go through the roots of large plants, leading to the elimination of weeds selectively in crops. These kinds of herbicides can have foliage activity.
Foliage applied herbicides are applied to the leaves of unwanted plants, either as contact or translocated and can have an activity on soil. The systemic herbicides are translocated in a vascular system of plant from point of absorption (leaf or root) to sites of action. However, the contact herbicides are not translocated and they cause damage to the top plant part where the spray solution contacts, but the perennial plants, at their underground partitions do not get affected and can fastly start new growth. Water or diesel are used to dilute the herbicides, which are foliar sprayed onto the plants foliage at a particular rate using spray equipment and the spraying is continued till every single leaf get wetted, but not dripped.

2.13 The Fate of Herbicides in Soil

As only a small amount of herbicides reach to the target organism (Pimentel., 1995) and their residues cause potential adverse effects on human, animal and crop health in soil and water, the herbicides environmental fate has been a huge concern. The most remarkable

factors influencing soil and water pollution are the capability of herbicides to adsorb and desorb on soils and sediments. The chemico-physical characteristics of the released herbicides determine their fate in the environment. Abiotic and biotic reactions play an important role in herbicides transformation in soils and waters. The behavior of herbicide in water and its mobility in soil is importantly related to the herbicide solubility. The restriction of herbicides environmental impact can be obtained by understanding of their movement and fate in soil. Herbicide has one of three fates after it has been applied to soil. It can remain in the soil solution (at dissolved status), adsorbed to soil colloids or absorbed by plant roots and shoots.

Adsorption refers to the removal of herbicides due to interaction with soils, plants, and sediments which is affected by Clay content, organic matter and moisture of soil. The herbicide Uptake by plant roots or animal ingestion is called absorption which is influenced by cell membrane transport and contact time. Runoff is the herbicide movement in water over a sloping surface. Spray drift refers to the movement of herbicides due to wind action which is affected by wind speed and drop sizes of herbicides molecules. Leaching is the horizontal and vertical movement of herbicides downward through the soil affected by water content, soil texture, clay and organic matter contents of soil. Volatilization, the transformation of herbicide molecules from aquatic into the atmosphere is of great significance to predict the quantity that is remained as residues, and therefore, its environmental persistence. The photolysis is the degradation of herbicide molecules on soil surfaces and in aquatic environments with photochemical reactions like sunlight radiation. Microorganisms activity which is influenced by properties such as temperature and pH leads to the herbicide biodegradation. Hydrolysis and redox reactions occur within chemical breakdown of herbicides (Braschi *et al*., 2011) (Figure 2.1).



Source: (British Columbia Ministry of Agriculture and Lands 2007)

Figure 2.1: Processes that affect environmental fate of herbicides

2.14 Persistence

The time that the herbicide molecule remains in soil is called soil persistence or soil residual life and half-life is usually used for the persistence expression. The half life ($t_{1/2}$) is the time that the concentration of the organic molecule requires to halve to its initial level. In order to understand the possible environmental effect of a chemical, half-life values should be taken into account importantly. If the degradation products of a chemical would be harmless, its impact on the environment is reduced as it degrades rapidly with a low $t_{1/2}$ value. On the contrary, despite of the moderate toxicity of a molecule, its environmental impact can be substantial, if it would have high $t_{1/2}$. As the persistence of herbicide provides information on the residual activity of agrochemicals, which can damage the following crops, the prediction of their half-life is of great importance (Braschi *et al*., 2011).

2.15 Factors Affecting the Persistence of Herbicides in Soil

Soil properties and environmental conditions influence the extent, which a residual herbicide can persist and harm the subsequent crops. The phytotoxicity and persistence of most herbicides in soil are influenced by the major factors, including soil organic matter content and texture (Rahman and James., 2002). As the soil pH influences microbial activity and hydrolysis process, its variation affects persistence of specific herbicides. Herbicide breakdown would decrease if the microbial activity responsible for herbicides breakdown become slow due to the soil pH conditions. High pH levels cause a decrease in hydrolysis rate (Franzen and Zollinger., 1997). Hydrolysis and/ or microbial degradation lead to the degradation of many herbicides with residual activity (Vencill., 2002). The increase in temperature results in herbicide degradation rate (Hamaker., 1972). In the degradation process, moisture content is considered a significant factor, as the oxygen level in soil is adjusted with competition for pore space. The organic matter incorporation might boost the microbial activity and decomposition in soil. The initial concentration of the herbicide has been shown to affect degradation kinetics.

2.16 Biodegradation Process

Biodegradation (degradation by soil microorganisms) among different processes leading to herbicide fate, is considered to be the most difficult to predict. Degradation in the environment is accompanied by some environmental procedures, including sorption, hydrolysis, volatilization, transport, and bound residues reservoir (Sims and Cupples., 1999). Soil microorganisms consume the herbicides as beneficial carbon and/ or nitrogen sources (Qiu *et al.*, 2009). The most remarkable agrochemicals degraders have been reported to be the soil microorganisms, particularly bacteria and fungi. The environmental circumstances, including temperature, moisture and aeration, which are suitable for chemical degradation are similar to those that microbes choose (Beulke et al, 2004).

2.17 Bioremediation Alternative

Microbial metabolism is used to detoxify contaminants in bioremediation which is carried out either in situ, using at the contamination site or ex situ, when the contamination was removed from the original site (ex situ). The introduced microorganisms to the polluted site which were isolated elsewhere or the indigenous microbes are used. Several mechanisms determine the bacterial tolerance or resistance to the noxious compounds including the elimination of toxic compounds by the cell (Silver and Phung., 1996), or producing compounds which degrade enzymes (Talaro., 2008). However, microbes are not capable of surviving, adapting and thriving in very few environments according to the researches. Bacteria and fungi or plants are used to degrade or eliminate harmful chemicals for human and the environment.

2.18 Effects of Herbicides on Soil Microbial Population

Non-target organisms might be affected by substantial quantity of herbicides, when applied off-target. Effect of pesticides on indigenous soil microbes has been reported in several studies. Microbial communities on soil surface might be naturally subjected to high pollutant concentrations of herbicides. It has been anticipated that the population of bacteria (Rajendran and Lourduraj., 1999), fungi (Shukla., 1997) and actinomycetes (Rajendran and Lourduraj., 1999) have been influenced adversely by herbicide application; however, herbicides generally do not cause detrimental effects at the recommended doses in soil (Selvamani and Sankaran., 1993). The naturally numerous PGPR communities in soil can be inactivated metabolically through the uptake of herbicides, if they applied at excessive rates to the soil (Singh and Wright., 2002). The negative influence of herbicides on microorganisms would be deteriorated with an increase in herbicide dosage.

Vlad *et al*. (2012) reported that nitrogen fixing aerobic bacteria were dead in experimental variants by Tribenuron-metyl and nicosulfuron, which induced a major toxic impact at high concentration. Cycoń *et al*. (2010) observed that indigenous soil microorganisms might be affected with linuron, particularly at high concentrations. Bacterial population was declined with high concentrations (× 1.5 and 2.0 recommended rates) of herbicides glyphosphate and paraquat compared to the control treatment (Ayansina and Amusan., 2013). According to Ayansina and Oso (2006) a decrease in microbial counts was observed with recommended and 1.5X recommended rates of atrazine and atrazine+metolachlor, with lower microbial counts with higher herbicides concentrations compared to the recommended dose, and some microbial species were eliminated. Latha and Gopal (2010) found that the bacterial population was reduced with application of herbicides viz., 2, 4-DEE, butachlor, pretilachlor and pyrazosulfuron ethyl, with highest reduction in population by butachlor and as the herbicides concentration increased the effect was stronger. The results from the trial done by Gürsoy and Padem (2012) revealed

that an increase in aclonifen herbicide dose decreased the number of *Rhizobium* bacteria, total mesophilic bacteria, yeast and molds.

In contrast, a few microorganisms show tolerance or resistance (moderately or not affected) to a specific herbicide. The soil microbial populations and enzyme activity can be qualitatively and quantitatively prompted by herbicides application (Saeki and Toyota., 2004). According to Raut *et al*. (1997) the microbial activity of rice rhizosphere soil was stimulated with herbicide butachlor; however, a little overwhelming effect was observed initially. The study by Sebiomo *et al*. (2011) discovered the significant response of soil microbial activity to atrazine, primeextra, paraquat and glyphosate and an increase in adaptation of microbial community to stress of higher concentration of herbicides was observed over weeks of treatment. The growth of soil bacteria, actinomycetes, mould, and the pure cultures of *Br.japonicum* and *A. chroococcum.* was stimulated by lower concentrations (0.5 mg. μg^{-1} dry soil) to below than 10 mg. μg^{-1} dry soil of herbicide trifluralin; however, microbial colonies improvements were inhibited in terms of both amount and size at higher concentrations of trifluralin (Hang *et al*., 2001).

2.19 Effects of Herbicides on N_2 Fixing Activity

There is insufficient literature on the effects of herbicides on N_2 fixation activity of pure cultures bacteria *in-vitro* conditions. The study on the effects of three pesticides (Imazetapir, Dimethoate and Bayleton 50) at the recommended concentration (in the field) on nitrogen fixation of pure cultures of *Azotobacter chroococcum* and *Azotobacter vinelandii* showed that Herbicide Imazetapir had no negative effect on N_2 fixation, while Dimethoate and Bayleton 50 exhibited inhibitory effect on N_2 fixation of studied bacterial species (Khudhur & Askar, 2013). The pesticides (Funaben T seed dressing and Pivot 100 SL herbicide) reduced the nitrogenase activity of *Rhizobium leguminosarum* bv.*trifolli* KGL, *Sinorhizobium melilotti* Bp and *Bradyrhizobium* sp. *Ornithopus* B bacteria (Niewiadomska & Klama, 2005). Among the four commonly used pesticides, Bagalol caused maximum inhibition of nitrogenase activity on *Nostoc ellipsosporum* and *Scytonema simplex*, while Thiodan and Phorate had maximum effect on *Tolypothrix tenuis*, and *Westiellopsis prolific* species of cyanobacteria and Mancozeb showed lesser effect on nitrogenase activity of tested species of cyanobacteria (Debnath *et al*., 2012).

2.20 Herbicide Phytotoxicity

The effect or harm by a compound on specific plant properties including growth rate, germination, or development of root and shoot is called phytotoxicity. The total herbicide phytotoxicity is specified with the quantity of herbicide sorbed to the soil and desorbed back into the soil solution. The herbicides become accessible and phytotoxic to sensitive species immediately after they move back into the soil solution. When the meristematic part of plant is influenced, root stunting and pruning occur, leading to the plant damage

(Vencill., 2002). In evaluation of pollutants effects, studies have demonstrated that as the root and shoot develop during seed germination, sensitivity in the root system increases (Wong and Bradshaw., 1982).

A probable initial injuries (30%) in rice disappear within 2-4 weeks in most rice herbicides (Moody., 1977). Moore and Kröger (2010) found out that metolachlor/ atrazine mixture, diazinon, and lambda-cyhalothrin significantly reduced coleoptile (shoot) growth of rice plant in comparison with controls, while fipronil significantly increased radicles (root) compared to controls. The pretilachlor concentration in soil water determines mostly its phytotoxic activity on rice seedlings in soil (Kobayashi *et al*., 1999). Seed germination and 1000 grain weight of rice were not negatively affected with application of diquat, ghlyphosate, paraquat or sodium chlorate (Eastin., 1980). When it was used in initial vegetative period, paraquat residues on vegetables were not considerable (Akinloye *et al*., 2011). Moyer and Esau (1996) found that 1 year after application of imazethapyr, canola was injured and imazamethabenz and imazethapyr application caused damage to sugar beet, and yield and quality of potatoes. According to Alonso-Prados *et al*. (2002) dark green coloration, stunting with a stem base redness and less bushy secondary root system symptoms emerged with sulfosulfuron residues. One year after application of sulfosulfuron, peas, canola and barley were still affected (Shinn *et al*., 1998).

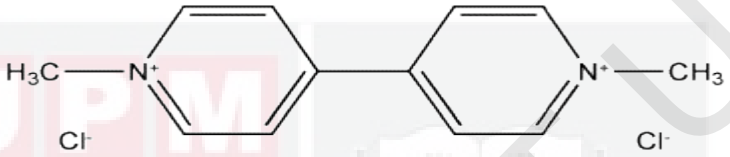
2.21 UV-VIS-NIR Spectroscopic Method for Herbicides Analysis

The understanding of herbicides dissipation pattern in soil is attained through the quantitative determination of their residues. Several analytical methods including gas liquid chromatography (GLC), high performance liquid chromatography (HPLC), radio techniques, immunochemical techniques and etc have been employed for herbicides analysis. The great sensitivity and reliability in analysis are the unique characteristics of these methods; however, they are complex, costly, time-consuming, great user of large quantities of organic solvents, and destructive of the sample. Therefore, rapid, cost-effective and non-destructive techniques for pesticide detection are required. Being non-destructive to the sample, cost-effective and rapid to operate made the near-infrared (NIR) spectroscopy the most popular analytical method (Armenta *et al*., 2007). The organic pesticides can be determined with NIRS method, given the presence of dipolar bonds in these chemicals. The pesticides atrazine and alachlor (1.25-100 ppm) in pure methanol/ water solutions, using a 1 mm transmittance cell and a Foss NIR System 6500 spectrophotometer were determined (Gowen *et al*., 2011). However, the tested herbicides in the present study, including paraquat, pretilachlor and 2, 4-D have not been analyzed with NIR-Spectrosccopy method previously.

2.22 Selected Herbicides in the Study

Paraquat, pretilachlor and 2, 4-D, the commonly used herbicides for rice cultivation areas in Malaysia were used in this study.

2.22.1 Paraquat



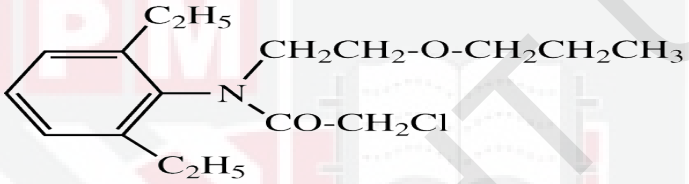
(Source: www.pesticideinfo.org)

Figure 2.2. Chemical stracture of Paraquat

Paraquat, an herbicide, is categorized in the heterocyclic organic compound in group of bipyridium herbicide or quaternary ammonium herbicide. Two pyridium rings with cationic characteristic form paraquat molecular structure (Figure 2.2). 1, 1'-dimethyl-4, 4'-bipyridinium dichloride is the International Union of Pure and Applied Chemistry (IUPAC) chemical name. Paraquat has been classified by the Weed Science Society of America (WSSA) as a group 22 with an inhibition of photosystem-1-electron diversion (Senseman., 2007). The only formulation type of paraquat registered for all uses is a soluble concentrate/ liquid (SC/L). A colorless crystal is a physical form of paraquat. In neutral and acidic media it shows stability; however, hydrolyzation would occur in an alkaline media. It is a polar organic compound with aqueous solubility of 700,000 mg/L at 20 ˚C, high solubility in water and very low (<10-3 and 10-5 Pa at 20˚C) vapor pressure. The extreme polar nature of paraquat leads to its high affinity and strong adsorption to soil. It is tightly adsorbed to soil particles, particularly clay minerals, leading to its inactivity in soil (Constenla *et al*., 1990). Thus, its detection cannot represent its normal use. Paraquat does not cause phytotoxic effects in condition of strong bounding to soil and may persist indefinitely (Mordaunt *et al*., 2005). The long half-life of paraquat mostly referred to its strong binding to clay minerals. Depending on soil type, it has a half-life of 16 months to 13 years in soil. Its bioavailability for absorption by living organisms is very restricted, leading to its very slow biodegradation. However, bacteria, fungi, and yeast are the microorganisms able to degrade paraquat and use as nitrogen sources. Paraquat is a non-selective contact herbicide which destroys all green plant tissues which have been contacted and effectively used as broad-spectrum herbicide for control of broad-leaved weeds and grasses. It is applied as pre-plant (at planting), pre-emergence, post-emergence or post-harvest. Both terrestrial and aquatic plants are effectively affected with paraquat. The vegetable, paddy rice, rubber, oil palm and cocoa are the common cultivations in Malaysia which use paraquat (Cheah *et al*., 1998).

2.22.2 Pretilachlor

Pretilachlor is a selective systemic pre-emergence herbicide from chloroacetanilide group. The International Union of Pure and Applied Chemistry (IUPAC) chemical name of pretilachlor is [2-chloro-2, 6-diethyl-N-(2-propoxyethyl) acetanilide (Figure 2.3). Pretilachlor has been classified by the Weed Science Society of America (WSSA) as a group 15 with very long chain fatty acids (VLCFAs) inhibiton (cell divison) (Senseman, 2007). Several grasses, broad-leaved weeds and sedges are controlled with pretilachlor. The pretilachlor in the liquid form is light yellow to yellowish brown in color and free from external impurities or added modifying agents. Pretilachlor 50% EC is a systemic herbicide with aqueous solubility of 50 mg/L at 20 ˚C and boiling point of 135 ˚C.



Source: www.farmchemicalsinternational.com

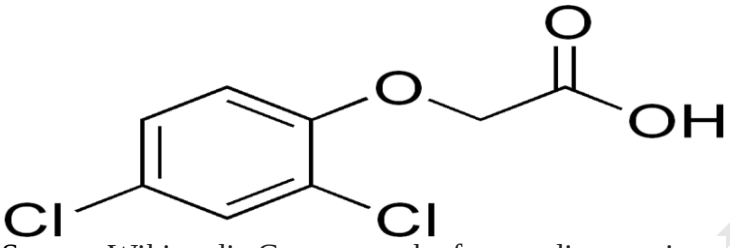
Figure 2.3. Chemical structure of pretilachlor

The germinating shoots are its primary absorption route, followed by the roots as a secondarily site and ended up to the thorough translocation in plant. The vegetative parts receive higher concentrations compared to reproductive parts. The growth of barnyard grass is retarded through the prevention of nutrient transportation from leaves to embryos via the α-amylase inhibition, leading to energy deficiency, within the weed germination, inhibiting the division, growth and differentiation of plant cells (Su., 1989). Photodecomposition, microbial degradation and volatilization are the mechanism of pretilachlor dissipation in rice fields and the soil characteristics and environmental contamination status do not get influenced by its recommended rate (Adachi *et al*., 2007). The half-life of 3.9-10 days is estimated for pretilachlor.

2.22.3 2, 4-D

2, 4-D is an herbicide and a plant growth regulator (Tomlin., 2006). The International Union of Pure and Applied Chemistry (IUPAC) chemical name of the 2, 4-D acid form is 2, 4-dichlorophenoxyacetic acid (Figure 2.4). 2, 4-D belongs to the phenoxy family of herbicides and has been classified by the Weed Science Society of America (WSSA) as a group 4 as synthetic auxins (Senseman., 2007). The chemical properties, environmental behavior and toxicity (to a less extent) differ in esters, acids, and several salts (mostly amine) of 2, 4-D formulations (WHO., 1989). The parent acid derivatives are the salt and ester forms (WHO., 1989). The liquids, water-soluble powders, dusts, granules, or

2, 4-D pellets are 2, 4-D formulations. Butoxyethyl ester (ester) or dimethylamine salt (amine) are the liquid formulations of 2, 4-D acid.



Source: Wikimedia Commons, the free media repository

Figure 2.4. Chemical structure of 2, 4-D

2, 4-D, a selective systemic and post-emergence herbicide is effective on a broad terrestrial and aquatic broadleaf varieties of weeds; however, grasses are not much affected. 2, 4-D is a polar herbicide with an aqueous solubility of 45000 mg/L. Its high water solubility and low soil adsorption coefficient makes 2, 4-D leach to the soil easily, leading to its probable percolation to groundwater (Balinova and Mondesky., 1999). 2, 4-D has a half life of around 7-10 days in soil.

Meristemic areas of shoots and roots are the places that 2, 4-D accumulate. The auxins effect or other plant growth regulating hormones are mimicked by 2, 4-D, leading to growth stimulation, old cells regeneration and abnormal growth and death in some plants that arise from young cells overstimulation (Mullison., 1987). 2, 4-D usually breaks down through microbial degradation in soils. The ambient pH determines the 2, 4-D fate in the environment (Aly and Faust., 1964). Hydroxylation, cleavage of the acid side-chain, decarboxylation, and ring opening occurred through 2, 4-D microbial degradation (Tomlin., 2006). Degradation rates would maximize in soil conditions that microbial populations maximize (i.e. warm and moist) (Foster and McKercher., 1973).

CHAPTER 3

EFFECT OF PARAQUAT, PRETILACHLOR AND 2, 4-D ON GROWTH AND N_2 FIXING ACTIVITY OF *Stenotrophomonas maltophilia* (Sb16)

3.1. Introduction

Diazotrophs associated in the rhizosphere can improve growth and development of rice plants. Aerobic rice, water-saving direct seeded rice cultivation is subjected to greater weed pressure with a wider weed spectrum compared to flood-irrigated rice (Balasubramanian and Hill., 2002). Herbicides have been the most efficient chemical weed management since they were introduced to agriculture. Herbicides may cause undesirable effects when applied at high concentrations. Herbicides applied to soil or plant might interfere with the microbial biofertilizer inoculated to crop plants, in case that they come into contact. There are studies reporting the effects of herbicides on N_2 fixing bacteria *in-vitro* conditions; however some contradictions have been observed in the results. The differences in chemicals, concentration of chemicals, bacterium strain and culture medium may be the reason of discrepancies. Moreover, the effects of herbicides on the bacterium strain should be determined in laboratory conditions to predict its ability in protection of plant from probable herbicidal damages under natural soil conditions.
The effect of herbicides on N_2 fixing activity of diazotrophs is major concern among researchers as it is vital to the soil fertility of rice fields. Research on the effect of herbicides on N_2 fixation *in-vitro* conditions is scarce. Besides, as the attributing of N_2 fixation to the specific bacterium in plant system is impossible, the effects of herbicides on N_2 fixation ability of the specific bacterium should be determined in laboratory conditions to predict its role in N_2 fixation in plant system under natural soil condition. Therefore, the present investigation was conducted to study the effects of three common rice herbicides i.e. paraquat, pretilachlor and 2, 4-D at different concentrations on growth and N_2 fixing activity of *Stenotrophomonas maltophilia* (Sb16) isolated by Naher *et al*. (2009) from Tanjong Karang, rice (*Oryza. Sativa* L.) growing area, Selangor, Malaysia.

3.2 Materials and Methods

3.2.1 Experimental Location

The experiment was conducted in Soil Microbiology Laboratory, Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia (UPM), Serdang, Selangor. The experiment tested the effects of paraquat, pretilachlor, and 2, 4-D at half, full and double doses of recommended rate on growth and N_2 fixing activity of Sb16.

3.2.2 Inoculum Preparation

Sb16 pure culture was obtained from the Soil Microbiology Laboratory. The bacteria were allowed to multiply on N-free agar plates within 4-5 days of incubation at temperature of 30°C. Pure colony of bacterial strain was used to prepare the inoculum. Jensen's nitrogen free broth (Jensen., 1951) was inoculated with a single pure colony and incubated at 35 °C for 3-4 days on a rotary shaker at 150 rpm. Approximately 10^8 cfu mL^{-1} of live cell, with adjusted optical density $(OD)_{600}$ of 1.05 was inoculated to each treatment.

3.2.3 Herbicides Solutions Preparation

The herbicides used in this study were paraquat dichloride (13 % w/w,), pretilachlor (28.7% w/w) and 2, 4-D isopropylamine (35.5% w/w) (Table 3.1). Concentrations of herbicides used were corresponding to half, full and double doses of recommended rate for each herbicide.

The recommended rate (12 mL/L) of paraquat considering 13% a.i. was used to prepare solutions of paraquat concentrations. Aliquots of 0.078, 0.156 and 0.312 mL of paraquat was dissolved in 100 mL sterilized distilled water to obtain concentrations of 0.78, 1.56 and 3.12 mg a.i/ml of paraquat corresponding to half, full and double of the recommended dose, respectively.

To prepare the solutions of pretilachlor concentrations, the recommended rate (5 mL/L) considering 28.7% a.i. was used. Aliquots of 0.072, 0.144 and 0.287 mL of pretilachlor was dissolved in 100 mL sterilized distilled water to get the concentrations of 0.72, 1.44 and 2.87 mg a.i/ml of pretilachlor corresponding to half, full and double of the recommended dose, respectively.

To obtain the concentrations of 1.42, 2.84 and 5.68 mg a.i/ml of 2, 4-D, corresponding to half, full and double doses of recommended rate (8 mL/L) considering 35.5 % a.i., aliquots of 0.142, 0.284 and 0.568 mL of 2, 4-D, respectively was dissolved in 100 mL sterilized distilled water. Herbicides solutions thereafter were sterilized by filtration (Millipore filter, 0.22 mm) aseptically in a laminar flow cabinet.

Table 3.1. Technical information of herbicides used in the study

Commercial Product	Technical product	Chemical group	Active ingredient of technical product (% a.i.)
Syngenta Capayam	Paraquat dichloride	Bipyridilium	13% w/w
Syngenta Sofit N300 EC	Pretilachlor	Chloroacetanilide	28.7% w/w
Kompressor Ancom Cropcare	2, 4-D isopropylamine	Phenoacetic	35.5% w/w

3.2.4 Sample Preparation

Culture flasks containing 75 mL Jensen's N-free media were sterilized by autoclaving at 15 lbs pressure (121˚C) for 15 minutes. An amount of 1 mL of each prepared solution of herbicides was added to flasks containing Jensen N-free broth with respect to the desired concentrations of each herbicide. Control flasks did not receive any herbicides. Before inoculum application, optical density (OD_{600}) of inoculum was checked and regulated to 1.05 and drop plate method for cell count was employed to confirm the population on N-free agar (Somasegaran and Hoben., 1985). Aliquot of 1 mL of the desired inoculum (approximately 10^8 cells.mL^{-1}) of live cells was transferred to Jensen broth using sterilized pipette. Flasks were incubated at 28 ˚C on rotary shaker for 7 days till the end of the experiment.

3.2.5 Experimental Design and Treatments

The treatments included three herbicides with four different concentrations, including 0 (control), half, full and double doses of the recommended rate. The study was conducted as factorial complete randomized design (CRD) with four replications. The factors were 3 types of herbicides with 4 different concentrations and 7 incubation periods.

3.2.6. Determination of Population

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

3.2.7 Estimation of N_2 Fixing Activity

The N_2 fixing activity of Sb16 in Jensen N-free broth amended with paraquat, pretilachlor and 2, 4-D was determined using acetylene reduction assay (ARA) based on the method by Hardy *et al.* (1968) and Somasegaran and Hoben (1985). The experiment was conducted in Laboratory of Physiology, Faculty of Veterinary, UPM. The procedure for the sample preparation was previously stated above (3.2.4). 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 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 hour. A sample of 1 mL of air from each incubated syringe was injected into a Gas Chromatography (HP 6890) equipped with Hydrogen Flame Ionization Detector (FID) with temperature of 120˚C, injector temperature 150 ˚C with Column (Agilent J&W GC Column, HP-PLOT/Q, 30M, 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 actual concentrations of ethylene were determined based on the standard curve of ethylene concentrations (Nmol C_2H_4) and the peak area (Appendix A.a).

3.2.8 Determination of pH

Changes in pH of Jensen N-free broth was determined using a standard pH meter (pHM 210, MeterLab®) equipped with a glass electrode at every 24-h interval.

3.2.9 Statistical analysis

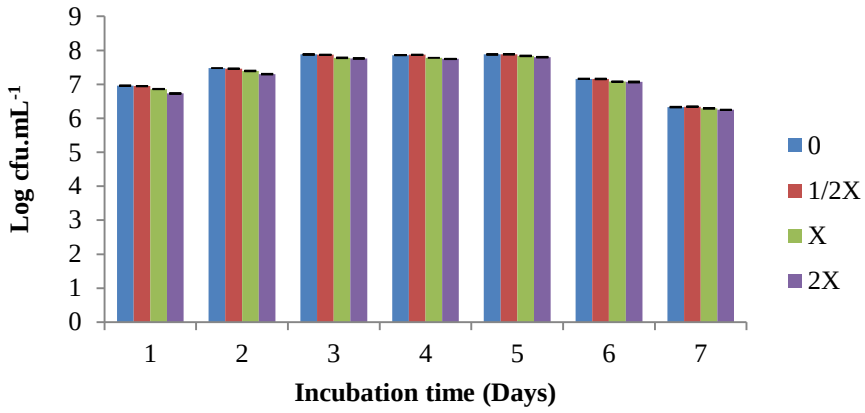
The data were subjected to analysis of variance (ANOVA) and analyzed using SAS (version 9.3). The treatment means were compared by Duncan's multiple range test (DMRT) ($P \leq 0.05$). The number of bacteria was $\log_{10}$ transformed before statistical analysis.

3.3 Results

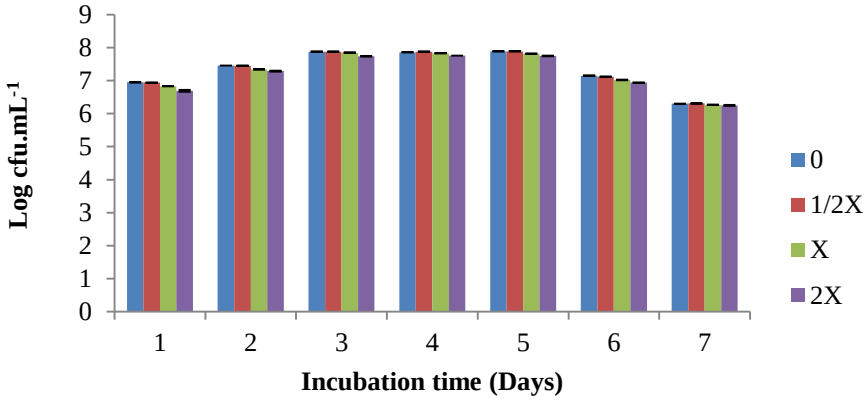
3.3.1 Population of Sb16

Analysis of variance (ANOVA) showed high significant differences among herbicides, concentrations and incubation time for Sb16 population. Three herbicides showed significant differences for Sb16 population, with the highest population in samples treated with 2, 4-D (7.32 $\log_{10}$ cfu.mL^{-1}), followed by paraquat (7.314 $\log_{10}$ cfu.mL^{-1}) and pretilachlor (7.3 $\log_{10}$ cfu.mL^{-1}). Half dose of herbicides and control (without herbicide) showed no significant effect for Sb16 population.

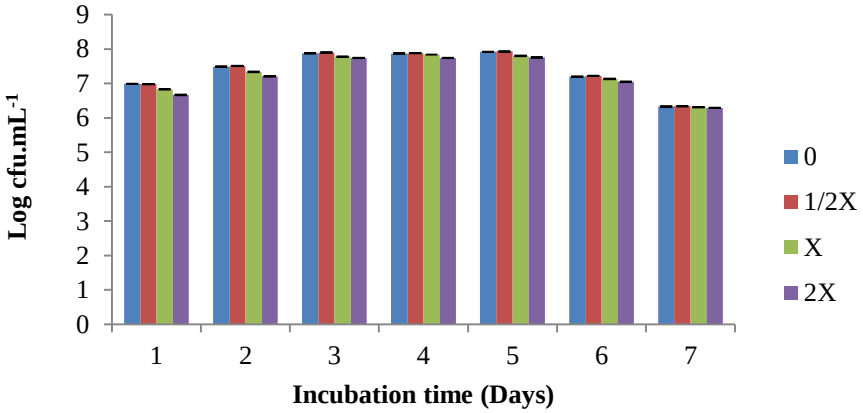
Sb16 Population significantly increased from day 1-3 of incubation period; however it decreased at 4th day, followed by a significant increase at 5th day. Population significantly declined thereafter from 6-7th day. Sb16 had the highest and lowest population at 5th and 7th day, respectively. An inhibition in growth of Sb16 in samples amended with different concentrations of herbicides was recovered and comparable to the control at day 7. Population in samples treated with three herbicides showed the similar trend. The highest population in samples amended with herbicides was obtained by 7.93 $\log_{10}$ cfu.mL^{-1} with half dose of 2, 4-D at 5th day Sb16 had the lowest population (6.25, 6.27 and 6.31 $\log_{10}$ cfu.mL^{-1}) in samples amended with double, full and half doses of pretilachlor, respectively at 7th day (Figure 3.1). There was a significant three way interaction effect between herbicides, concentration and incubation time on Sb16 population (Appendix B.a).



(a) Paraquat



(b) Pretilachlor



(c) 2, 4-D

Figure 3.1. Effect of different concentrations of herbicides on population of Sb16 in Jensen N-free medium during 7 days of incubation period; Bars indicate standard error (n=4)

3.3.2 N_2 Fixing Activity of Sb16

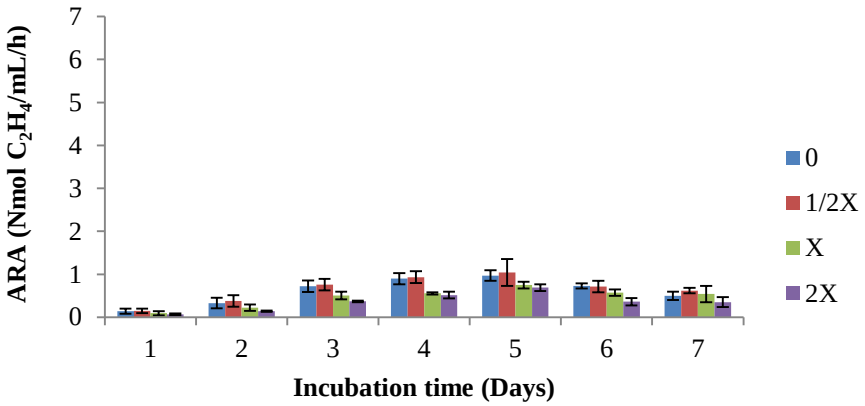
There were significant differences among herbicides, concentrations and incubation time for nitrogenase activity of Sb16. The samples amended with 2, 4-D had the highest nitrogenase activity (1.35 Nmol C_2H_4/mL/h), followed by paraquat (0.52 Nmol C_2H_4/mL/h) and pretilachlor (0.47 Nmol C_2H_4/mL/h). However, there was not significant difference between paraquat and pretilachlor for nitrogenase activity of Sb16. Samples amended with half dose of herbicides showed the highest nitrogeanse activity, followed by control, full and double doses. There were not significant differences between control with full doses and full with double doses of herbicides for nitrogenase activity. Nitrogenase activity significantly increased from 1st to 5th incubation day; however day 4 and 5 did not show significant differences. It declined thereafter till day 7; however there was not significant difference between day 6 and 7.

The highest and lowest nitrogenase activity was obtained at 5th and 1st day, respectively. The highest nitrogenase activity (5.23 Nmol C_2H_4/mL/h) was obtained in samples amended with half dose of 2, 4-D at 4th day. However, the lowest nitrogenase activity (0.09 and 0.07 Nmol C_2H_4/mL/h) was recorded in samples amended with double and full doses of paraquat, respectively at 1st day (Figure 3.2). Herbicides, concentrations and incubation time showed a significant three way interaction effect on nitrogenase activity of Sb16, indicating that the factors were dependent (Appendix B.a).

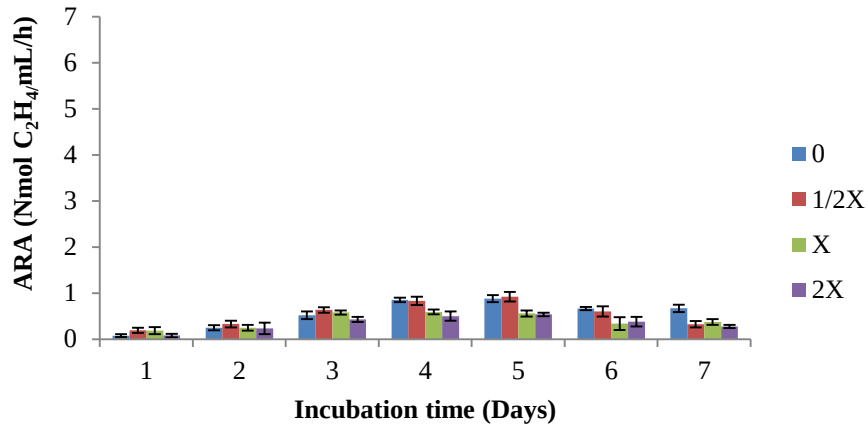
3.3.3. pH of Jensen N-free Medium

There were significant differences among herbicides, concentrations and incubation time for pH of Jensen N-free broth. There was not a significant difference among paraquat and pretilachlor for pH. The samples amended with 2, 4-D showed the highest pH (7.29), followed by paraquat (7.28) and pretilachlor (7.28). Control with half dose of herbicides did not have significant difference for pH. Acidity (pH) of Jensen broth significantly increased from day 1-5 of incubation period, followed by a significant decline till day 7, with the highest and lowest pH at 5th and 1st day, respectively.

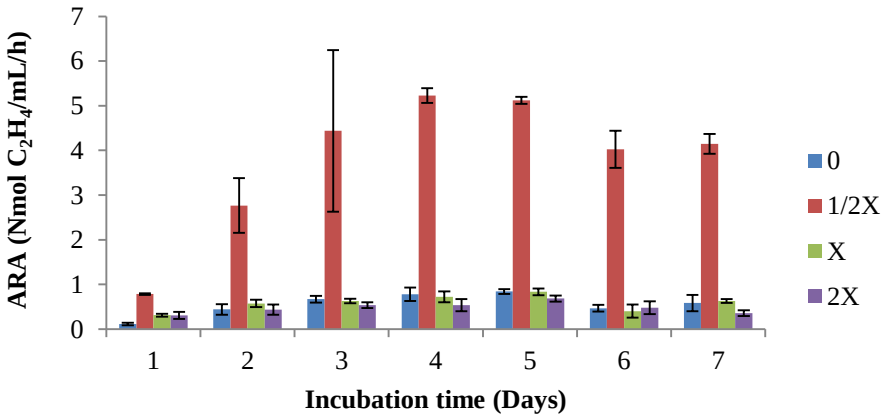
The highest pH in herbicide amended samples was recorded by 7.46 with pretilachlor at half dose and by 7.45 and 7.42 with 2, 4-D at full and double doses, respectively at 5th day. However, the lowest pH were obtained by 6.92 and 6.8 in samples amended with full dose of pretilachlor and double dose of 2, 4-D, respectively (Figure 3.3). There was a significant three way interaction effect between herbicides, concentrations and incubation time on pH of Jensen broth (Appendix B.a).



(a) Paraquat

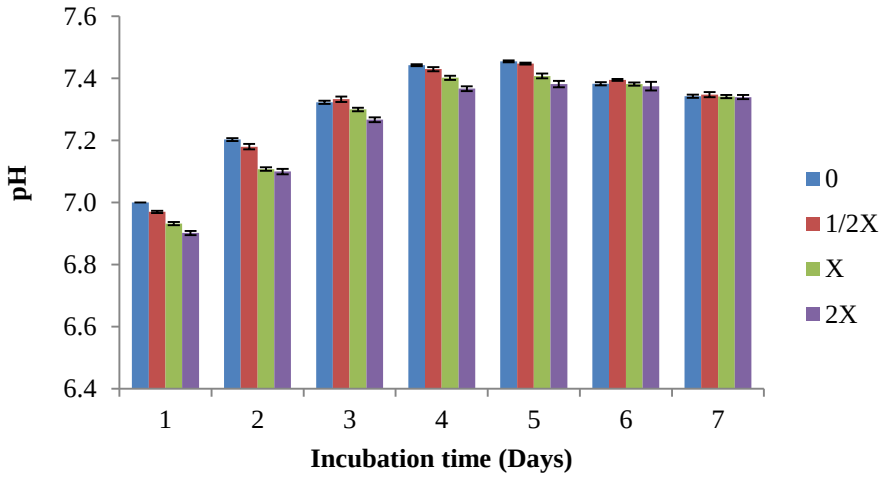


(b) Pretilachlor

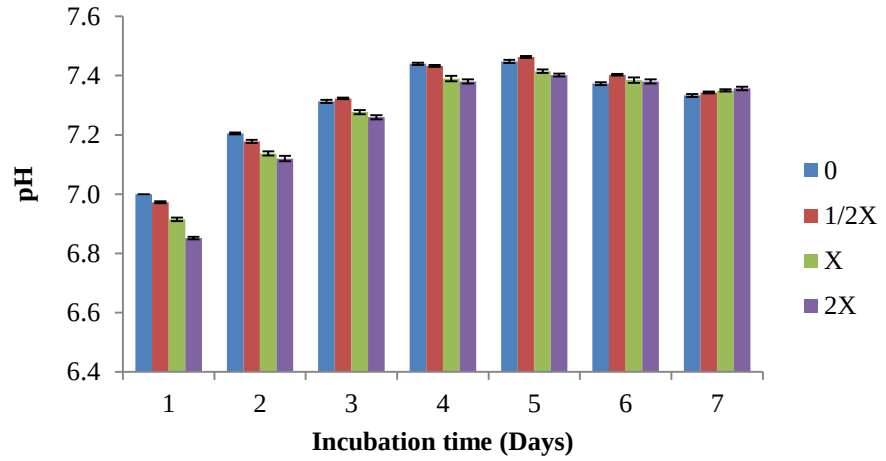


(c) 2, 4-D

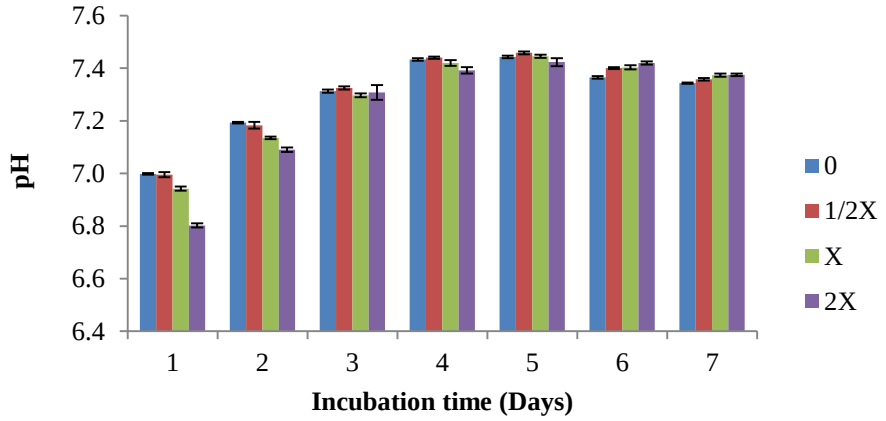
Figure 3.2. Effect of different concentrations of herbicides on N_2 fixing activity of Sb16 in Jensen N-free medium during 7 days of incubation period; Bars indicate standard error (n=4)



(a) Paraquat



(b) Pretilachlor



(c) 2, 4-D

Figure 3.3. Effect of different concentrations of herbicides on pH of Jensen N-free medium inoculated with Sb16 during 7 days of incubation period; Bars indicate standard error (n=4)

The correlation coefficient analysis showed a highly significant positive correlation (≤0.01) between population and N_2 fixing activity of Sb16, population and pH of Jensen medium and between nitrogenase activity and pH of Jensen medium (Table 3.2).

Table 3.2. The correlation coefficient between population and N_2 fixing activity of Sb16 and pH of Jensen N-free medium

	Population	N_2 fixing Activity	pH
Population	1.00	0.193**	0.345**
N_2 fixing activity		1.00	0.291**
pH			1.00

****** significant at $P \leq 0.01$

3.4 Discussion

The recovery from initial inhibition of growth of Sb16 in broth amended with herbicides after 6th incubation day in the present study concurs with the study by Latha and Gopal (2010) who observed an initial decrease in growth of *A. lipoferum* with herbicides 2, 4-D, butachlor, pretilachlor and pyrazosulfuron compared to control treatment after 24h of incubation, followed by an increase over time. Stanley *et al*. (2013) reported a decline in bacterial population with presence of half and recommended rate of atrazine and paraquat at 4th week of post treatment, followed by a subsequent progressive increase in soil at 6th and 8th week. The increase in bacterial population over time can be due to the mineralization of herbicides by the bacteria as energy and carbon sources. There are several experiments which have shown the use of herbicides as carbon source by microbes (Radosevich *et al*., 1995).

The growth was affected adversely at higher concentrations of herbicides, which agrees with the study by Ayansina and Oso (2006) who found that microbial counts were lower in higher concentrations of herbicides compared to recommended doses. Earlier study by Othman *et al.* (2012) showed no adverse effect of paraquat on population and activity of N_2 fixing bacteria at recommended dose. The results from present study agrees with the study made by Drouin *et al.* (2010) who observed an inhibition of growth of three strains of *Bradyrhizobium* and strains of *Rhizobium* by paraquat. An inhibitory effect of paraquat on *B. megaterium* and *B. subtilis* was reported by Smith and Fletcher (1964). Adeleye *et al.* (2004) studied the toxicity of 2, 4-D amine to *B. subtilis* and found out that a decrease in the survival percentage occurred at higher herbicide concentrations which is similar to the results of the present study. Hinteregger *et al.* (1995) stated that microorganisms might be affected by toxic effect of high concentrations of phenoxy herbicides and their derivatives. It has been pointed out that 2, 4-D penetrates to the cell envelope of *Rhizobium*

and accumulates in the cytosol causing toxic effects (Fabra *et al.*, 1997). Jaiswal *et al*. (2002) opined that 2, 4-D induces the cytotoxicity and mutagenicity to *Rhizobium* and *Bradyrhizobium* species.

The reduction in nitrogenase activity of Sb16 with higher concentrations of herbicides in present study is in accordance with the study by Debnath *et al.* (2012) who found that Bagalol, Mancozeb (fungicides), Thiodan and Phorate (insecticides) at EC 50 concentration inhibited the nitrogenase activity in four species of cyanobacteria including *N. ellipsosporum*, *S. simplex*, *T. tenuis*, and *W. prolific*. Several studies have reported the decline of nitrogenase activity by pesticides. However, the inhibition was recovered over incubation period, indicating the relationship between population and nitrogenase activity and utilization of herbicides as source of nutrients and energy for growth and activity.
Population of Sb16 in presence of half dose of herbicides was not significantly different to control (without herbicides) in present study. The stimulation in growth of Sb16 with half dose of herbicides can be explained by alteration in the medium nutrient, chemical structure and herbicide degradation by the bacteria itself. A significant stimulation in nitrogenase activity of Sb16 in presence of lower concentration of 2, 4-D in present study corresponds to the study by Saikia *et al*. (2014) who reported the higher rate of acetylene reduction in seedling roots of citronella inoculated with *A. brasilense* and treated with 2, 4-D than in *A. brasilense* alone. 2, 4-D at low concentrations might stimulate growth of organism by cell division and elongation; however it may induce abnormalities at high concentrations. The effects of herbicides on growth and activity of bacterial strain depend on several factors including herbicides types, dosages, bacterial species, properties of culture medium and incubation time.

In present study, pH of Jensen N-free medium decreased in presence of herbicides; however, it increased at last incubation days. The decrease of pH with herbicides can be due to reduction of bacterial growth and activity following the contact with herbicides, leading to more acidic medium. The recovery of inhibition in pH of medium at last incubation days is related to an increase in growth and activity of Sb16 in Jensen broth. Bacteria consumed and decreased the organic acids and NH_4^+ concentrations in medium and altered the acidic medium to more basic.

There were highly positive correlations between growth and nitrogen fixing activity of Sb16 and pH of broth. The decrease in Sb16 population at initial incubation time following the herbicides application led to the reduction of nitrogenase activity and alteration of Jensen medium to more acidic due to lack of bacterial growth and activity. Thus, an increase in Sb16 bacterial population over incubation time, resulted in increase of nitrogenase activity and more basic medium.

As microorganisms are the most sensitive factor in evaluation of the pollutants and perform a great deal of specific functions in all ecosystems, they can be used in microbial toxicity tests for evaluation of the hazards of environmental contaminants (Van Beelen

and Doelman., 1997). Herbicides toxicity and their microbial biodegradability play a vital role to assess their environmental impact and performance.

However, the result of present study cannot be directly applied to the field conditions where a multitude of environmental factors like climate, soil type, agricultural activities and the microbial communities' constitution interact simultaneously. The effect of pesticides on bacterial species either positive or negative may not be significant in soil, as several factors including adsorption, volatility, photodecomposition, leaching and microbial degradation determine the herbicide persistence (Cork and Krueger., 1991), leading to a decrease in actual effect of these chemicals to soil microflora.

3.5 Conclusion

The study showed that growth and activity of Sb16 were stimulated by half dose of tested herbicides. Full and double doses of herbicides significantly decreased population and nitrogenase activity of Sb16. However, the activity and growth inhibition were recovered over incubation time. It can be concluded that the tested herbicides at recommended doses might have insignificant effect on growth and nitrogenase activity of Sb16 under natural field conditions.

CHAPTER 4

EFFECTS OF *Stenotrophomonas maltophilia* (Sb16) AND HERBICIDES ON GROWTH OF AEROBIC RICE AND ON THE SOIL MICROBIAL AND CHEMICAL PROPERTIES

4.1 Introduction

Aerobic rice (*Oryza sativa* L.), a direct seeded and water-saving rice cultivation system, has become successful in sustainable agriculture recently. As environmental concerns increase, several options are being applied to make the plant less dependent on nitrogen fertilizer for its nutrient requirement. The use of diazotrophs as bio-fertilizer has gained a great significance in agricultural practices.

Besides, aerobic rice suffers from a huge weed pressure; thus an efficient weed management approach is required. The herbicide is being considered as the most practical, effective and economical means of weed management in rice (De Datta., 1981). The use of herbicides in agriculture causes some non-target effects on environment. The non-target effects of herbicides on microbial communities in soils, besides their effects on target organisms (weeds) may lead to reducing the performance of important soil functions (Sebiomo *et al*., 2011). The nutrients production and other soil functions are indirectly influenced as the alteration of microbial population and activity affects the nutrient cycling and accessibility (Wang *et al*., 2008). Some microbial strains have the capability to degrade (utilize) the chemicals and alter them to beneficial compounds, leading to microbial multiplication. They will further promote crop production through the protection of plant from hazardous effects of herbicides and stimulating phytohormones to the plant. There are studies on effects of herbicides application and bacterial inoculation on plants and soil microorganisms. However there is no study conducted on effects of herbicides and N_2 fixing bacteria on growth of aerobic rice and alterations in soil microbial population and chemical properties. Therefore, the present study was carried out with the following objectives:

1. To determine the effect of Sb16 bacterial inoculation and application of three common rice herbicides on growth of aerobic rice

2. To determine the effect of Sb16 bacterial inoculation and application of three common rice herbicides on microbial population and chemical properties of aerobic rice soil

4.2 Materials and Methods

4.2.1 Experimental Setup and Treatments

A pot experiment was conducted under glasshouse conditions at 25 ± 2 ˚C and 60% relative humidity at Ladang 2, UPM 43400, Serdang, Selangor. The soil with sandy clay loam in texture was collected from Ladang 2, UPM, 43400, Serdang, Selangor. Soils were taken to the laboratory, air-dried at 25 ˚C during 48 hour, crushed, homogenized and sieved through a 2 mm mesh to clear the soil from stones and plant debris. The physico-chemical and microbial parameters of studied soil were determined (Table 4.1).

The soils were divided into two parts and the second sets of soil samples were autoclaved 2 times for 15 min (121 ˚C, 150 psi) in order to sterilize the soils. About 2 kg of prepared soil were poured in each plastic pot. The pots were sterilized with alcohol prior pouring the soil. The experiment was laid out as factorial randomized complete block design (RCBD) with three replications. Treatment combinations were assigned at random within a block. A series of the same treatments were run for sterilized soil samples.

4.2.2 Herbicides treatments

The herbicides used in this study were paraquat dichloride (13 % w/w), pretilachlor (28.7% w/w) and 2, 4-D isopropylamine (35.5% w/w). The herbicides solutions were prepared based on the area of pot (227 cm^2).

The recommended rate of 0.702 kg a.i. ha^{-1} of paraquat was used to prepare the solutions. Aliquots of $6.12\times10^{-3,}$ 1.225×10^{-2} and 2.45×10^{-2} mL of paraquat were applied to each pot to obtain concentrations corresponding to half, full and double doses of recommended rates, respectively.

The recommended rate of 0.430 kg a.i. ha^{-1} of pretilachlor was used to prepare the solutions. Aliquots of 1.7×10^{-3}, 3.4×10^{-3} and 6.8×10^{-3} mL of pretilachlor were applied to each pot to obtain concentrations corresponding to half, full and double doses of recommended rates, respectively.

Aliquots of 2.25×10^{-3}, 4.5×10^{-3} and 9×10^{-3} mL of 2, 4-D with recommended rate of 710 kg a.i. ha^{-1} were applied to pots to get the doses corresponded to half, full and double doses of recommended rates, respectively.

Table 4.1. Physico-chemical and microbial characteristics of soil used in the study

Soil Characteristics	Results
Sand (%)	49.12
Silt (%)	19.38
Clay (%)	31.5
Soil pH (1: 2.5 w/v) in water	5.3
Cation exchange capacity (cmol(+) kg^{-1})	12.1
Organic matter (%)	2.2
Total nitrogen (%)	0.291
Available phosphorus ($mg.kg^{-1}$)	27.87
Exchangeable potassium (cmol(+) kg^{-1})	0.269
Total bacteria (log_{10} $cfu.g^{-1}$ dry soil)	5.71
Diazotrophs (log_{10} $cfu.g^{-1}$ dry soil)	5.01
Fungi (log_{10} $cfu.g^{-1}$ dry soil)	3.59

Herbicides solutions for sterilized soil samples were prepared in sterilized distilled water. The herbicides solutions were applied pre-plant, incorporated to soil and mixed comprehensively with a sterile spatula to disperse the herbicides homogenously. The untreated soil samples (control) received the same amount of distilled water. The soil moisture content was adjusted to 60% of water holding capacity (WHC) by adding appropriate amount of distilled water. The soil moisture level was checked regularly by weighing the soil and adjusted to 60% of WHC by adding the necessary quantity of distilled water. The sterilized soil treatments received regular sterilized distilled water based on WHC.

4.2.3 Seed Surface Sterilization and Germination

Aerobic rice line MR 219-9 mutant (M-9) was obtained from laboratory of Microbiology. The seeds were surface sterilized by 70% (v/v) ethanol for 1 minute, immersed in 4% (v/v) sodium hypochlorite for 3 minutes and drenched 6 times with sterilized distilled water (Somasegaran and Hoben., 1994). Seeds were soaked with sterilized distilled water and allowed to germinate on filter paper in Petri dishes. In order to wet the seeds, sterilized distilled water was added regularly.

4.2.4 Preparation and Application of Inoculum

The diazotrophic Sb16 was used as an inoculum source. The strain was grown in Erlenmeyer flasks containing 100 mL Jensen's N-free medium. The culture was shaken on orbital shaker at 150 rpm at 37°C for 3-4 days. The optical density (OD_{600}) was checked regularly (≈1.05) and colony-forming units (CFU) was done using drop plate method to confirm the population (10^7cells.mL^{-1}). Seven days old seedlingss were soaked in cultured inoculum (10^7cfu.mL^{-1}) for 30 minutes and five seeds were transplanted in each pot at depth of 5 cm. After germination of seedlings, the plants were thinned to 2 plants per pot. The pots were hand weeded at regular intervals.

4.2.5 Sample Collection

Soil samples were collected from each respective pot at each 15-day interval from day 0 (1 h) till 60 after transplanting to carry out the microbial and chemical analysis. The samples were taken from the surface layer (0-15 cm depth) of the pot, placed separately in plastic bags and transferred immediately to laboratory. Soil samples were sieved through a 2 mm pore size and divided into two sub-samples. One portion was air-dried for chemical analysis. Another portion was sieved through a 2 mm pore size sieve and frozen for microbial and residual analysis. Plant samples were collected at 60th day of transplanting to measure the growth and root morphological parameters.

4.2.6 Soil Microbial Enumeration

The microbial population count was performed using spread plate method. The plates were inverted and incubated at 30°C and population of total bacteria, diazotrophs and fungi were enumerated. Nutrient agar (NA) medium was used for total bacterial determination; however N-free semi-solid malate medium (NFb) (Gyaneshwar *et al.*, 2001) and PDA agar were used for diazotrophs and fungal population, respectively. Colony forming units (CFU) per gram of dry soil was employed to express the population counts. The data then were $\log_{10}$ transformed before analysis.

4.2.7 Soil Chemical Properties

The acidity (pH) of soil samples was measured in 1:2.5 soil: H2O (w/v) suspensions using a pH meter fitted with a glass electrode.

The Kjeldahl method (Bremner., 1996) and the sodium bicarbonate method (Olsen *et al.*, 1954) were used to determine total N and available P, respectively.

The cation exchange capacity (CEC) of soils was determined by the NH_4OAc method (Gillman., 1979). To determine the exchangeable potassium (K), the ammonium acetate buffered at pH 7 (Thomas., 1982) was used. Organic matter (%) of soils was determined by WB-T method (Nelson and Sommers., 1982).

4.2.8 Measurement the Growth Parameters of Aerobic Rice

The data on plant height and leaf chlorophyll content was collected before plant sampling. Plant height was measured at each interval from the base of plant to the tip of the uppermost part of plant and the data was expressed as centimeter (cm). The leaf chlorophyll content was estimated with chlorophyll meter (Minolta SPAD 502) and expressed in SPAD units.

The plant samples were collected at 60^{th} day after transplanting (DAT), placed individually in plastic bags and transported immediately to laboratory for the measurements of growth parameters. In lab, the plant was shaken to remove the residual soil and shoot and root portions were separated. To determine the root and shoot dry weight, the plant fractions were air-dried and then oven dried at 70 $^{\circ}$C till constant weight obtained. Laboratory balance was used for weighing the biomass of shoot and roots. To measure the leaf area per plant, leaves from each plant were randomly taken and total surface area of each leaf was measured using a leaf area meter, (Licor, Model LI-3100 Area Meter, LI-COR Inc. Lincoln, Nebraska, USA). The Kjeldahl method (Bremner., 1996) was used to determine the nitrogen concentration (%) in plant tissue.

4.2.9 Measurement the Root Morphological Parameters

After separating the root from the shoot parts in the lab, the fresh parts of root were used immediately to determinate the root morphological parameters using the Root Scanner Image Analyzer Win Rhizo STD1600 WIA - EPSON EXPRESSION 1680. Fresh roots were washed thoroughly with distilled water and placed on the root scanner. Total root length (cm), surface area (cm^2), volume (cm^3) and average diameter (mm) of each root were measured.

4.2.10 Statistical Analysis

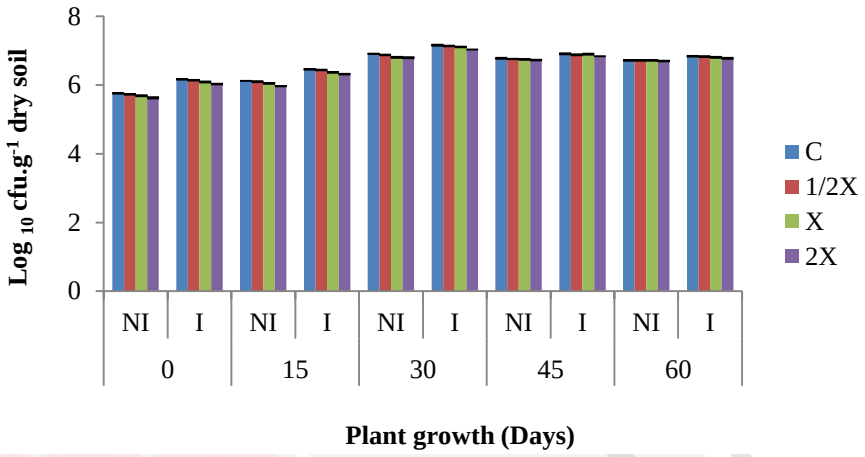
Data were subjected to analysis of variance (ANOVA) using SAS program version 9.3 and the mean comparison was separated using Duncan Multiple Range Test (DMRT) at $P<0.05$ (Gomez and Gomez, 1984). The Pearson's correlation coefficients were performed using SPSS software version 21.

4.3 Results

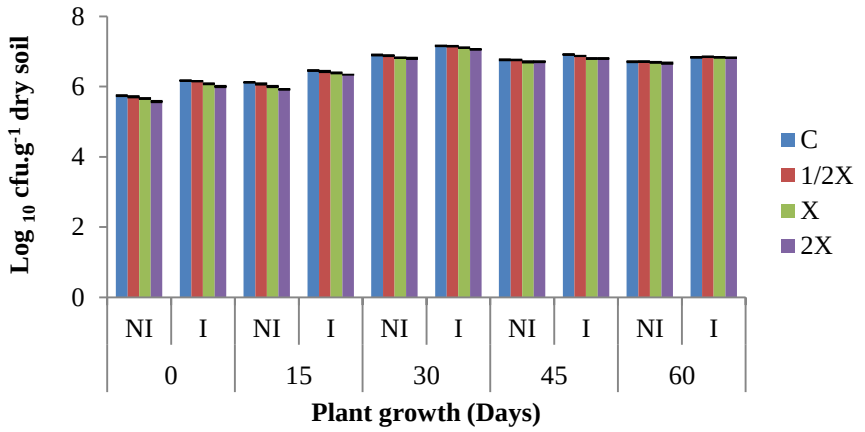
4.3.1 Total Bacterial Population

There were significant differences among bacterial inoculation, herbicides, concentrations and sampling dates for total bacterial population in soil. In general, population was significantly greater in inoculated compared to non-inoculated samples. Soil samples treated with paraquat had the highest population (6.54 $\log_{10}$ cfu.g^{-1} dry soil), followed by pretilachlor (6.53 $\log_{10}$ cfu.g^{-1} dry soil) and 2, 4-D (6.52 $\log_{10}$ cfu.g^{-1} dry soil). Control samples (without herbicides) had the highest population, followed by the half, full and double doses. Population significantly increased from day 0-30 of treatment, followed by a significant decline thereafter, with the lowest and highest population at day 0 and 30 of treatment, respectively.

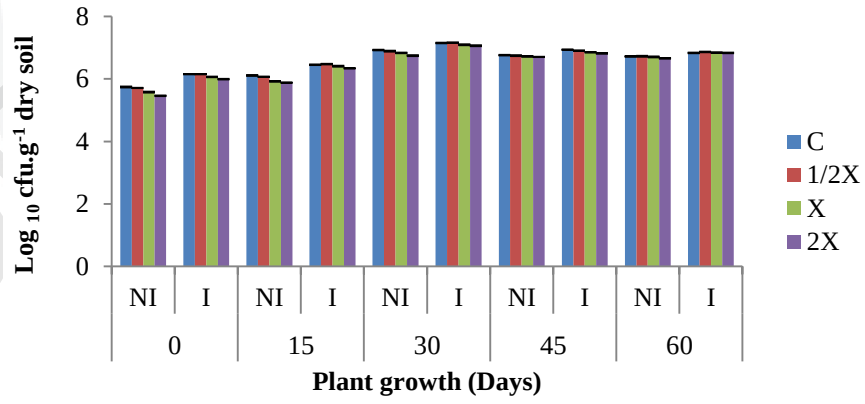
The lowest total bacterial population (5.46, 5.58 and 5.71 $\log_{10}$ cfu.g^{-1} dry soil) was obtained in non-inoculated samples treated with double, full and half doses of 2, 4-D, respectively at day 0 after treatment. The maximum increase of population in inoculated samples treated with herbicides was recorded by 9.71, 8.6 and 7.71% with double, full and half doses of 2, 4-D, respectively over non-inoculated treatments at day 0 after treatment (Figure 4.1). There was a significant four way interaction effect of inoculation, herbicides, concentrations and sampling dates on total bacterial population in soil (Appendix B. b) For instance, population was higher in soil samples inoculated with bacteria and treated with half dose of paraquat at 1 hour after treatment than in non-inoculated samples.



(a) Paraquat



(b) pretilachlor



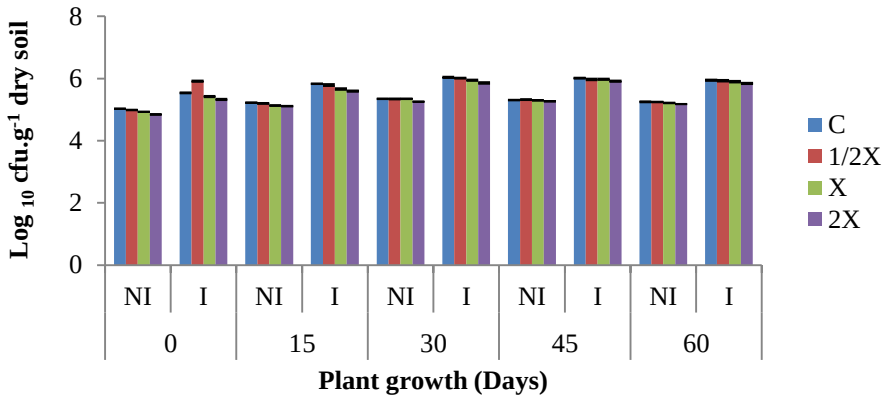
(c) 2, 4-D

Figure 4.1. Effect of Sb16 bacterial inoculation and herbicides application on total bacterial population of soil within 60 days of aerobic rice growth; (a) paraquat, (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose; Bars indicate standard error (n=3)

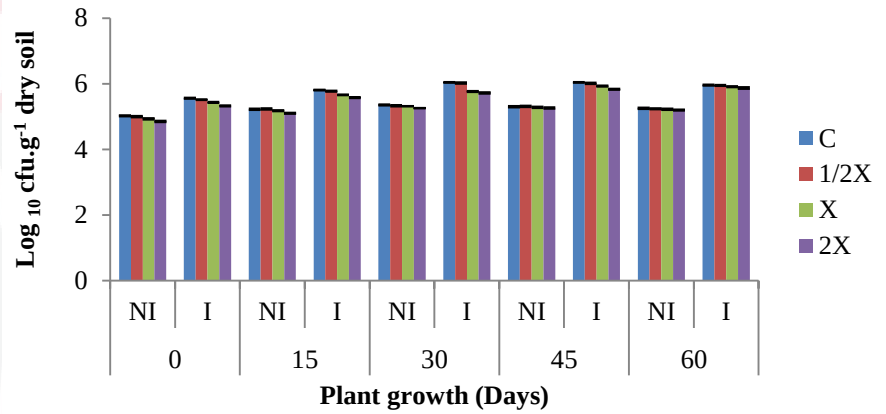
4.3.2 Diazotrophs Population

There were highly significant differences among bacterial inoculation, herbicides, concentrations and sampling dates for diazotrophs population in soil. In general, population significantly increased with inoculation. There were significant differences between paraquat (5.5 $\log_{10}$ cfu.g^{-1} dry soil), pretilachlor (5.49 $\log_{10}$ cfu.g^{-1} dry soil) and 2, 4-D (5.47 $\log_{10}$ cfu.g^{-1} dry soil) for diazotrophs population. Control samples (without herbicides) had the highest population, followed by the half, full and double doses. Population significantly increased from day 0-45 of treatment, followed by a significant decline till day 60, with the highest and lowest population at day 45 and 0 of treatment, respectively.

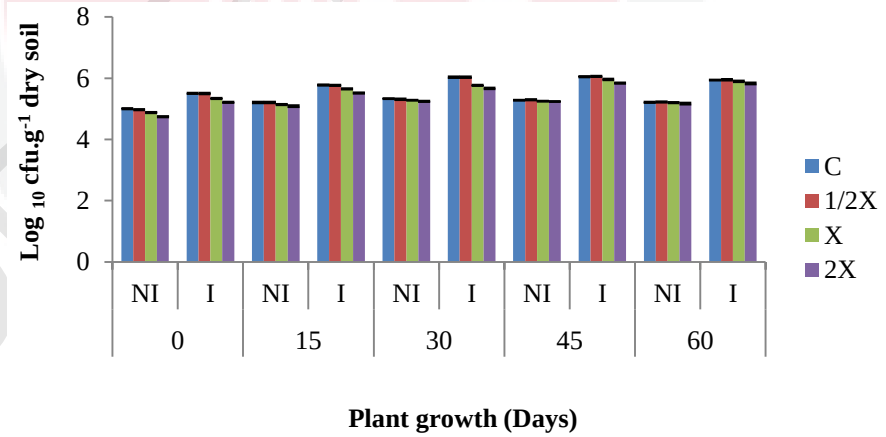
The lowest diazotrophs population (4.74, 4.88 and 4.97 $\log_{10}$ cfu.g^{-1} dry soil) was obtained in non-inoculated samples treated with double, full and half doses of 2, 4-D, respectively at day 0 after treatment. However, the highest population in inoculated samples treated with herbicides was obtained by 6.06 log cfu.g^{-1} dry soil with half dose of 2, 4-D and by 5.98 and 5.92 $\log_{10}$ cfu.g^{-1} dry soil with full and double doses of paraquat, respectively (Figure 4.2). There was a significant four way interaction effects between bacterial inoculation, herbicides, concentrations and sampling dates on diazotrophs population in soil, indicating that population was affected by dependent effect of all factors (Appendix B. b). For example, population was higher in inoculated soil samples treated with half dose of paraquat at 45^{th} day after treatment than in non-inoculated samples.



(a) paraquat



(b) pretilachlor



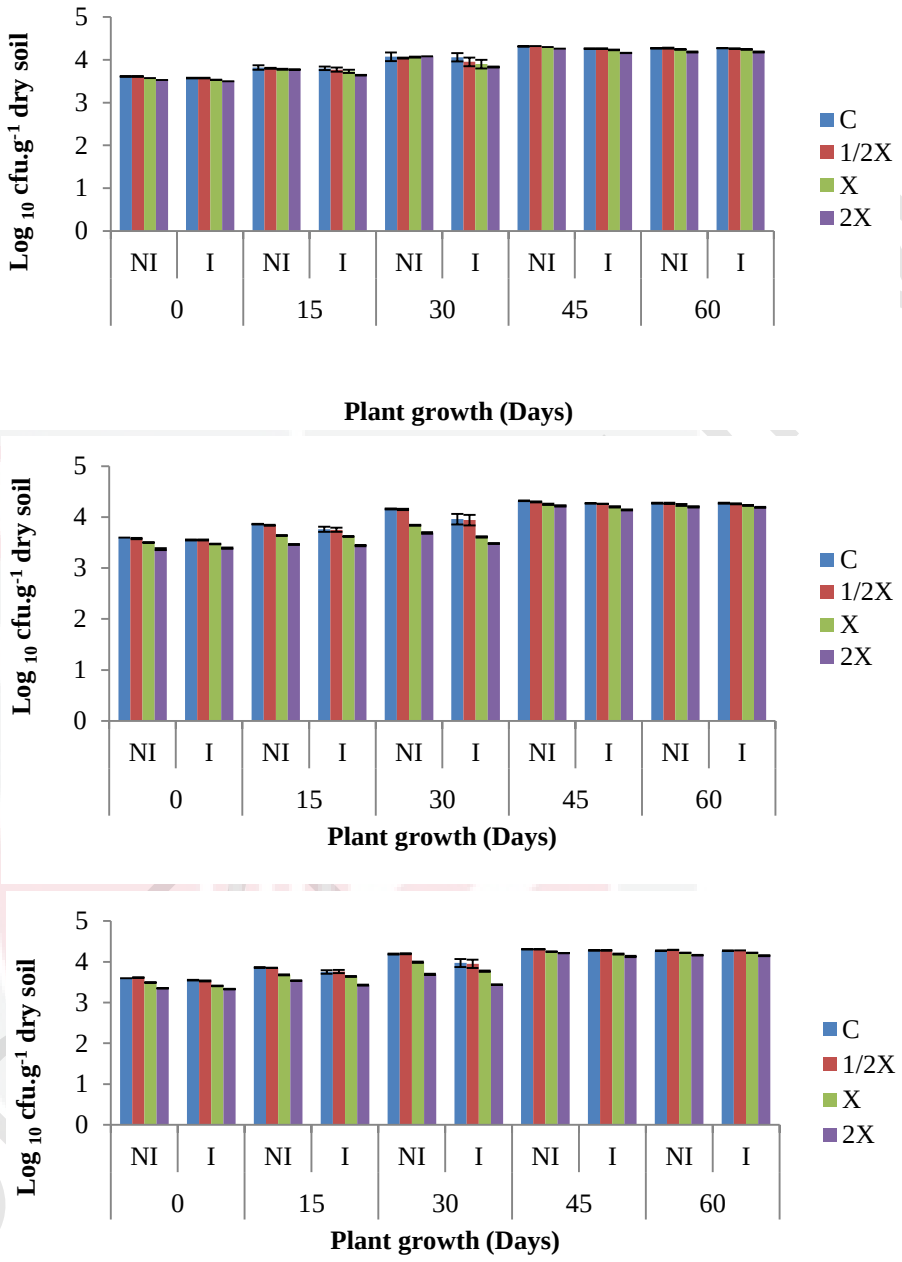
(c) 2, 4-D

Figure 4.2. Effect of Sb16 bacterial inoculation and herbicides application on diazotrophs population of soil within 60 days of aerobic rice growth; (a) paraquat, (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose; Bars indicate standard error (n=3)

4.3.3 Fungal Population

There were significant differences among bacterial inoculation, herbicides, concentrations and sampling dates for fungal population in soil. In general, fungal population significantly decreased with bacterial inoculation. There was no significant difference between pretilachlor and 2, 4-D. Fungal population in samples treated with paraquat had the highest values (3.96 $\log_{10}$ cfu.gr^{-1} dry soil), followed by pretilachlor (3.91 $\log_{10}$ cfu.gr^{-1} dry soil) and 2, 4-D (3.9 $\log_{10}$ cfu.gr^{-1} dry soil); however, pretilachlor and 2, 4-D did not show significant difference for fungal population. Half dose of herbicides did not show significant difference with control treatments for fungal population. The population significantly increased from day 0 till 45 after treatment, followed by a decrease at 60^{th} DAT, however, there was not a significant difference between 45^{th} and 60^{th} day.

The lowest fungal population (3.33, 3.41 and 3.53 $\log_{10}$ cfu. g^{-1} dry soil) were obtained in inoculated samples treated with double, full and half doses of 2, 4-D, respectively. However, population showed the highest values (4.32, 4.3 and 4.26 $\log_{10}$ cfu.g^{-1} dry soil) in non-inoculated samples treated with half, full and double doses of paraquat, respectively (Figure 4.3). There was significant three way interactions effect between bacterial inoculation, herbicides and sampling dates and between herbicides, concentrations and sampling dates on fungal population in soil (Appendix B.b). For example, non-inoculated samples treated with paraquat at 45^{th} DAT had higher population than inoculated samples. Population was higher in samples treated with paraquat at half dose at 45^{th} DAT than at full or double doses.



(a) paraquat

(b) pretilachlor

(c) 2, 4-D

Figure 4.3. Effect of Sb16 bacterial inoculation and herbicides application on fungal population of soil within 60 days of aerobic rice growth; (a) paraquat, (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose; Bars indicate standard error (n=3)

4.3.4 Growth Parameters of Aerobic Rice in Non-sterilized Soil

There were significant differences among bacterial inoculation and concentrations for plant height of aerobic rice in non-sterilized soil. In general, bacterial inoculation significantly increased the plant height. Three herbicides did not show significant differences for plant height of aerobic rice. There was no significant difference between full and double dose of herbicides for plant height. The highest plant height (71.67 cm) was obtained in inoculated control samples (without herbicides). The highest plant height in inoculated samples treated with herbicides was recorded by 71.53 cm with half dose of paraquat and by 71.27 and 71.17 cm with full and double doses of 2, 4-D, respectively. The shortest plant height (70.5 cm) was obtained in non-inoculated samples treated with double dose of 2, 4-D and paraquat (Table 4.2). There were significant interaction effects between inoculation with herbicides and herbicides with concentrations on height of aerobic rice plant in non-sterilized soil (Appendix B.c).

There were significant differences among inoculation and concentrations for leaf area of aerobic rice in non-sterilized soil. In general, inoculation significantly increased the leaf area. There was no significant difference between three herbicides for leaf area of aerobic rice in non-sterilized soil. Control with half dose of herbicides did not show significant difference. The highest leaf area of rice (182.55 cm^2) was obtained in inoculated samples treated with half dose of pretilachlor. The highest leaf area of aerobic rice in inoculated samples treated with herbicides at different concentrations was recorded by 174.02 cm^2 with full dose of pretilachlor and by 163.4 cm^2 with double dose of 2, 4-D. The lowest leaf area (144.55 cm^2) was obtained in non-inoculated samples treated with double dose of pretilachlor (Table 4.2). There was a significant two way interaction effect between bacterial inoculation and concentrations on leaf area of aerobic rice in non-sterilized soil (Appendix B.c).

There were significant differences among bacterial inoculation, herbicides and concentrations on leaf chlorophyll content of aerobic rice in non-sterilized soil. In general, inoculation significantly increased the chlorophyll content of rice in non-sterilized soil. Pretilachlor caused the lowest chlorophyll content (43.6), followed by 2, 4-D and paraquat (43.74); however, paraquat and 2, 4-D did not show significant difference for chlorophyll content. There was no significant difference between control with half dose of herbicides for chlorophyll content of rice in non-sterilized soil. The highest chlorophyll content of rice (44.57) was obtained in inoculated samples without herbicides. The highest chlorophyll content of rice in herbicides treated samples was obtained by 43.67 and 43.4 in inoculated samples with full and double doses of pretilachlor, respectively. The lowest chlorophyll content (43.03) was obtained in non-inoculated non-sterilized soil samples treated with double dose of paraquat (Table 4.2). There was no interaction effect of factors on chlorophyll content of aerobic rice in non-sterilized soil, indicating the independent effect of factors.

Table 4.2. Mean comparison of interaction effect of Sb16 bacterial inoculation, herbicides types and concentrations on growth parameters of aerobic rice in non-sterilized soil

Herbicides	Doses	Plant height (cm)		Leaf area (cm^2)		Chlorophyll content (SPAD Units)		Plant N content (%)		Shoot biomass (g/ plant)	
		NI	I	NI	I	NI	I	NI	I	NI	I
Paraquat	0	71.47^{b}	71.57^{ab}	150.48^{e}	177.67^{a}	43.9^{b}	44.57^{a}	2.24^{d}	2.49^{a}	4.576^{abc}	4.579^{a}
	1/2X	71.37^{ab}	71.53^{ab}	152.35^{de}	175.28^{ab}	43.8^{b}	44.33^{ab}	2.14^{cde}	2.47^{ab}	4.573^{bcd}	4.575^{c}
	X	70.73^{c}	70.97^{cd}	150.15^{def}	161.04^{bcd}	43.37^{c}	43.6^{bcd}	2.09^{efg}	2.32^{c}	4.57^{cde}	4.571^{bcdef}
	2X	70.5^{cd}	70.63^{d}	147.69^{f}	161.69^{c}	43.03^{d}	43.3^{cd}	1.89^{g}	2.21^{de}	4.561^{ef}	4.566^{def}
Pretilachlor	0	71.47^{b}	71.57^{ab}	153.37^{d}	180.02^{a}	43.6^{bc}	44.17^{ab}	2.21^{de}	2.54^{a}	4.577^{abc}	4.576^{b}
	1/2X	71.13^{bcd}	71.4^{b}	150.19^{e}	182.55^{a}	43.63^{b}	44^{ab}	2.16^{e}	2.41^{b}	4.574^{abcd}	4.574^{bc}
	X	71.07^{c}	70.93^{cd}	150.08^{def}	174.02^{ab}	43.17^{cd}	43.67^{bc}	2.11^{ef}	2.4^{b}	4.562^{def}	4.564^{def}
	2X	71.17^{c}	71.1^{c}	144.55^{ef}	151.07^{cdf}	43.13^{cd}	43.4^{cd}	1.99^{fg}	2.13^{ef}	4.556^{f}	4.558^{ef}
2, 4-D	0	71.57^{ab}	71.67^{a}	149.63^{e}	180.73^{a}	43.97^{ab}	44.13^{ab}	2.25^{cde}	2.54^{a}	4.578^{abc}	4.577^{ab}
	1/2X	71.3^{bc}	71.5^{abc}	151.79^{de}	178.67^{abc}	43.8^{b}	44.17^{ab}	2.2^{de}	2.53^{ab}	4.573^{bcd}	4.577^{abc}
	X	70.47^{d}	71.27^{bc}	151.01^{de}	168.51^{b}	43.43^{c}	43.63^{bc}	2.02^{efg}	2.42^{ab}	4.571^{d}	4.567^{def}
	2X	70.5^{d}	71.17^{c}	145.56^{f}	163.74^{bc}	43.4^{c}	43.37^{c}	2.08^{ef}	2.28^{cde}	4.561^{ef}	4.556^{ef}

Block	*	*	NS	NS	NS	
Inoc	**	**	**	**	NS	
Herb	NS	NS	*	NS	NS	
Conc	**	**	**	**	**	
Inoc*Herb	*	NS	NS	NS	NS	
Inoc*Conc	NS	**	NS	NS	NS	
Herb*Conc	*	NS	NS	NS	NS	
Inoc*Herb*Conc	NS	NS	NS	NS	NS	

NI: Non-Inoculated, I: Inoculated; Inoc: Inoculation, Herb: Herbicides, Conc: Concentrations; 1/2X: Half dose, X: Full dose, 2X: Double dose; Significant levels are * at ≤0.05, ** at ≤0.01 and NS=Not Significant at $p \leq 0.05$, No significant difference among means with same letters in each two column

There were significant differences among bacterial inoculation and concentrations for N content of aerobic rice in non-sterilized soil. Inoculation significantly increased the N content of rice in non-sterilized soil. There was significant difference between 2, 4-D and paraquat for N content. The N content in samples treated with 2, 4-D was highest (2.29%), followed by pretilachlor (2.24%) and paraquat (2.23%). Control samples (without herbicides) inoculated with Sb16 had the highest N content by 2.54%. The highest N content in herbicides treated samples were recorded by 2.53, 2.42 and 2.28% in inoculated samples treated with half, full and double doses of 2, 4-D, respectively. Non-inoculated samples treated with double dose of paraquat had the lowest N content by 1.89% (Table 4.2). There was no significant interaction effect found between the factors on N content of aerobic rice in non-sterilized soil.

There was significant difference among concentrations for aerobic rice dry weight in non-sterilized soil. Inoculation did not have significant effect on shoot dry weight of aerobic rice. Samples treated with paraquat had the highest shoot dry weight by 4.57 g/plant, followed by 2, 4-D (4.57 g/plant) and pretilachlor (4.57 g/plant). There was significant difference found between paraquat and pretilachlor for shoot dry weight of aerobic rice in non-sterilized soil. Control with half dose of herbicides did not show significant differences for shoot dry weight. Shoot dry weight had the highest value (4.58 g/plant) in inoculated samples without herbicides. The highest shoot dry weight in herbicides treated samples were recorded by 4.58 g/plant with half dose of 2, 4-D and by 4.57 and 4.57 g/plant with full and double doses of paraquat, respectively. The lowest shoot dry weight of aerobic rice (4.56 g/plant) in non-sterilized soil was found in non-inoculated samples treated with double dose of pretilachlor and in inoculated samples treated with double dose of 2, 4-D (Table 4.2). There was no significant interaction found between the factors on shoot dry weight of aerobic rice in non-sterilized soil.

4.3.5. Growth Parameters of Aerobic Rice in Sterilized Soil

There was a high significant difference among concentrations for aerobic rice height in sterilized soil; however control with half dose and full with double dose did not show significant difference. There was no significant difference found among three herbicides for aerobic rice plant height. Bacterial inoculation did not have significant effect on aerobic rice height in sterilized soil. The tallest plant height (49.53 cm) was obtained in inoculated samples without herbicides. The highest plant height in inoculated samples treated with herbicides was recorded by 49.3, 48.7 and 48.67 cm with half, full and double doses of 2, 4-D, respectively. However the shortest plant height (48.17 cm) was obtained in non-inoculated samples treated with double dose of paraquat (Table 4.3). There was no significant interaction effect between the factors on height of aerobic rice in sterilized soil. There were significant differences among bacterial inoculation, herbicides and concentrations on leaf area of aerobic rice in sterilized soil. In general, bacterial inoculation significantly increased the leaf area of rice in sterilized soil. Leaf area was higher in samples treated with paraquat (62.49 cm^2), followed by pretilachlor (59.46 cm^2) and 2, 4-D (59.02 cm^2); however, pretilachlor and 2, 4-D did not show significant

difference for leaf area of rice. There were no significant differences between control with half dose and full with double doses of herbicides for leaf area of rice in sterilized soil. The highest leaf area of rice (74.48 cm^2) was obtained in inoculated sterilized soil samples treated with half dose of 2, 4-D. The lowest leaf area of rice in herbicides treated samples was recorded by 46.73, 51.8 and 57.63 cm^2 in non-inoculated sterilized soil samples treated with 2, 4-D at double, full and half doses, respectively (Table 4.3). There was a significant interaction effect between inoculation and concentrations on leaf area of aerobic rice in sterilized soil (Appendix B. d).

There were significant differences among inoculation, herbicides and concentrations for chlorophyll content of aerobic rice in sterilized soil. Bacterial inoculation significantly increased the chlorophyll content of rice in sterilized soil. Samples treated with paraquat had the highest chlorophyll content (35.24), followed by 2, 4-D (34.93) and pretilachlor (34.82); however 2, 4-D and pretilachlor did not show significant difference for chlorophyll content. The non-inoculated control samples (without herbicides) had the highest chlorophyll content by 35.67. The highest chlorophyll content in herbicides treated sterilized soil samples were recorded by 35.53 with half dose of 2, 4-D and by 35.03 and 34.93 with full and double doses of paraquat, respectively. Non-inoculated samples treated with double, full and half doses of pretilachlor had the lowest chlorophyll content by 34.07, 34.2 and 34.7, respectively. The lowest chlorophyll content was obtained by 34.07 in non-inoculated samples treated with double dose of pretilachlor (Table 4.3). There was a significant two way interaction effects between inoculation and herbicides on chlorophyll content of aerobic rice in sterilized soil (Appendix B.d).

Table 4.3. Mean comparison of interaction effect of Sb16 bacterial inoculation, herbicides types and concentrations on growth parameters of aerobic rice in sterilized soil

Herbicides	Doses	Plant height (cm)		Leaf area (cm^2)		Chlorophyll content (SPAD Units)		Plant N content (%)		Shoot biomass (g/ plant)	
		NI	I	NI	I	NI	I	NI	I	NI	I
Paraquat	0	49.27^{ab}	49.43^{ab}	64.7^{b}	70.31^{a}	35.67^{a}	35.63^{a}	1.36^{b}	1.44^{ab}	1.695^{a}	1.694^{a}
	1/2X	49.23^{ab}	49.07^{abcd}	61.56^{bc}	70.4^{ab}	35.5^{ab}	35.5^{ab}	1.34^{bc}	1.39^{abc}	1.694^{a}	1.693^{a}
	X	48.4^{cd}	48.53^{cd}	60.09^{c}	55.94^{bcd}	35^{cd}	35.03^{bcd}	1.15^{d}	1.2^{cd}	1.686^{b}	1.683^{bc}
	2X	48.17^{cd}	48.2^{cd}	58.46^{cd}	58.42^{bcd}	34.67^{cd}	34.93^{bcd}	0.92^{f}	1.17^{cde}	1.677^{bcd}	1.674^{de}
Pretilachlor	0	49.3^{ab}	49.4^{ab}	62.93^{bc}	72.05^{a}	35.3^{b}	35.6^{a}	1.38^{b}	1.46^{a}	1.694^{a}	1.694^{a}
	1/2X	49.17^{ab}	49.1^{bc}	59.12^{cd}	70.44^{a}	34.7^{c}	35.2^{bc}	1.3^{bc}	1.35^{bc}	1.692^{a}	1.693^{a}
	X	48.37^{cd}	48.43^{cd}	54.09^{de}	51.2^{de}	34.2^{de}	34.8^{cd}	1.21^{d}	1.23^{c}	1.684^{bc}	1.68^{bcde}
	2X	48.4^{cd}	48.27^{d}	50.79^{de}	55.07^{cd}	34.07^{de}	34.67^{cde}	1.05^{def}	1^{ef}	1.676^{cd}	1.675^{de}
2, 4-D	0	49.43^{ab}	49.53^{a}	62.66^{bc}	72.92^{ab}	35.37^{ab}	35.5^{ab}	1.33^{b}	1.46^{ab}	1.695^{a}	1.695^{a}
	1/2X	49.43^{ab}	49.3^{b}	57.63^{c}	74.48^{ab}	34.8^{bc}	35.53^{ab}	1.33^{bc}	1.43^{ab}	1.695^{a}	1.695^{a}
	X	48.33^{cd}	48.7^{c}	51.8^{d}	52.64^{d}	34.43^{cde}	34.97^{cd}	1.23^{cde}	1.27^{bcd}	1.68^{bcde}	1.686^{b}
	2X	48.33^{cd}	48.67^{cd}	46.73^{e}	53.31^{cde}	34.07^{e}	34.8^{cde}	1.14^{cde}	1.2^{cd}	1.667^{e}	1.681^{c}
Block		NS		NS		**		NS		*	
Inoc		NS		**		**		**		NS	
Herb		NS		*		**		*		NS	
Conc		**		**		**		**		**	
Inoc*Herb		NS		NS		**		NS		**	
Inoc*Conc		NS		**		NS		NS		NS	
Herb*Conc		NS		NS		NS		NS		NS	
Inoc*Herb*Conc		NS		NS		NS		NS		*	

NI: Non-Inoculated, I: Inoculated; Inoc: Inoculation, Herb: Herbicides, Conc: Concentrations; 1/2X: Half dose, X: Full dose, 2X: Double dose; Significant levels are * at ≤0.05, ** at ≤0.01 and NS=Not Significant at $p \leq 0.05$, No significant difference among means with same letters in each two column

There were significant differences among inoculation, herbicides and concentrations for N content of aerobic rice in sterilized soil. Inoculation significantly increased the N content in sterilized soil. Samples treated with 2, 4-D had the highest N content by 1.3%, followed by pretilachlor (1.25%) and paraquat (1.25%). There was no significant difference between paraquat and pretilachlor for N content of aerobic rice in sterilized soil. Control and half dose of herbicides did not show significant difference for N content. The highest N content (1.46%) was found in inoculated samples without herbicides. The highest N content in herbicides treated samples were recorded by 1.43, 1.27 and 1.2% in inoculated samples treated with half, full and double doses of 2, 4-D, respectively. The lowest N content (0.92%) of aerobic rice in sterilized soil was obtained in non-inoculated samples treated with double dose of paraquat (Table 4.3). There was no significant interaction effect between the factors on N content of aerobic rice in sterilized soil.

There was highly significant difference found among concentrations for shoot dry weight of aerobic rice in sterilized soil. Inoculation did not have a significant effect on shoot dry weight of aerobic rice in sterilized soil. There were no significant differences found between three herbicides for shoot dry weight. Control with half dose of herbicides did not show significant difference for shoot dry weight. Control treatments (without herbicides) irrespective of bacterial inoculation had the highest shoot dry weight (1.7 g/plant). The inoculated samples treated with half, full and double dose of 2, 4-D had the highest shoot dry weight by 1.7, 1.69 and 1.68 g/plant, respectively. The lowest shoot dry weight in herbicides treated samples were recorded by 1.67 and 1.68 g/plant in non-inoculated samples treated with double and full doses of 2, 4-D, respectively (Table 4.3). There was significant three way interaction effects between bacterial inoculation, herbicides and concentrations on shoot dry weight of aerobic rice in sterilized soil (Appendix B.d).

4.3.6 Root Morphological Parameters of Aerobic Rice in Non-sterilized Soil

There were significant differences among bacterial inoculation and concentrations for root dry weight of aerobic rice in non-sterilized soil. Inoculation significantly increased root dry weight. There was no significant difference found between three herbicides for root dry weight. Control with half dose of herbicides did not show significant difference for root dry weight. The highest root dry weight of aerobic rice (1.16 g/plant) was obtained in inoculated samples without herbicides. The highest root dry weight in inoculated samples treated with herbicides were recorded by 1.16 and 1.15 g/plant with half and full doses of 2, 4-D and by 1.15 with double dose of paraquat. However, the lowest root dry weight in non-inoculated samples treated with herbicides were recorded by 1.14, 1.15 and 1.15 g/plant with double, full and half doses of paraquat, respectively (Table 4.4). There was a significant interaction effect between bacterial inoculation and herbicides on root dry weight of aerobic rice in non-sterilized soil (Appendix B.e).

There were significant differences among bacterial inoculation, herbicides and concentrations for root length of aerobic rice in non-sterilized soil. Root length was significantly longer in inoculated compared to non-inoculated samples. Paraquat and pretilachlor showed no significant difference for root length. Soil samples treated with paraquat had the longest root length (1393.11 cm), followed by pretilachlor (1389.7 cm) and 2, 4-D (1371.98 cm). There was no significant difference between control and half dose of herbicides for root length of aerobic rice in non-sterilized soil. Control soil samples inoculated with Sb16 had the highest root length (1455.83 cm). The longest root length (1455.83 cm) was obtained in inoculated control samples. The shortest root length in herbicides treated samples were recorded by 1287, 1320.98 and 1397.16 cm in non-inoculated samples with double, full and half doses of 2, 4-D, respectively. Moreover, the shortest root length in inoculated samples treated with herbicides were recorded by 1350.85 and 1357.03 cm with double and full and doses of 2, 4-D, respectively (Table 4.4). There was no significant interaction effect between factors on root length of aerobic rice in non-sterilized soil.

There was significant difference found among concentrations for root surface area of aerobic rice in non-sterilized soil. Inoculation did not have a significant effect on root surface area of rice in non-sterilized soil. Three herbicides did not show significant differences for root surface area. The highest root surface area (341.94 cm^2) was found in inoculated control samples. Root surface area had the highest values by 325.87, 305.01 and 279.42 cm^2 in inoculated samples treated with half, full and double doses of 2, 4-D, respectively. Moreover, the lowest root surface area (253.06, 272.22 and 311.04 cm^2) was found in non-inoculated samples treated with 2, 4-D at double, full and half doses, respectively (Table 4.4). There was a significant two way interaction effect between bacterial inoculation and herbicides on root surface area of aerobic rice in non-sterilized soil, indicating the dependent effect of these factors (Appendix B.e).

Table 4.4. Mean comparison of interaction effect of Sb16 bacterial inoculation, herbicides types and concentrations on root morphological parameters of aerobic rice in non-sterilized soil

Herbicides	Doses	Root biomass (g/ plant)		Root length (cm)		Root surface area (cm^2)		Root volume (cm^3)		Root average diameter (mm)	
		NI	I	NI	I	NI	I	NI	I	NI	I
	0	1.153^{b}	1.158^{a}	1399.45^{bc}	1455.83^{a}	327.01^{a}	329.54^{a}	14.19^{c}	15.78^{ab}	1.179^{abc}	1.224^{ab}
	1/2X	1.152^{b}	1.156^{ab}	1408.52^{b}	1432.53^{abc}	312.78^{b}	312.91^{b}	14.64^{bc}	14.03^{cde}	1.132^{cd}	1.181^{abc}
Paraquat	X	1.146^{d}	1.15^{c}	1372.26^{bcd}	1372.73^{bcde}	304.86^{b}	276.69^{cd}	13.76^{d}	12.67^{ef}	1.117^{def}	1.156^{bcd}
	2X	1.141^{e}	1.15^{cd}	1334.02^{def}	1369.51^{d}	81.04^{c}	247.41^{de}	12.16^{ef}	12.34^{ef}	1.122^{cdef}	1.131^{cd}
	0	1.154^{b}	1.157^{ab}	1391.88^{bc}	1442.2^{abc}	336.98^{a}	341.94^{a}	15.18^{b}	15.37^{b}	1.178^{bc}	1.187^{b}
	1/2X	1.154^{b}	1.153^{b}	1400.34^{b}	1407.09^{abc}	322.05^{ab}	323.73^{ab}	13.52^{cdef}	15.11^{bc}	1.161^{bcd}	1.198^{b}
Pretilachlor	X	1.147^{d}	1.152^{bc}	1386.82^{c}	1387.34^{bcde}	294.96^{bc}	276.35^{cde}	13.17^{de}	13.47^{cde}	1.111^{ef}	1.131^{d}
	2X	1.145^{de}	1.149^{cd}	1338.28^{e}	1363.68^{de}	278.98^{c}	262.29^{de}	12.19^{ef}	13.28^{de}	1.091^{f}	1.112^{def}
	0	1.153^{b}	1.155^{ab}	1407.7^{abc}	1426.61^{ab}	319.37^{ab}	338.47^{a}	13.88^{b}	15.84^{a}	1.181^{bc}	1.259^{ab}
	1/2X	1.153^{bc}	1.157^{a}	1397.16^{bc}	1428.49^{abc}	311.04^{b}	325.87^{ab}	13.52^{cdef}	15.57^{abcde}	1.151^{cd}	1.252^{a}
2, 4-D	X	1.147^{d}	1.152^{bcd}	1320.98^{ef}	1357.03^{de}	272.22^{cd}	305.01^{abcde}	12.83^{ef}	13.32^{e}	1.116^{ef}	1.169^{bcd}
	2X	1.145^{dc}	1.146^{d}	1287^{f}	350.85^{e}	253.06^{d}	279.42^{bcde}	12.1^{f}	12.87^{ef}	1.111^{ef}	1.15^{cd}
Block		NS		*		NS		NS		NS	
Inoc		**		**		NS		**		**	
Herb		NS		*		NS		NS		**	
Conc		**		**		**		**		**	
Inoc*Herb		*		NS		**		NS		**	
Inoc*Conc		NS		NS		NS		NS		NS	
Herb*Conc		NS		NS		NS		NS		NS	
Inoc*Herb*Conc		NS		NS		NS		NS		NS	

NI: Non-Inoculated, I: Inoculated; Inoc: Inoculation, Herb: Herbicides, Conc: Concentrations; 1/2X: Half dose, X: Full dose, 2X: Double dose; Significant levels are * at ≤0.05, ** at ≤0.01 and NS=Not Significant at $p \leq 0.05$, No significant difference among means with same letters in each two column

There were significant differences among bacterial inoculation and concentrations for root volume of aerobic rice in non-sterilized soil. In general, inoculation significantly increased the root volume in non-sterilized soil. Three herbicides did not show significant differences for root volume. There were not significant differences between control and half dose and between full and double doses of herbicides for root volume of aerobic rice in non-sterilized soil. The highest root volume (15.84 cm^3) was obtained in inoculated control samples. The highest root volume in herbicides treated samples were obtained by 15.57 cm^3 with half dose of 2, 4-D and by 13.47 and 13.28 cm^3 with full and double doses of pretilachlor, respectively. The lowest root volume (12.1, 12.83 and 13.52 cm^3) were found in non-inoculated samples treated with 2, 4-D at double, full and half doses, respectively (Table 4.4). There was no significant interaction effect found between the factors on root volume of aerobic rice in non-sterilized soil (Appendix B. e).

There were significant differences among bacterial inoculation, herbicides and concentrations for root average diameter of aerobic rice in non-sterilized soil. In general, inoculation significantly increased the root average diameter of rice in non-sterilized soil. There was no significant difference between paraquat and pretilachlor for root diameter of rice in non-sterilized soil. The samples treated with 2, 4-D had the highest root diameter (1.17 mm), followed by paraquat (1.16 mm) and pretilachlor (1.15 mm). Full and double doses of herbicides did not show significant difference for root diameter. The highest root diameter (1.26 mm) was recorded in inoculated control soil samples. The highest root diameter in inoculated samples were found by 1.25, 1.17 and 1.15 mm with half, full and double doses of 2, 4-D, respectively. The lowest root diameter in non-inoculated samples were recorded by 1.09 and 1.11 mm in samples treated with double and full doses of pretilachlor, respectively and by 1.13 mm with half dose of paraquat (Table 4.4). Inoculation and herbicides had a significant interaction effect on root average diameter of aerobic rice in non-sterilized soil (Appendix B.e).

4.3.7. Root Morphological Parameters of Aerobic Rice in Sterilized Soil

There were significant differences among bacterial inoculation, herbicides and concentrations for root dry weight of aerobic rice in sterilized soil. Inoculation significantly increased the root dry weight of rice in sterilized soil. There was significant difference found between paraquat and 2, 4-D for root dry weight. Samples treated with paraquat had the highest root dry weight (0.37 g/plant), followed by pretilachlor (0.37 g/plant) and 2, 4-D (0.36 g/plant). The half and full doses of herbicides did not show significant difference for root dry weight of aerobic rice in sterilized soil samples. The highest root dry weight (0.38 g/plant) was obtained in inoculated control samples. The highest root dry weight in herbicides treated samples were obtained by 0.37, 0.37 and 0.37 g/plant in inoculated sterilized soil samples treated with half, full and double doses of paraquat, respectively. However, the lowest root dry weight (0.34 g/plant) was obtained in non-inoculated samples treated with double dose of 2, 4-D (Table 4.5). There was a significant two way interaction effect between inoculation and concentrations on root dry weight of aerobic rice in sterilized soil (Appendix B.f).

There were significant differences found among bacterial inoculation and concentrations for root length of aerobic rice in sterilized soil. In general, inoculation significantly increased the root length. Herbicides did not show significant differences for root length. Inoculated samples without herbicides showed the longest root length (950.11 cm). Root length in herbicides treated samples inoculated with Sb16 had the highest values by 923.69 and 861.03 cm with half and full doses of 2, 4-D, respectively and by 843.61 cm with double dose of paraquat. However, the shortest root length in non-inoculated samples treated with herbicides were recorded by 789.44 and 827.42 cm with double and full doses of 2, 4-D, respectively (Table 4.5). There were significant two way interaction effects between bacterial inoculation with herbicides, bacterial inoculation with concentrations and herbicides with concentrations on root length of aerobic rice in sterilized soil samples (Appendix B.f).

There was significant difference among the concentrations for root surface area of aerobic rice in sterilized soil. Inoculation did not have a significant effect on root surface area of rice in sterilized soil. There was significant difference between 2, 4-D and pretilachlor for root surface area. 2, 4-D treated samples showed the highest root surface area (215.53 cm^2), followed by paraquat (210.21 cm^2) and pretilachlor (204.27 cm^2). Control with half dose of herbicides did not show a significant difference for root surface area. The highest root surface area (277.83 cm^2) was obtained in inoculated samples without herbicides. The highest root surface area in herbicides treated samples were recorded by 252.02 cm^2 with half dose of 2, 4-D and by 197.43 and 179.82 cm^2 with full and double doses of paraquat, respectively. However, root surface area showed the lowest values by 139.29 and 179.47 cm^2 in non-inoculated samples treated with double and full doses of paraquat, respectively and by 237.79 cm^2 with half dose of pretilachlor (Table 4.5). A significant interaction effect between inoculation and herbicides on root surface area of aerobic rice in sterilized soil was observed (Appendix B.f).

Table 4.5. Mean comparison of interaction effect of Sb16 bacterial inoculation, herbicides types and concentrations on root morphological parameters of aerobic rice in sterilized soil

Herbicides	Doses	Root biomass (g/ plant)		Root length (cm)		Root surface area(cm^2)		Root volume (cm^3)		Root average diameter(mm)	
		NI	I	NI	I	NI	I	NI	I	NI	I
Paraquat	0	0.374^{ab}	0.376^{a}	905.56^{b}	909.61^{bc}	241.55^{c}	255.13^{abc}	10.32^{b}	10.49^{ab}	1.285^{ab}	1.264^{abc}
	1/2X	0.371^{bc}	0.374^{b}	895.67^{bc}	886.23^{c}	238.84^{cd}	250.1^{b}	8.66^{cdef}	10.55^{ab}	1.283^{ab}	1.231^{abc}
	X	0.366^{cd}	0.372^{c}	869.98^{cd}	853.46^{de}	179.47^{e}	197.43^{e}	7.87^{ef}	9.11^{c}	1.166^{bcde}	1.212^{cde}
	2X	0.36^{de}	0.365^{de}	824.03^{f}	843.61^{e}	139.29^{g}	179.82^{e}	7.65^{ef}	8^{de}	1.201^{cde}	1.216^{c}
Pretilachlor	0	0.375^{abc}	0.376^{ab}	899.65^{bc}	950.11^{a}	241.91^{bcd}	277.83^{a}	10.52^{ab}	10.7^{a}	1.281^{b}	1.302^{ab}
	1/2X	0.373^{bc}	0.373^{bc}	911.42^{b}	896.97^{bc}	237.79^{d}	233^{cd}	9.91^{abc}	9.46^{c}	1.264^{abc}	1.301^{a}
	X	0.358^{e}	0.37^{cd}	859.47^{d}	831.6^{ef}	181.16^{e}	179.97^{ef}	8.33^{cdef}	8.57^{d}	1.148^{de}	1.201^{c}
	2X	0.345^{f}	0.365^{d}	805.03^{fg}	842.63^{def}	144.86^{fg}	137.61^{g}	8.38^{cdef}	6.93^{ef}	1.132^{e}	1.176^{cde}
2, 4-D	0	0.374^{ab}	0.375^{ab}	910.73^{bc}	941.38^{a}	251.26^{bc}	258.31^{abcd}	10.11^{abc}	10.31^{ab}	1.262^{bc}	1.283^{abc}
	1/2X	0.369^{bcd}	0.358^{bcdef}	898.73^{c}	923.69^{abcd}	256.39^{b}	252.02^{bcd}	8.3^{bcde}	9.09^{c}	1.296^{abc}	1.282^{abc}
	X	0.362^{d}	0.366^{cd}	827.42^{ef}	861.03^{cdef}	195.13^{ef}	178.11^{e}	7.56^{ef}	7.84^{de}	1.228^{c}	1.234^{c}
	2X	0.343^{f}	0.364^{cde}	789.44^{g}	834.42^{def}	172.48^{ef}	160.57^{efg}	6.6^{ef}	6.53^{f}	1.169^{cde}	1.18^{cde}

Block	*	NS	NS	NS	*
Inoc	**	**	NS	NS	NS
Herb	*	NS	NS	**	NS
Conc	**	**	**	**	**
Inoc*Herb	NS	**	*	*	NS
Inoc*Conc	**	**	NS	NS	NS
Herb*Conc	NS	*	NS	NS	NS
Inoc*Herb*Conc	NS	NS	NS	NS	NS

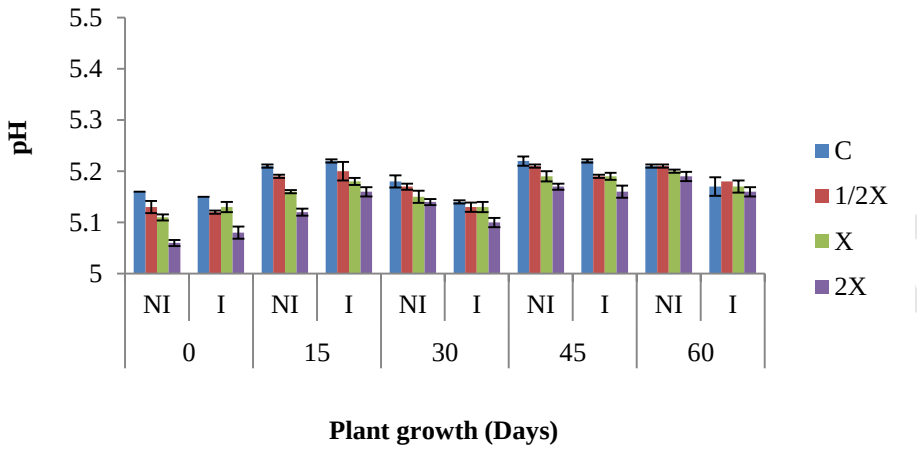
NI: Non-Inoculated, I: Inoculated; Inoc: Inoculation, Herb: Herbicides, Conc: Concentrations; 1/2X: Half dose, X: Full dose, 2X: Double dose; Significant levels are * at ≤0.05, ** at ≤0.01 and NS=Not Significant at $p \leq 0.05$, No significant difference among means with same letters in each two column

There were significant differences among the herbicides and concentrations for root volume of aerobic rice in sterilized soil. Inoculation did not significantly affect the root volume of aerobic rice in sterilized soil. Paraquat and pretilachlor did not show significant differences for root volume of aerobic rice in sterilized soil. Samples treated with pretilachlor had the highest root volume (9.1cm^3), followed by paraquat (9.08 cm^3) and 2, 4-D (8.29 cm^3). The highest root volume (10.7 cm^3) was obtained in inoculated control samples. The highest root volume by 10.55, 9.11 and 8 cm^3 were recorded in inoculated samples with half, full and double doses of paraquat, respectively. The lowest root volume in non-inoculated sterilized soil samples were recorded by 6.6, 7.56 and 8.3 cm^3 in samples treated with 2, 4-D at double, full and half doses, respectively (Table 4.5). Bacterial inoculation and herbicides had a significant interactive effect on root volume of aerobic rice in sterilized soil (Appendix B.f).

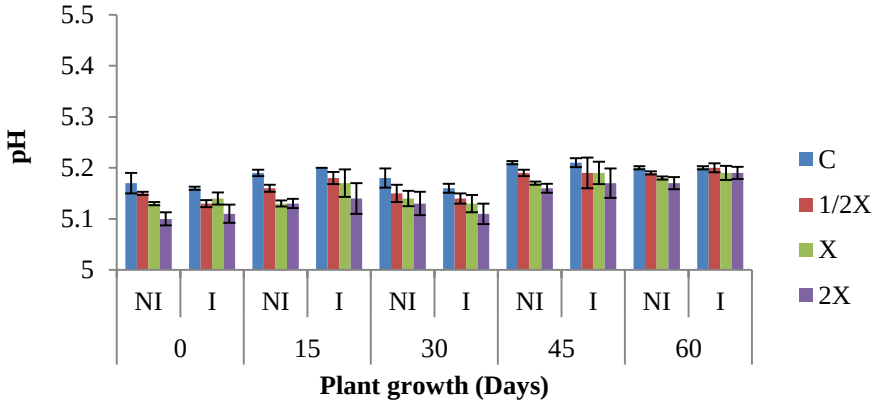
There was a significant difference among the concentrations for root average diameter of aerobic rice in sterilized soil. Inoculation did not significantly affect the root diameter. Three herbicides did not show significant differences for root diameter. There were not significant differences among control with half dose and full with double doses of herbicides for root diameter. The highest root diameter (1.3 mm) was obtained in inoculated samples without herbicides. Root diameter had the lowest values (1.13, 1.15 and 1.26 mm) in non-inoculated sterilized samples treated with double, full and half doses of pretilachlor, respectively (Table 4.5). There was no significant interaction effect found between factors on root diameter of aerobic rice in sterilized soil (Appendix B. f).

4.3.8 Soil pH

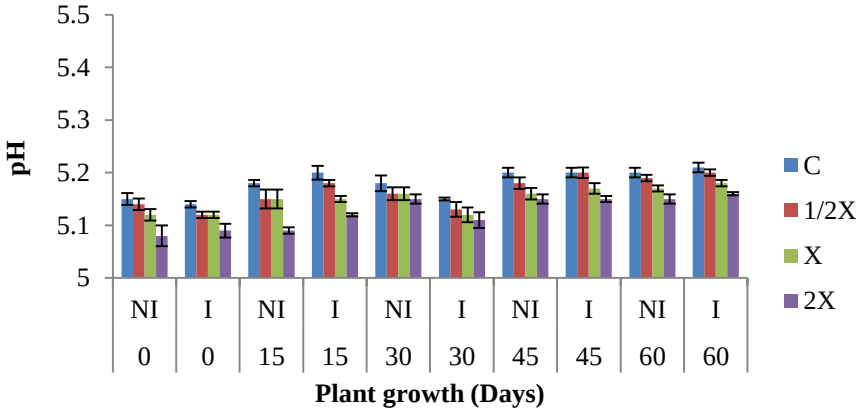
There were significant differences among herbicides, concentrations and sampling dates for pH of non-sterilized soil. Bacterial inoculation did not have a significant effect on pH of non-sterilized soil. Paraquat and pretilachlor did not show significant differences for pH of non-sterilized soil. Samples treated with paraquat had the highest pH (5.164), followed by (5.163) and 2, 4-D (5.155). There were significant differences among the concentrations of herbicides for pH of non-sterilized soil. pH significantly increased from day 0-15 after treatment; followed by a significant decline at day 30 and an increase till 60th DAT, with highest and lowest value at day 60 and 0 after treatment, respectively. There was no significant difference between 45th and 60th DAT for pH of non-sterilized soil. The lowest pH (5.06, 5.11 and 5.13) was obtained in non-inoculated samples treated with double, full and half doses of paraquat, respectively at day 0 after treatment. The highest pH (5.21, 5.2 and 5.19) were obtained in non-inoculated samples treated with half, full and double doses of paraquat, respectively at 60th DAT (Figure 4.4). There was a significant three way interaction effect between bacterial inoculation, herbicides and sampling dates on pH of non-sterilized soil (Appendix B



(a) paraquat



(b) pretilachlor



(c) 2, 4-D

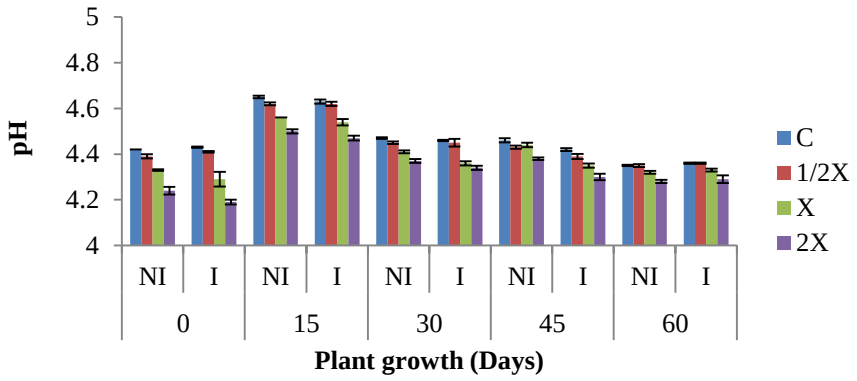
Figure 4.4. Effect of Sb16 bacterial inoculation and herbicides application on pH of non-sterilized soil within 60 days of aerobic rice growth; (a) paraquat; (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose Bars indicate standard error (n=3)

There were significant differences among herbicides, concentrations and sampling dates for pH of sterilized soil. Inoculation did not have a significant effect on pH of sterilized soil. Paraquat and 2, 4-D did not show significant difference for pH of sterilized soil. Samples treated with paraquat had the highest pH (4.409), followed by 2, 4-D (4.406) and pretilachlor (4.40). There were significant differences between different concentrations of herbicides for pH of sterilized soil. In general, pH significantly increased from day 0-15 after treatment, followed by a significant decline thereafter till 60th day, with highest and lowest pH at 15th and 60th DAT, respectively.

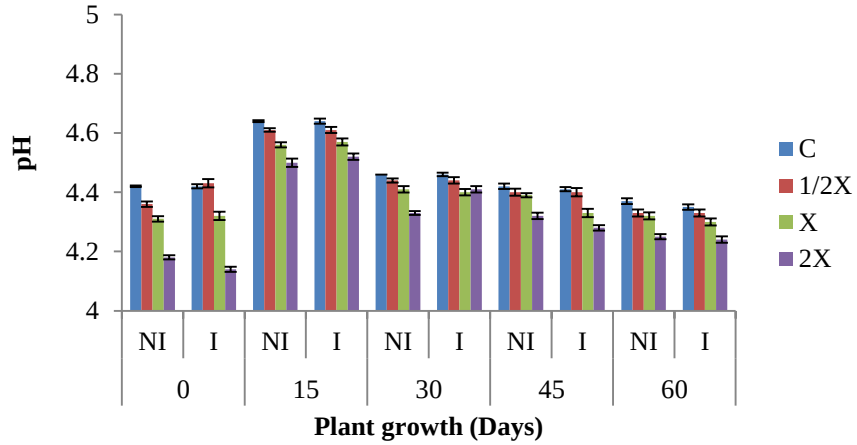
The highest pH in herbicides treated samples were obtained by 4.64 and 4.59 in inoculated samples treated with half and double doses of 2, 4-D, respectively and by 4.57 with full dose of pretilachlor at 15th DAT. However, the lowest pH (4.14) was obtained in inoculated samples treated with double dose pretilachlor at day 0 after treatment (Figure 4.5). There were significant four way interaction effects between bacterial inoculation, herbicides, concentrations and sampling date on pH of sterilized soil (Appendix B. h).

4.3.9 Soil Organic Matter (OM)

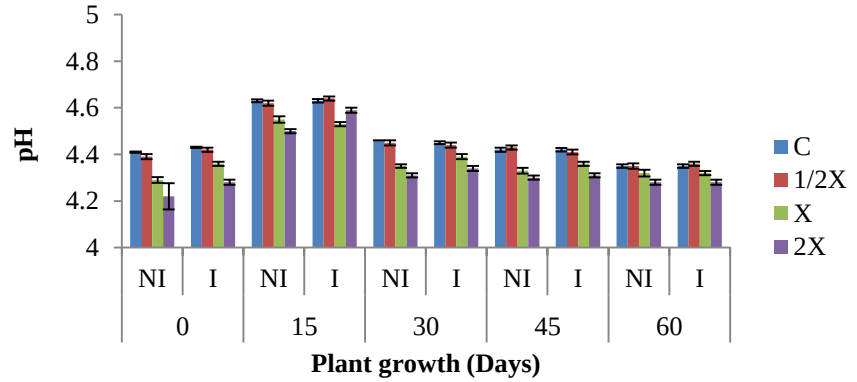
There were significant differences among bacterial inoculation, herbicides, concentrations and sampling dates for organic matter (OM) of non-sterilized soil. In general, inoculation significantly increased OM of non-sterilized soil. Samples treated with paraquat had the highest OM by (2.28%), followed by 2, 4-D (2.27%) and pretilachlor (2.26%). Different concentrations of herbicides showed significant differences for OM of non-sterilized soil. OM significantly increased from day 0-60 after treatment, with the highest and lowest values at day 60 and 0 after treatment, respectively. The highest OM of non-sterilized soil (2.76, 2.74 and 2.68%) were obtained in inoculated samples treated with half, full and double doses of 2, 4-D, respectively at 60th DAT. However, the lowest OM of non-sterilized soil in herbicides treated samples were recorded by 1.38 and 1.81% with double and half doses of pretilachlor, respectively and by 1.57% with full dose of 2, 4-D (Figure 4.6). There was a significant four way interaction effect between bacterial inoculation, herbicides, concentrations and sampling dates on OM of non-sterilized soil (Appendix B.g).



(a) paraquat

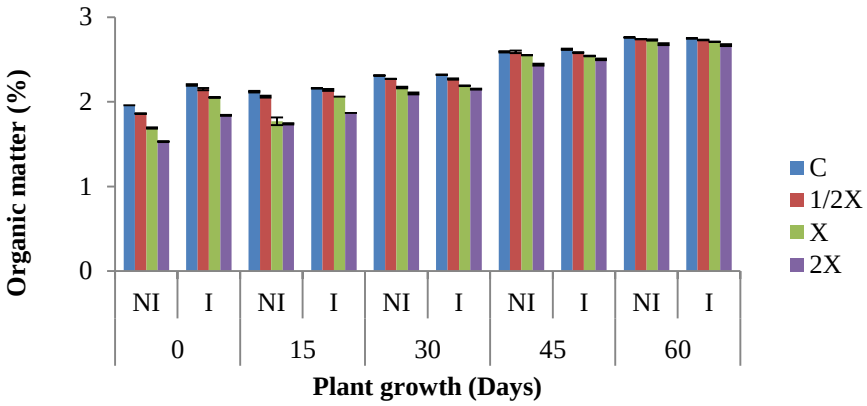


(b) pretilachlor

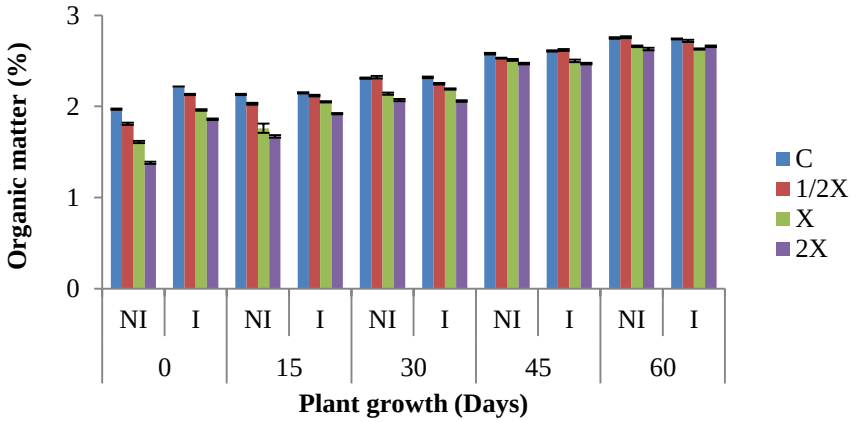


(c) 2, 4-D

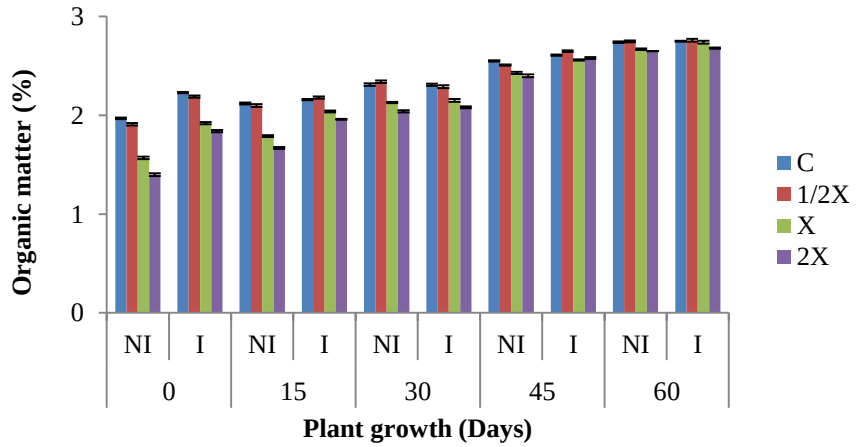
Figure 4.5. Effect of Sb16 bacterial inoculation and herbicides application on pH of sterilized soil within 60 days of aerobic rice growth; (a) paraquat; (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose Bars indicate standard error (n=3)



(a) paraquat



(b) pretilachlor



(c) 2, 4-D

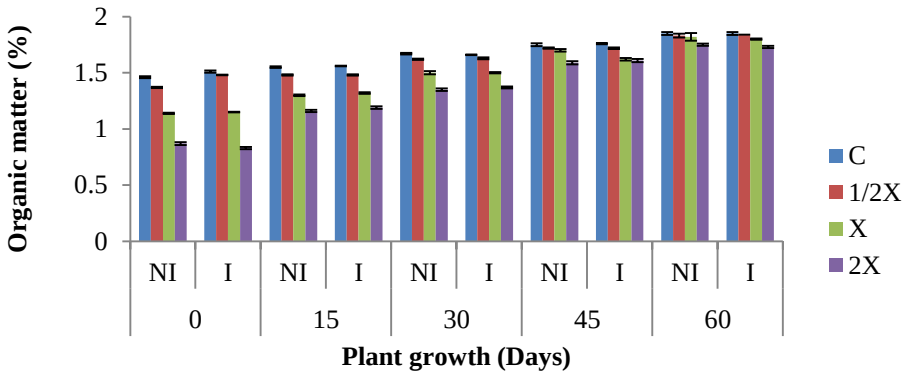
Figure 4.6. Effect of Sb16 bacterial inoculation and herbicides application on organic matter of non-sterilized soil within 60 days of aerobic rice growth; (a) paraquat; (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose Bars indicate standard error (n=3)

There were significant differences found among the bacterial inoculation, herbicides, concentrations and sampling dates for OM of sterilized soil. Bacterial Inoculation significantly increased OM of sterilized soil. Samples treated with paraquat showed the highest OM of sterilized soil (1.53%), followed by pretilachlor (1.5%) and 2, 4-D (1.47%). Control samples had the highest OM, followed by half, full and double doses. In general, OM of sterilized soil significantly increased from day 0-60 after treatment.

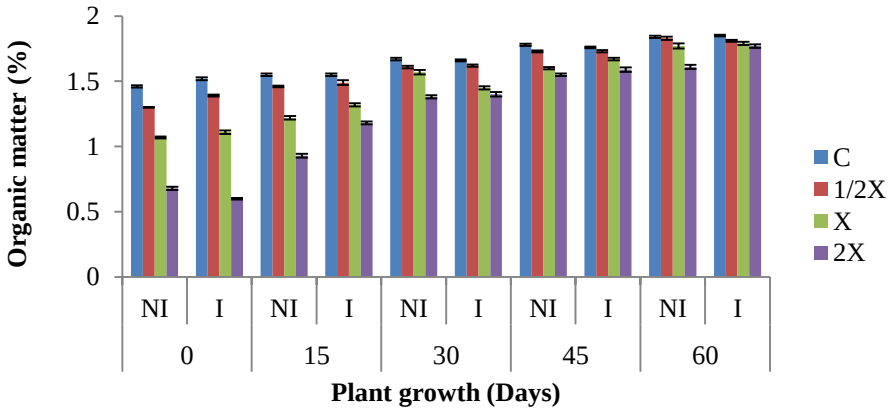
The lowest OM in herbicides treated samples were recorded by 0.51 and 1.29% in non-inoculated samples treated with double and half doses of 2, 4-D, respectively and by 0.75% with full dose of 2, 4-D in inoculated samples. The highest OM values were recorded by 1.87 and 1.8% in inoculated samples treated with half and full doses of 2, 4-D and by 1.77% with double dose of pretilachlor at 60th DAT (Figure 4.7). Bacterial inoculation, herbicides, concentrations and sampling dates had a significant four way interaction effect on OM of sterilized soil (Appendix B. h).

4.3.10 Soil Cation Exchange Capacity (CEC)

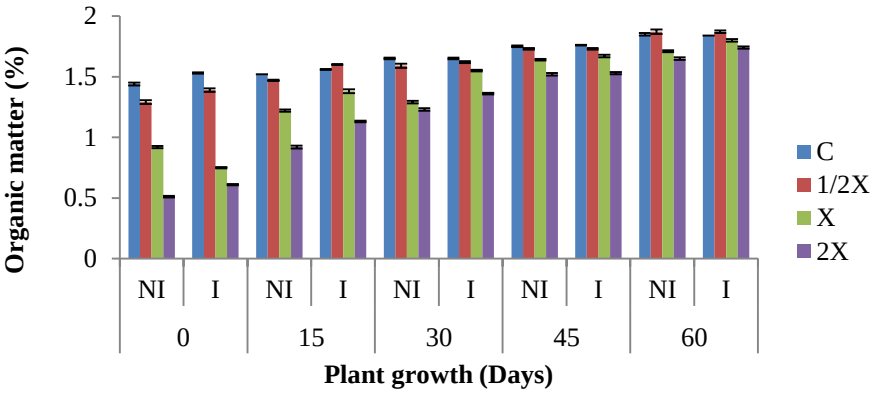
There were significant differences among bacterial inoculation, herbicides, concentrations and sampling dates on cation exchange capacity (CEC) of non-sterilized soil. Inoculation significantly increased the CEC of non-sterilized soil. Samples treated with paraquat showed the highest CEC (12.64 cmol(+) kg^{-1})), followed by pretilachlor (12.18 cmol(+) kg^{-1})) and 2, 4-D (12.09 cmol(+) kg^{-1})). Control samples had the highest CEC, followed by half, full and double doses of herbicides. CEC of non-sterilized soil significantly increased from day 0-60 after treatment. The highest CEC (15.07, 14.63 and 14.42 cmol(+) kg^{-1}) were obtained in inoculated samples treated with 2, 4-D at half, full and double doses, respectively at 60th DAT. However, the lowest CEC in herbicides treated samples were recorded by 6.97 and 10.74 cmol(+) kg^{-1}) in non-inoculated samples treated with double and half doses of pretilachlor, respectively and by 8.55 cmol(+) kg^{-1}) with full dose of 2, 4-D at day 0 after treatment (Figure 4.8). There was a significant four way interaction effect between bacterial inoculation, herbicides, concentrations and sampling dates on CEC of non-sterilized soil (Appendix B.g).



(a) paraquat

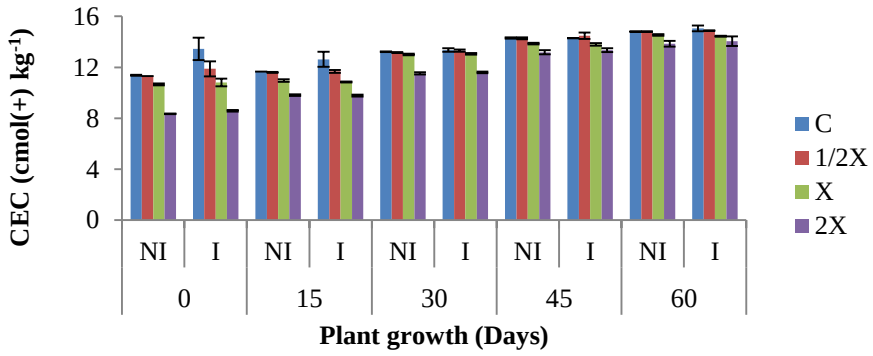


(b) pretilachlor

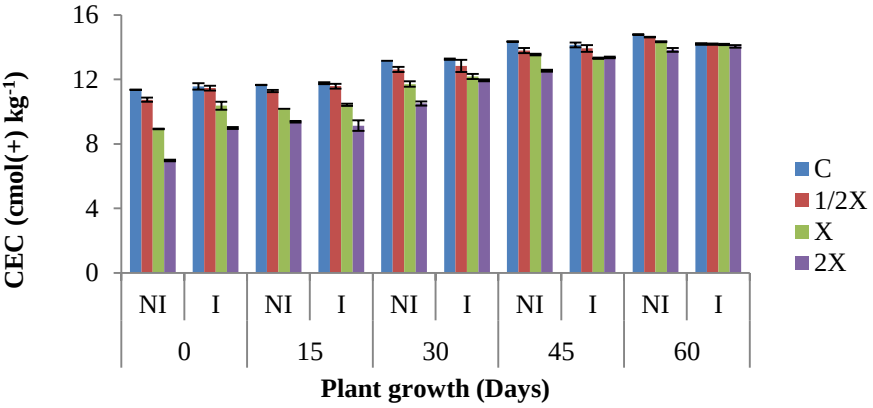


(c) 2, 4-D

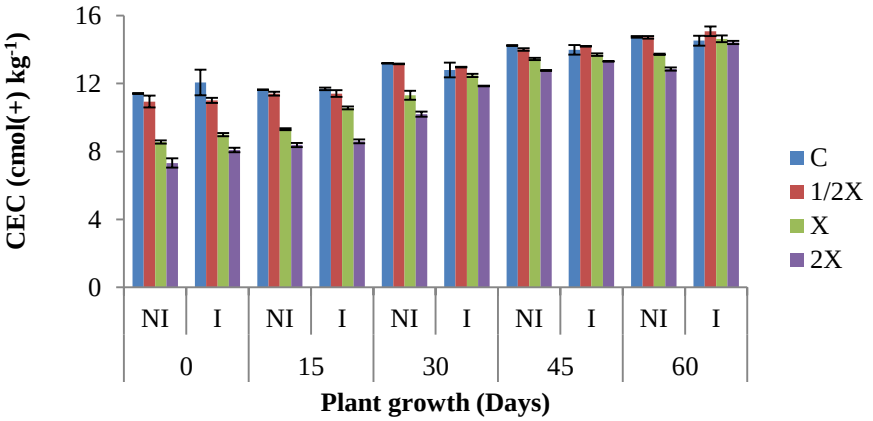
Figure 4.7. Effect of Sb16 bacterial inoculation and herbicides application on organic matter of sterilized soil within 60 days of aerobic rice growth; (a) paraquat; (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose Bars indicate standard error (n=3)



(a) paraquat



(b) pretilachlor



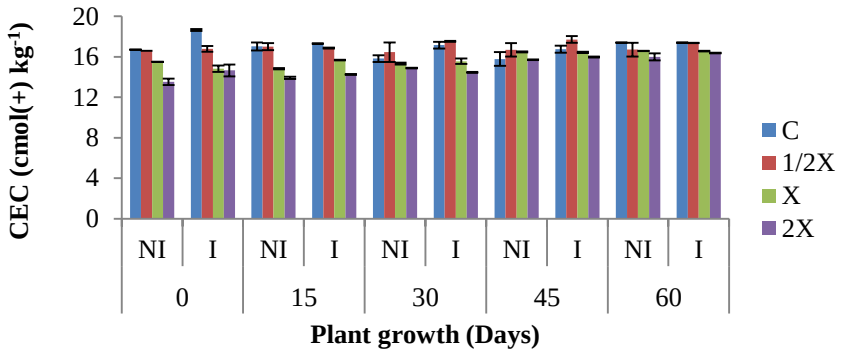
(c) 2, 4-D

Figure 4.8. Effect of Sb16 bacterial inoculation and herbicides application on cation exchange capacity of non-sterilized soil within 60 days of aerobic rice growth; (a) paraquat; (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose Bars indicate standard error (n=3)

There were significant differences among bacterial inoculation, herbicides, concentrations and sampling dates for CEC of sterilized soil. Bacterial inoculation significantly increased the CEC of sterilized soil. There was no significant difference between pretilachlor and paraquat for CEC of sterilized soil. Samples treated with pretilachlor showed the highest CEC by 16.21 cmol(+) kg^{-1}, followed by paraquat (16.19 cmol(+) kg^{-1}) and 2, 4-D (15.89 cmol(+) kg^{-1}). Control with half doses of herbicides did not show significant differences for CEC of sterilized soil. In general, CEC of sterilized soil significantly increased from day 0-60 after treatment. The highest CEC were recorded by 17.55, 17.29 and 16.86 cmol(+) kg^{-1}) in inoculated samples treated with half, full and double doses of pretilachlor, respectively at 60th DAT. However, the lowest CEC in herbicides treated samples were recorded by 8.35 and 16.1 cmol(+) kg^{-1}) in inoculated samples treated with double and half doses of 2, 4-D and by 13.41 cmol(+) kg^{-1}) in non-inoculated samples treated with full dose of pretilachlor at day 0 after treatment (Figure 4.9). There was significant four way interaction effect between bacterial inoculation, herbicides, concentrations and sampling dates on CEC of sterilized soil (Appendix B.h).

4.3.11 Soil Total N

There were significant differences among bacterial inoculation, herbicides, concentrations and sampling dates for soil total N. Soil total N significantly increased with bacterial inoculation. Three herbicides showed significant differences for soil total N, with the highest N (0.334%) for pretilachlor, followed by paraquat (0.333%) and 2, 4-D (0.332%). Control samples had the highest total N of soil, followed by half, full and double doses of herbicides. In general, total N of soil significantly increased from day 0-60 after treatment. The highest N in inoculated samples were obtained by 0.374% with half dose of pretilachlor and by 0.373 and 0.37% with full and double doses of paraquat, respectively at 60th DAT. However, N had the lowest values by 0.226, 0.255 and 0.282% in non-inoculated samples treated with double, full and half doses of 2, 4-D, respectively at day 0 after treatment (Figure 4.10). There were significant four way interaction effects between bacterial inoculation, herbicides, concentrations and sampling dates on total N of soil (Appendix B.i).



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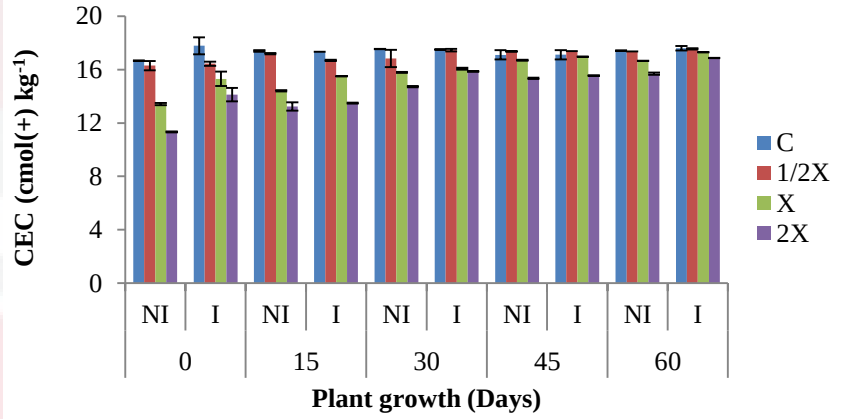
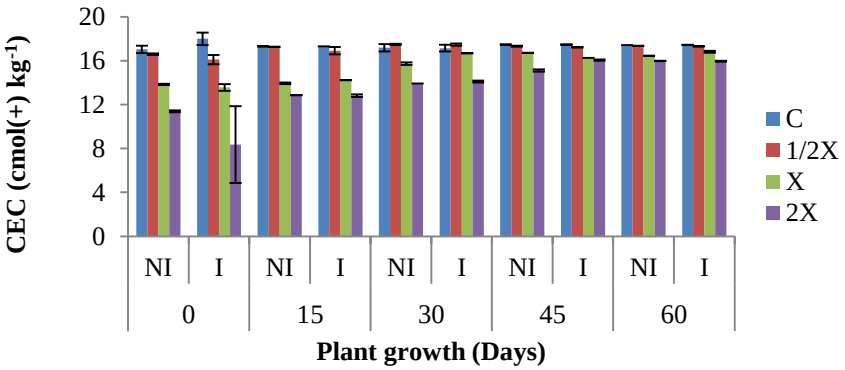
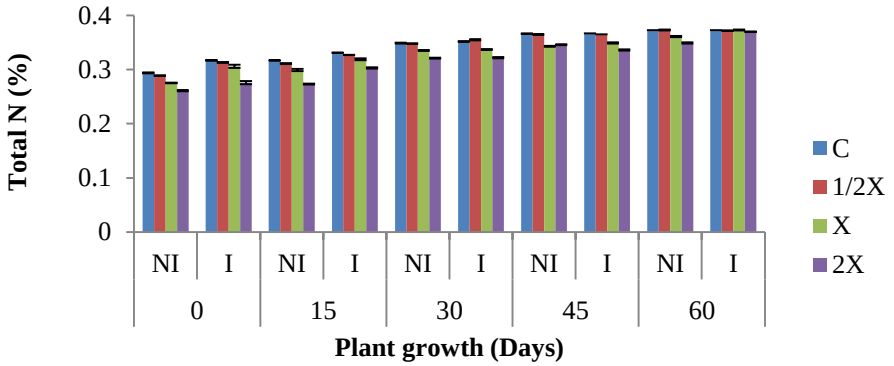


(b) pretilachlor

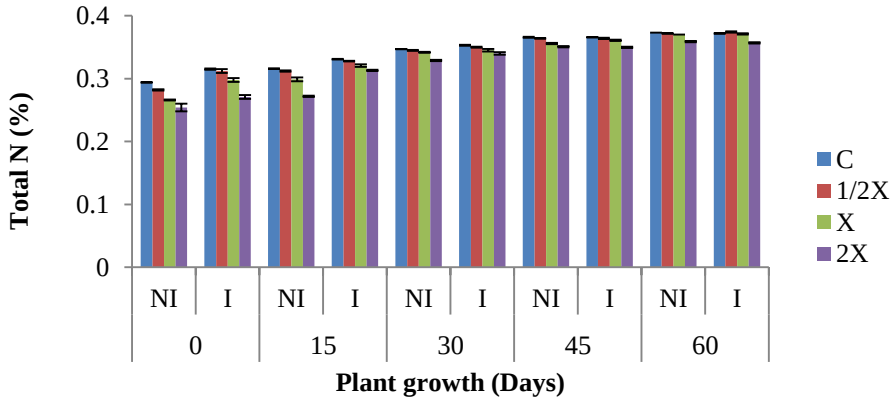


(c) 2, 4-D

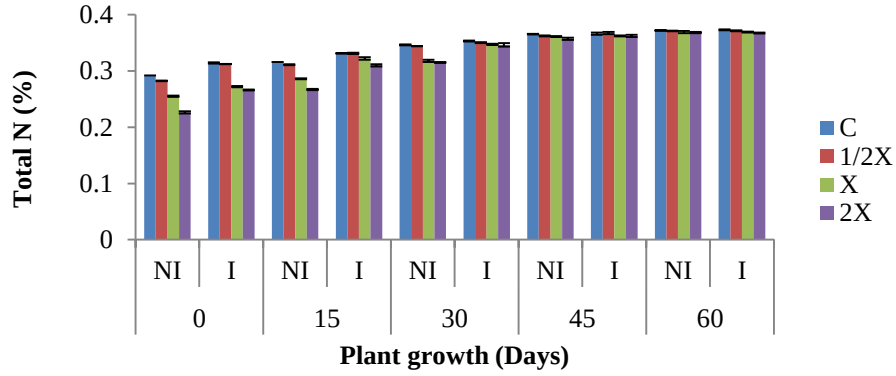
Figure 4.9. Effect of Sb16 bacterial inoculation and herbicides application on cation exchange capacity of sterilized soil within 60 days of aerobic rice growth; (a) paraquat; (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose Bars indicate standard error (n=3)



(a) paraquat



(b) pretilachlor



(c) 2, 4-D

Figure 4.10. Effect of Sb16 bacterial inoculation and herbicides application on soil total N within 60 days of aerobic rice growth; (a) paraquat; (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose Bars indicate standard error (n=3)

4.3.12 Soil Available P

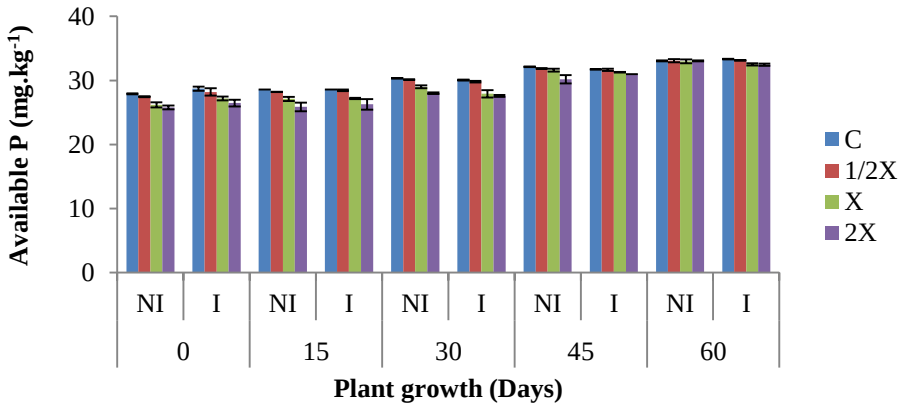
There were significant differences among bacterial inoculation, herbicides, concentrations and sampling dates for soil available P. Soil P significantly increased with inoculation. There were significant differences between herbicides, with the highest P in soil treated with paraquat (29.66 $mg.kg^{-1}$), followed by pretilachlor (29.3 $mg.kg^{-1}$) and 2, 4-D (29.12 $mg.kg^{-1}$). Control samples had the highest P, followed by half, full and double doses of herbicides. Soil available P significantly increased from day 0-60 after planting.

The highest soil available P in herbicides treated samples were recorded by 33.2 $mg.kg^{-1}$ in inoculated samples treated with half dose of pretilachlor and by 33.07 and 33 $mg.kg^{-1}$ in non-inoculated samples treated with double and full doses of paraquat, respectively at 60^{th} DAT. However, P had the lowest values by 22.67, 24.87 and 26.73 $mg.kg^{-1}$ in non-inoculated samples treated with double, full and half doses of pretilachlor, respectively at day 0 after treatment (Figure 4.11). Bacterial inoculation, herbicides and sampling dates showed a significant interactive effect on soil available P. There was significant interaction effect between inoculation, concentration and sampling dates on soil available P. Herbicides, concentrations and sampling dates showed significant interaction effect on soil available P (Appendix B.i).

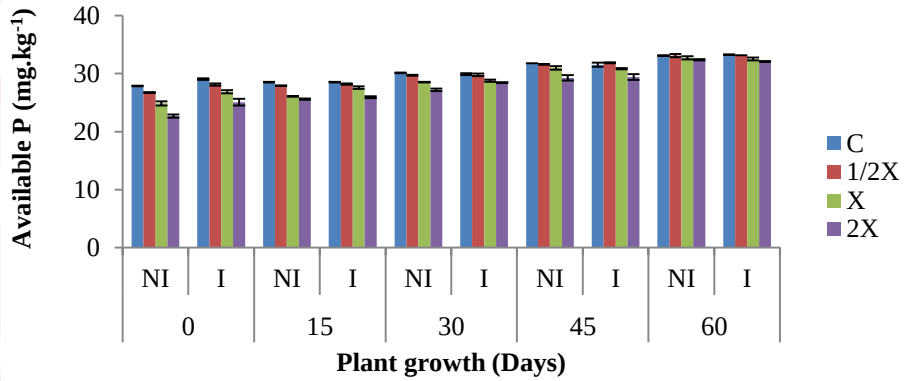
4.3.13 Soil Exchangeable K

There were significant differences among bacterial inoculation, herbicides, concentrations and sampling dates for soil exchangeable K. Exchangeable K significantly increased with bacterial inoculation. Pretilachlor and 2, 4-D showed no significant difference for soil exchangeable K. Samples treated with paraquat had the highest K (0.369 $cmol(+)\ kg^{-1}$), followed by pretilachlor (0.365 $cmol(+)\ kg^{-1}$) and 2, 4-D (0.365 $cmol(+)\ kg^{-1}$). Soil exchangeable K was highest in control samples, followed by half, full and double doses. K significantly enhanced from day 0-60 after planting.

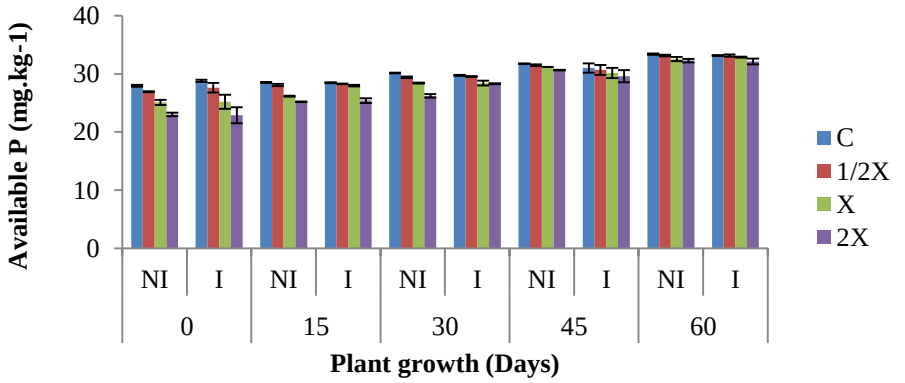
The highest K were recorded by 0.471, 0.466 and 0.464 $cmol(+)\ kg^{-1}$ in inoculated samples treated with half, full and double doses of paraquat, respectively at 60^{th} DAT. However, the lowest K in herbicides treated soils were recorded by 0.16 and 0.27 $cmol(+)\ kg^{-1}$ in non-inoculated samples treated with double and half doses of 2, 4-D, respectively and by 0.22 $cmol(+)\ kg^{-1}$ in non-inoculated samples treated with full dose of pretilachlor at day 0 after treatment (Figure 4.12). There were significant four way interaction effects between inoculation, herbicides, concentrations and sampling dates on soil exchangeable K (Appendi B.i).



(a) paraquat

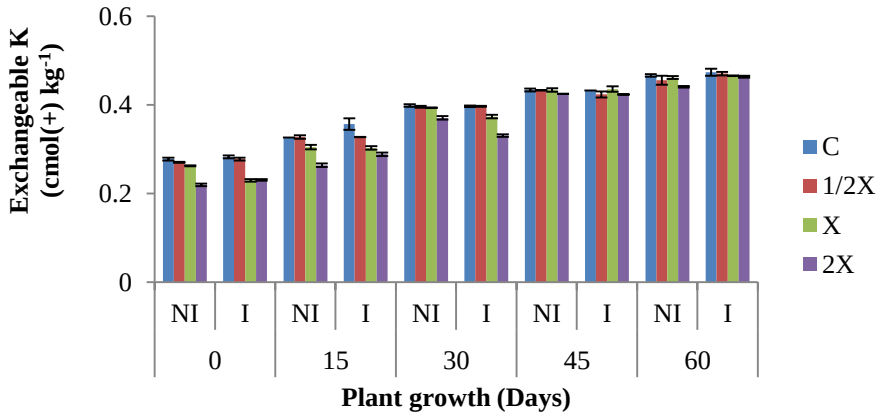


(b) pretilachlor

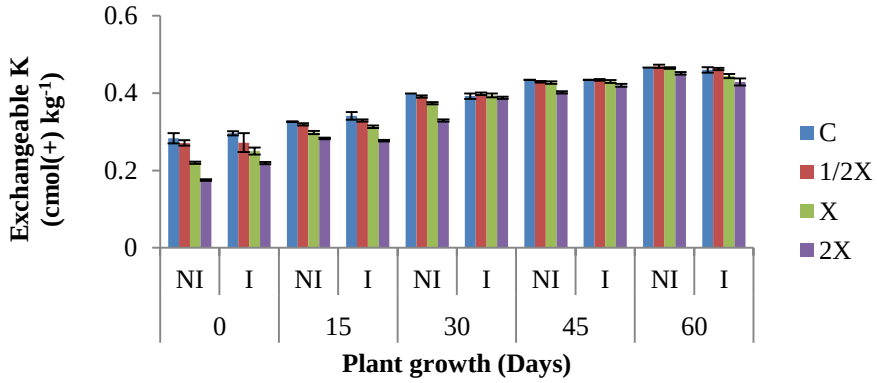


(c) 2, 4-D

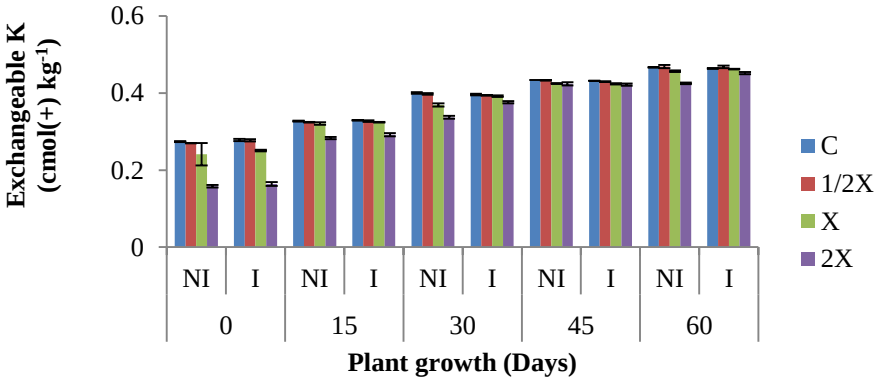
Figure 4.11. Effect of Sb16 bacterial inoculation and herbicides application on soil available P within 60 days of aerobic rice growth; (a) paraquat; (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose Bars indicate standard error (n=3)



(a) paraquat



(b) pretilachlor



(c) 2, 4-D

Figure 4.12. Effect of Sb16 bacterial inoculation and herbicides application on soil exchangeable K within 60 days of aerobic rice growth; (a) paraquat; (b) pretilachlor; (c) 2, 4-D; NI:non-inoculated, I:inoculated; C:control, 1/2X: half dose, X: full dose, 2X: double dose Bars indicate standard error (n=3)

4.3.14 Correlation Coefficient Analysis

Results from Pearson correlation analysis showed that there were significant positive correlation between plant height with leaf area per plant, chlorophyll content, plant N content, root and shoot dry mass, root length, root surface area , root volume and root diameter (Table 4.6). This result is in agreement with a study by Molazem *et al*. (2014) who found a positive correlation between plant height with leaf area and chlorophyll a of maize plant. Pervez *et al.* (2006) observed a highly significant ($p<0.01$) correlation between leaf area index and plant height and total dry weights of Black pepper (*Piper nigrum*). Araujo *et al*. (2014) punctuated the correlation of chlorophyll contents in the leaf with the increase in the plant nitrogen content. The improvements in growth parameters of aerobic rice plant were observed with an increase in N content.

There were significant positive relationships between soil chemical properties i.e. pH, OM and CEC with soil nutrients i.e. N, P, K (Table 4.7). This is similar to the study made by Das and Dey (2013) who found a positive relationship between total N and available P in soil treated with herbicides fenoxaprop, pendimethalin and paraquat. Kujur and Patel (2012) reported a strong positive correlation between soil pH and available P. The available P was found to have positive correlation with soil CEC and pH (Yang *et al*., 2013).

There were significant positive correlations between microbial populations i.e. total bacteria, diazotrophs and fungi with soil chemical properties and nutrients (Table 4.7). This agrees with the result from the study by Rigobelo and Nahas (2004) who found a highly strong correlation between soil organic matter and bacterial population and between organic matter and pH. However, population of total bacteria had a highly strong relationship with soil pH in the present study which is in contradiction with the result by Rigobelo and Nahas (2004).

An enhancement in soil nutrients with plant growth due to the root exudation of plant rhizosphere, leads to the increase in microbial population, indicating the usage of nutrients by microbes for their growth. Microbial populations were affected by the nutrients status and OM, which is in close relationship with soil pH and CEC. Sebiomo *et al*. (2011) also found a correlation between microbial population and soil organic matter. Swer *et al*. (2011) observed that the population of fungi was positively correlated with soil $_{Corg}$, total N, available P, exchangeable K and pH in farm yard manure (FYM) amended plots, indicating the vital role of available nutrients for fungal growth.

Table 4.6. Pearson Correlation coefficient of growth parameters of aerobic rice in non-sterilized soil inoculated with Sb16 and treated with paraquat, pretilachlor and 2, 4-D

	Plant height	Leaf area	chlorophyll content	N content	root weight	shoot weight	root length	root surface area	root volume	root average diameter
Plant height	1.00	0.458**	0.599**	0.526**	0.653**	0.427**	0.544**	0.539**	0.649**	0.563**
Leaf area		1.00	0.558**	0.808**	0.626**	0.308**	0.483**	0.351**	0.543**	0.616**
Chlorophyll content			1.00	0.718**	0.745**	0.402**	0.637**	0.574**	0.557**	0.701**
N content				1.00	0.718**	0.410**	0.589**	0.453**	0.521**	0.699**
Root weight					1.00	0.676**	0.673*	0.615**	0.662**	0.647**
Shoot weight						1.00	0.537**	0.645**	0.549**	0.582**
Root length							1.00	0.585**	0.530**	0.660**
Root surface area								1.00	0.553**	0.602**
Root volume									1.00	0.625**
Root average diameter										1.00

* Significant at p≤0.05; ** Significant at p≤0.01

Table 4.7. Pearson Correlation coefficient of microbial and chemical properties of non-sterilized soil inoculated with Sb16 and treated with paraquat, pretilachlor and 2, 4-D

	N	P	K	pH	CEC	OM	Total bacteria	Diazotrophs	Fungi
N	1.00	0.91**	0.951**	0.633**	0.924**	0.945**	0.865**	0.572**	0.863**
P		1.00	0.917**	0.667**	0.934**	0.946**	0.681**	0.403**	0.879**
K			1.00	0.631**	0.915**	0.924**	0.825**	0.465**	0.914**
pH				1.00	0.661**	0.657**	0.386**	0.273**	0.651**
CEC					1.00	0.935**	0.739**	0.476**	0.878**
OM						1.00	0.744**	0.495**	0.885**
Total bacteria							1.00	0.678**	0.702**
Diazotrophs								1.00	0.332**
Fungi									1.00

* Significant at p≤0.05; ** Significant at p≤0.01

4.4 Discussion

Inoculation of diazotrophic Sb16 significantly increased the population of total bacteria and diazotrophs in soil; however, the growth of microbes in herbicides treated samples was not as fast as in non-treated samples; suggesting faster adaptation of microbes to the non-treated soils. Sb16 inoculation significantly decreased the fungal population in soil, indicating the antagonistic effect of Sb16 on indigenous fungal communities in soil.

Results showed that herbicides, depending on type and dosage might decrease or increase the microbial counts. Total bacterial, diazotrophic and fungal population decreased after application of herbicides and higher doses resulted in lower counts in present study which is similar to the study by Ayansina and Oso (2006) who found the lower microbial counts with higher doses of herbicides compared to the recommended doses. The microbial population in soil was recovered after initial inhibition over time during aerobic rice plant growth in present study. This was due to either the microbial adaptation to the stressful conditions caused by chemicals or degradation of the chemicals by microbes. Herbicides may be utilized as carbon, energy and nutrients supplies by microbes, leading to an increase in their growth. Killed microorganisms by herbicides also can be used as nutrients sources for microbial multiplication (Latha and Gopal., 2010).

Total bacterial and diazotrophs population in herbicides treated samples were comparable to the control at 45^{th} day after planting; however fungal population in herbicides treated samples were not still comparable to the control at 60^{th} day after planting. The recovery of fungi from the harmful effects of herbicides requires more time in contrast to bacteria (Shukla and Mishra., 1997). Furthermore, fungal count can be influenced directly or indirectly by the effect of herbicides on the interaction between fungi and other microorganisms (Araujo *et al*., 2003).

As it is impossible to attribute the N_2 fixing activity in plant to any particular bacterium, N_2 fixing ability of inoculated bacterium (Sb16) was determined in laboratory conditions in present study (Chapter 3). The increase in nitrogenase activity and population of Sb16 with half dose of herbicides can justify an enhancement in microbial population and chemical properties of soil and growth parameters of aerobic rice following the Sb16 inoculation and application of herbicides at half dose.

An improvement in growth parameters of aerobic rice following inoculation with Sb16 strain indicates the useful symbiosis of Sb16 strain with root of aerobic rice plant and consequently increase in nutrients uptake, particularly N, leading to the overall enhancement in plant growth. This also could be attributed to an increase in total microbial population from root exudation alteration following the Sb16 inoculation. The increase in aerobic rice plant height following the inoculation with Sb16 in the present study agrees with the observations made by Sakthivel and Gnanamanickam (1987) who reported a

significant improvement in rice plant height with inoculation of *P. fluorescens*. The enhancement of plant height can be due to the atmospheric N fixation by inoculated diazotrophic Sb16 in plant roots, leading to overall growth of plant.

An increase in leaf area, chlorophyll content and N content of aerobic rice following inoculation of Sb16 in the present study is similar to the study by Sheng (2005) who observed the higher N content of above ground plant components in cotton and ripe plants with inoculation of bacterial strain *B. edaphicus* NBT. An increase in chlorophyll content of aerobic rice leaves in the present study corroborated with the results of a study by Keyeo *et al*. (2011) who reported an increase in chlorophyll content of rice plant inoculated with *Enterobacter* sp. Redžepović *et al.* (2006) also found an increase in chlorophyll a, b and a+b content with inoculation of *B. japonicum* strain.

An increase in N content of aerobic rice with an inoculation of Sb16 in the present study could occur through the BNF procedure, leading to an increase in chlorophyll content, leaf area and shoot and root dry weight of plant. Naher *et al*. (2011) also found an increase in plant biomass of Mayang Segumpal rice genotype in association with Sb16 by 195 ± 40 % and 36 ± 19.8 % over control and 60 kg $ha^{-1}$of N fertilizer, respectively. Inoculation of biofertilizer, containing 7 endophytic rhizobial strains with *R. leguminosarum bv. trifolii* to 5 rice varieties during 5 growing season in large-scale field experiments resulted in an enhancement of rice production, thus the need for additional chemical N fertilizer usage to maintain sustainable agriculture and economy production was reduced (Yanni and Dazzo., 2010).

The decrease in growth parameters and morphological parameters of aerobic rice with herbicides application in the present study is in line with the study by Zaidi *et al.* (2005) who observed the higher reduction of N contents in the whole plant at 60^{th} day of planting with higher doses of herbicides glyphosate, metribuzin, fluchloralin and 2, 4-D. They also observed that shoot and root dry weight of greengram were significantly reduced with 2 µg a.i.$g^{-1}$of fluchloralin and metribuzin and 2, 4-D at all tested doses compared to control treatment. The decrease in chlorophyll content of aerobic rice plant following application of herbicides could be due to photosynthetic inhibition, leading to an inhibition of photosynthetic pigments. An inhibition in shoot growth and chlorophyll concentration of soybean was observed after pre-emergence and post-emergence applications of imazaquin at four doses (Alonge., 2000).

The decrease in leaf area of aerobic rice with herbicides application could be due to decrease in chlorophyll content and N content of plant; which are interrelated. Besides, an inhibition of effective N_2 fixation following the herbicides application at higher dosages resulted in decrease of overall growth of plant; however lower reduction occurred in samples inoculated with Sb16, indicating the role of bacteria to promote the plant strength and protect it from hazardous herbicidal effects. Ahemad and Saghir Khan (2011) found that inoculation of *Bradyrhizobium* sp. (vigna) strain MRM6 together with application of

herbicides quizalafop-p-ethyl and clodinafop at any concentrations led to better response of greengram plant in terms of its whole biomass and N in root and shoot compared to non-inoculated soil treated with the same concentration of herbicides.

Root morphological parameters of aerobic rice increased following an increase in N content and subsequently overall growth parameters in inoculated samples in present study. This is in accordance with the study made by Dawwam *et al*. (2013) who reported that seven bacterial isolates including P18, P19, P31, P32, P35, P39, and P42 isolated from surface sterilized roots of sweet potato showed significant differences in all vegetative parameters of potato, photosynthetic pigments and N, P and K concentrations compared to control. The number of roots per plant, the total root length per plant, the total root volume and total root surface area of maize plant were positively influenced by *A. lipoferum* CRT1 inoculation, while it did not have an effect on root average diameter (El Zemrany *et al*., 2006). This result agrees with the result of present study; however root average diameter of aerobic rice significantly increased with Sb16 bacterial inoculation. PGPR can develop the root growth with the ability to produce phytohormones, plant tissue extension or other root morphological alterations (Salisbury., 1994).

In general, the plant growth and soil chemical properties showed better performance in non-sterilized compared to sterilized soil treatments in present study. This result favor the observations made by Nezarat and Gholami (2009), who found better growth and development of maize plant inoculated with PGPR strains in non-sterilized compared to sterilized soil treatments. Qiu *et al*. (2008) also observed that the plants grew better in non-sterilized than in sterilized soil in condition of a high level of nitrogen nutrient medium. This is due to the presence of indigenous microbes and soil nutrients in natural non-sterilized soil conditions which promote the plant growth and nutrients uptake compared to sterilized soil without existed microbes.

Moreover, inoculated Sb16 could establish a beneficial interaction with indigenous microbes in natural soil conditions. Khalid *et al*. (2004) pointed out that inoculated bacteria could be able to compete, survive and influence the host plant in the condition that indigenous microflora exist. However the result of the present study contradicts the report made by Al-Khaliel (2010) who found that peanut plants represented better growth in sterilized soil compared to non-autoclaved soil, due to the absence of pathogens that cause diseases to the plants.

pH in sterilized soil treatments was lower compared to non-sterilized treatments, which is in line with the study by Shaw *et al*. (1999) who investigated that the soil pH significantly decreased by autoclaving sterilization; however, CEC did not significantly change. Organic acids solubilization in autoclaved soil may lead to a declination in pH; however, the soil acid buffering capacity determines the decline extent (Shaw *et al*., 1999). The increase in CEC of sterilized soil samples in the present study might be due to an increase

in pH dependent charge of the soils. However, OM decreased with soil sterilization, which can be resulted from the absence of microbes in sterilized conditions.

The significant increase in soil chemical properties including total N, available P, exchangeable K, OM and CEC following Sb16 inoculation in non-sterilized soil treatments in present study is in line with the study by Esitken *et al*. (2010) who reported that soil nutrient element contents, including total N, available P and K, as well as soil pH, OM and CEC were significantly affected by PGPR bacterial applications. Siderophores production and enzymatic nutrients mobilization from organic matter lead to an increase in nutrients availability by PGPR (Jing *et al*., 2007). The enhancement in organic complex mineralization and organic acid production by plants and bacteria in the rhizosphere can lead to an increase in mineral contents accessibility in soils within the plantation and bacterial inoculation in soil (Shen *et al.*, 2004).

The increase in available P and exchangeable K can be attributed to an increase in phosphate solubilizing and potassium solubilizing bacteria, respectively following inoculation with Sb16 that established an interaction with these bacteria. The decrease in soil pH following Sb16 inoculation in present study can be due to an increase in organic matter, leading to the release of organic acid. Herbicides application significantly reduced the soil chemical properties i.e. total N, available P, exchangeable K, pH, CEC and OM in both soil conditions in present study, which can be due to an adverse effect of herbicides on soil microbes and plant. Moreover, soil nutrients may be negatively affected by the herbicides through the disturbing of soil physicochemical and biological properties.

4.5 Conclusion

Inoculation of Sb16 strain resulted in an enhancement of overall growth of aerobic rice, improvement in nutrients uptake, increase total bacterial and diazotrophs population and decrease fungal population and protection the plant from potential toxicity of herbicides. The tested parameters in herbicides treated samples showed greater performance in inoculated compared to non-inoculated samples. Moreover, growth parameters of aerobic rice and soil chemical properties showed better performance in natural non-sterilized compared to sterilized soil conditions. Therefore, Sb16 inoculant is recommended for cultivation of aerobic rice plant in natural soil applied with recommended doses of paraquat, pretilachlor and 2, 4-D.

CHAPTER 5

EFFECT OF *Stenotrophomonas maltophilia* (Sb16) ON PERSISTENCE OF PARAQUAT, PRETILACHLOR AND 2, 4-D IN AEROBIC RICE SOIL

5.1 Introduction

Herbicides are considered essential parts of sustainable agriculture. The concern about the environmental effects of herbicides has driven an attempt to research into the environmental fates of herbicides in soil. The chemical, physical and microbial factors affect the herbicide persistence in soil. Soil microorganisms, particularly bacteria and fungi are the most essential degraders of these agrochemicals in soil. Paraquat, pretilachlor and 2, 4-D, three common rice herbicides were tested for their persistence in aerobic rice soil inoculated with Sb16 in sterilized and non-sterilized conditions to predict their fates in rice field soils of Malaysia. Several studies have investigated the effects of soil microorganisms on degradation of pesticides in soil in laboratory and glasshouse conditions. However, an inconsistency has been observed among the results (Kruglov., 1991). This study was conducted with the aim to determine the effect of Sb16 on persistence of paraquat, pretilachlor and 2, 4-D in aerobic rice soil.

5.2 Materials and Methods

5.2.1 Soil Samples Preparation

Soil samples for herbicides determination were collected from pot experiments under glasshouse condition in second experiment (Chapter 4). At each 15-day interval from day 0 (1 h) till 60 of plant growth, soil samples were taken from the surface layer (0-15 cm depth) of each respective pot with minimum disturbance of the surroundings and placed in plastic bags, tightened and immediately transferred to the laboratory for herbicides analysis. In lab, soil samples were frozen and kept until the analysis using UV-VIS-NIR-Spectrophotometer.

5.2.2. UV-VIS-NIR-Spectroscopic Method for Herbicides Analysis

UV-VIS-NIR-Scanning Spectrophotometer Shimadzu (model UV-3101PC) with 1cm matched quartz Cuvettes, located at Department of Crop Science, Faculty of Agriculture, University Putra Malaysia, was used for determination of herbicides residues in soil samples using the absorbance spectra.

5.2.3. Preparation of the Standard Stock Solution

The reagents of methanol, acetone and hexane were provided from Laboratory of Toxicology, Plant Protection Department, Faculty of Agriculture, UPM.

The standard stock solution (1000 ppm) of paraquat was prepared by dissolving 0.1 mL paraquat in 100 mL of methanol and the working standards (0.5, 1, 2, 3, 4, 5 ppm) were prepared from the stock solution by serial dilutions. Aliquots of 0.1 mL of pretilachlor was added to 100 mL distilled acetone to obtain a 1000 ppm stock solution. Solutions of desired concentrations of pretilachlor (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4 ppm) were prepared from the stock. The same amount of 2, 4-D (0.1 mL) was added to 100 mL methanol to obtain the stock solution of 1000 ppm, followed by the preparation of working concentration (0.05, 0.1, 0.2, 0.4, 0.8, 1, and 2 ppm) from the stock.

5.2.4 Determination of Maximum Wavelength

A total of 5 mL of paraquat 20 ppm was added with methanol and the solution was diluted up to 10 mL. An amount of 5 mL of 20 ppm pretilachlor was added to the acetone and diluted to 10 mL. The same procedure was taken to prepare the 2, 4-D solution. Methanol, acetone and methanol were the blank solutions of paraquat, pretilachlor and 2, 4-D, respectively. The solutions containing herbicides were scanned within 250-400 nm to find the maximum absorption wavelength of herbicides. The maximum wavelength found for each herbicide was used throughout the study.

The absorbance of prepared standard solutions of paraquat, pretilachlor and 2, 4-D was measured based on the maximum wavelength obtained from UV-VIS-NIR-Spectrophotometer. Calibration curves of herbicides were plotted using concentrations (ppm) on the X axis and absorbance (nm) on the Y axis and linear regressions were employed (Appendix A.b)

5.2.5 Extraction Procedure

An amount of 15 mL of methanol regarding its high capability in extracting paraquat due to its high solubility was added to an equivalent of 5 g of prepared soil samples (< 2 mm) and vigorously shaken on the shaker at 150 rpm for 1-2 hours. The samples were removed from the shaker and filtered using Whatman filter paper. The filtrate was transformed to a rotary evaporator and evaporated to obtain the final volume of 3-4 ml. The concentrated filtrate was reconstituted with methanol up to 30 mL and the sample was taken for the measurement of absorbance of paraquat based on the maximum wavelength obtained.

For the analysis of pretilachlor, 5 g of each soil sample (< 2 mm) was added to 15 mL acetone in 50 mL conical flasks and shaken for 1-2 hours. After the decantation of the suspended material phase with a Whatman filter paper, the liquid phase was transferred to 100 mL round bottom flask and concentrated under a rotary evaporator to 2-3 mL. The sample was eluted with 10 mL of hexane and the hexane layer was collected in round bottom flask, evaporated at 45 ˚C and concentrated to the final volume of 3-4 mL. The concentrated filtrate was then diluted to 30 mL with acetone. The liquid sample was taken for UV-VIS-NIR-Spectrophotometric analysis to estimate the absorbance based on obtained wavelength for pretilachlor.

To analyze the 2, 4-D, 5 g of each soil sample (< 2 mm) was added to 15 mL methanol in separating funnel and shaken on a rotary shaker for 1-2 hours. Aliquots of 10 mL of the suspension was extracted and transferred to a 50 mL vial, followed by the filtration with Whatman filter paper. The extract was allowed to evaporate under rotary evaporator to get the solution of 3-4 mL and redissolved with methanol up to 30 mL before the analysis with UV-VIS-NIR-Spectrophotometer using the obtained maximum wavelength for 2, 4-D.

The concentration of paraquat, pretilachlor and 2, 4-D in soil was determined according to obtained calibration curve for each herbicide.

5.2.6 Method Validation

The performance of UV-VIS-NIR-Spectrophotometric method for detection of paraquat, pretilachlor and 2, 4-D was validated by evaluating the linearity range, limit of detection (LOD), limit of quantification (LOQ), recovery and the selectivity.

The linearity of the method was assessed using calibration curve of each herbicide.

The Limit of detection (LOD) and limit of quantification (LOQ) for each herbicide were calculated from the residual standard deviation of the linear regression obtained from the calibration curve for each herbicide according to the following formula:

LOD/ LOQ= (F*SD)/b

Where:

F: Factor of 3.3 and 10 for LOD and LOQ, respectively

SD: Residual standard deviation of the linear regression

b: Slope of the regression line

The recovery study was performed in six replicates (n=6) of soil samples spiked at three known concentrations of 0.5, 1 and 10 ppm. An equivalent of 5 g of each soil sample was fortified with 1 mL of standard solutions of each herbicide at 2.5, 5 and 50 ppm to obtain the spiking levels of 0.5, 1 and 10 ppm, respectively. The spiked soils in the flasks were allowed to stand for 30 min before the extraction. Extraction was performed by adding 15 mL of methanol, acetone and methanol to spiked soil samples with paraquat, pretilachlor and 2, 4-D, respectively. The flasks were shaken at 250 rpm for 2 hours on a shaker. The solutions were then filtered using Whatman filter paper and the supernatants were transferred to a round bottomed flask to evaporate the vapour phase. The filtrates were concentrated up to 3-4 mL and redissolved with methanol, acetone and methanol up to 30 mL for the samples spiked with paraquat, pretilachlor and 2, 4-D, respectively. The absorbance of each prepared solution was measured using spectrophotometer. The percent recovery was calculated based on the initial concentration and the concentration recovered after the shaking process.

The recovery percentage was calculated according to the following formula:

% recovery = (A fortified/ A standard) * 100

Where,

A fortified = Concentration of fortified sample

A standard = concentration of standard

5.2.7 Degradation Study

The degradation rates of paraquat, pretiachlor and 2, 4-D were determined under glasshouse conditions on sterilized and non-sterilized soils samples at 0 (1 hour), 15, 30, 45 and 60 days after herbicides application. For most pesticides, the rate of degradation is proportional to concentration, thus the results can often be interpreted using first-order kinetics (Hurle and Walker., 1980). The metabolism rate constant (k) and half-life ($t_{1/2}$) of paraquat, pretilachlor and 2, 4-D were calculated using equation (1) derived from the law of first-order kinetics as follows:

$\text{Ln}\ (C_t/C_0) = -k_1\ (t_1-t_2)$

Where:

C_0: the initial concentration of herbicide (ppm) in soil at time zero,

C_t: the concentration of herbicide (ppm) at time t

t_1 and t_2: the periods in days at time t and t=0, respectively

k: the herbicide metabolism rate constant (d^{-1})

The plots of logarithm of the concentrations against time for each herbicide were drawn with the slope of plot proportional to the rate constant (k). $T_{1/2}$ is the time taken for degradation of 50 % of chemicals and calculated with this equation.

$T_{1/2}=Ln2/k$

5.2.8. Statistical Analysis

The data were subjected to analysis of variance (ANOVA) and the mean comparisons were separated with Duncan Multiple Range Test (DMRT). The calculations for the concentrations and half lives of herbicides were done using Microsoft Excel 2007.

5.3 Results

5.3.1 Determination of Maximum Wavelength

The UV-VIS-NIR-Spectrophotometeric chromatograms of paraquat, pretilachlor and 2, 4-D solutions showed the maximum wavelength of 300, 305 and 292 nm, respectively (Figure 5.1). The maximum wavelength obtained for each herbicide was used for determination of residues and half lives throughout the study.

5.3.2 Method Validation

The linearity of method was confirmed with calibration curve of herbicides (Appendix B). The mean regression coefficient for all herbicides was linear ($\geq$ 0.99).

The limit of detection (LOD) and limit of quantification (LOQ) of herbicides were less than 0.1 and 0.3ppm, respectively (Table 5.1).

Table 5.1. Limit of detection (LOD) and limit of quantification (LOQ) of herbicides

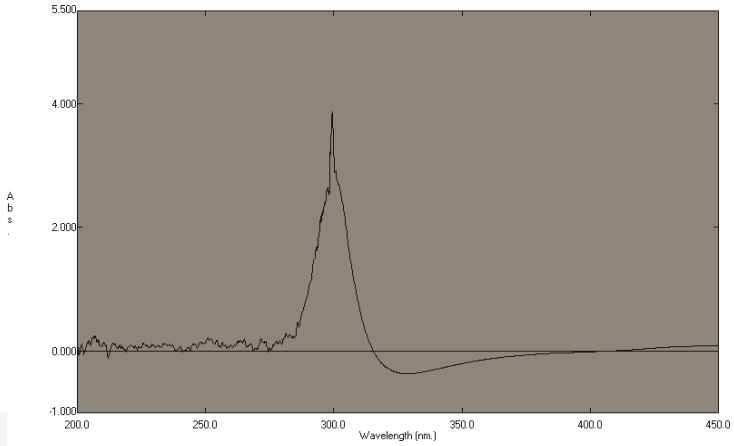
Herbicides	LOD (ppm)	LOQ (ppm)
Paraquat	0.041	0.124
Pretilachlor	0.034	0.102
2, 4-D	0.098	0.297

Table 5.2. Percentage of recovery of soil samples fortified with three spiking levels of paraquat, pretilachlor and 2, 4-D

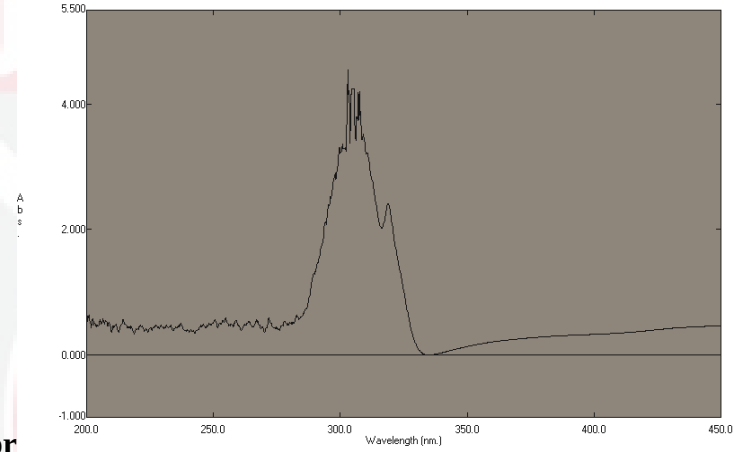
Herbicide	Fortification levels (ppm)	Recovery in spiked soil sample (%) ± standard error
Paraquat	0.5	92.59±1.11
	1	94.83±0.44
	10	100.5±0.08
Pretilachlor	0.5	96.63±0.47
	1	97.25±0.34
	10	98.89±0.37
2, 4-D	0.5	90.66±2.19
	1	91.62±1.19
	10	97.24±0.08

Values are mean± standard error

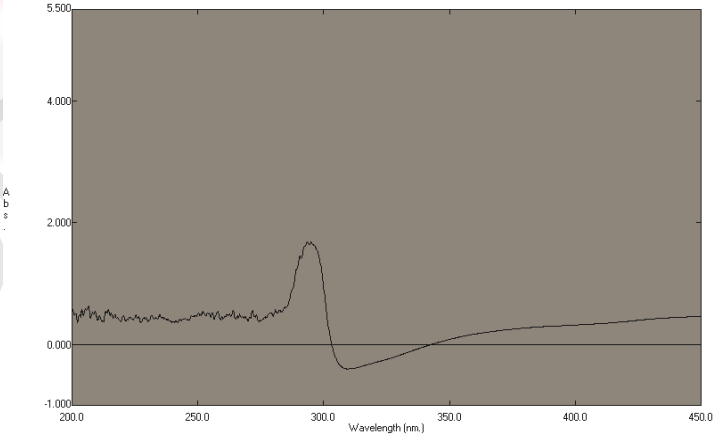
The recoveries of spiked soil samples with herbicides standard solutions at spiking levels of 0.5, 1 and 10 ppm were higher than 89% with standard error of less than 3% for three herbicides (Table 5.2).



(a) Paraquat



(b) Pretilachlor



(c) 2, 4-D

Figure 5.1. The UV spectra of the herbicides solutions using UV-VIS-NIR-Spectrophotometer

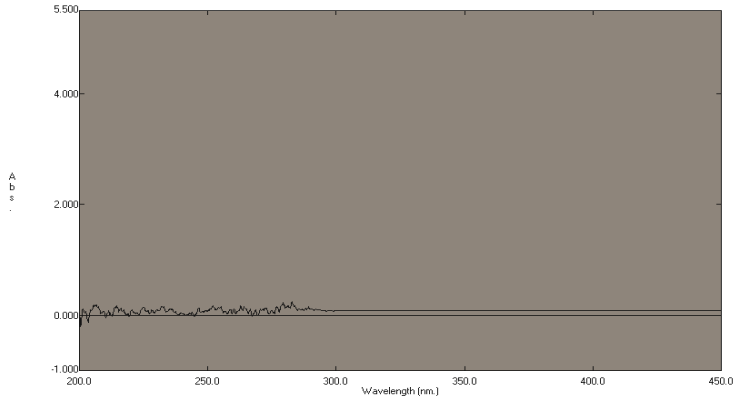


Figure 5.2. The chromatogram of the blank soil sample using UV-VIS-NIR-Spectrophotometer

Selectivity of method was verified by analyzing the blank soil sample, which hardly shows any interferences from the chromatogram (Figure 5.2).

5.3.3 Persistence

According to ANOVA, there were highly significant differences among bacterial inoculation, herbicides, concentrations and sampling dates for persistence of herbicides in sterilized and non-sterilized soils. In general, non-inoculated samples had higher residues of herbicides than inoculated samples in sterilized and non-sterilized samples. Three herbicides showed significant differences for persistence in sterilized and non-sterilized soil, with the highest persistence with paraquat, followed by 2, 4-D and pretilachlor. There were significant differences between double, full and half doses of herbicides for persistence, with the highest persistence in samples treated with double, followed by full and half doses. Herbicides residues decreased from day 0-60 of treatment, with highest and lowest residues at day 0 and 60 after treatment, respectively in sterilized and non-sterilized samples. There was significant four way interaction effect between bacterial inoculation, herbicides, concentrations and sampling dates on persistence of herbicides in sterilized and non-sterilized soil, indicating the dependent effect of factors on persistence of herbicides (Appendix B. j).

5.3.4. Persistence of Paraquat

The residues of paraquat were greater in sterilized compared to non-sterilized soil. The highest level of paraquat (0.385 μg/g) was detected in non-sterilized non-inoculated soil treated with double dose at day 0 (1 h) after application (DAA). The lowest paraquat content (0.033 μg/g) was obtained in non-sterilized inoculated samples treated with half

dose at 60th DAA. The highest reductions of paraquat residues in non-sterilized samples by 54.17, 41.56, 37.43, 32.75, 19.51 and 13.54 % were obtained in inoculated samples with half dose, non-inoculated with half dose, inoculated with full dose, non-inoculated with full dose, inoculated with double dose and non-inoculated with double dose, respectively compared to sterilized soil at 60th DAA; however, for inoculated samples treated with double dose occurred at 45th DAA (Table 5.3).

5.3.5 Persistence of Pretilachlor

The dissipation rates of pretilachlor in sterilized and non-sterilized soil samples showed higher quantities of pretilachlor at difference dosages in sterilized compared to non-sterilized soil samples irrespective of Sb16 inoculation. The residues of pretilachlor applied at half dose were not detectable at 30th and 60th DAA in inoculated non-sterilized and inoculated sterilized soil samples, respectively; however, they disappeared at 45th DAA in non-inoculated non-sterilized soil samples and were detectable until 60th DAA in sterilized soil samples. The residues of pretilachlor in inoculated non-sterilized soil samples treated with full and double doses were below the detectable limit (BDL) at 45th and 60th DAA, respectively, while they were detected until 60th DAA in non-inoculated non-sterilized and sterilized soil samples. The highest dissipations of pretilachlor in non-inoculated samples treated with full and double doses were recorded by 65.93 and 65.19%, respectively at 60th DAA in non-sterilized compared to sterilized soil samples. The degradation of pretilachlor applied at half, full and double doses was faster in inoculated compared to non-inoculated sterilized soil samples, with the highest dissipation rate by 35.29, 27.47 and 23.76% at 30th, 60th and 60th DAA, respectively (Table 5.4).

5.3.6 Persistence of 2, 4-D

The residues of 2, 4-D in all non-sterilized samples except in non-inoculated samples treated with double dose were below detectable limit at 45th DAA; however they were detectable until 60th DAA in all treatments in sterilized soil samples. The maximum dissipation rate of 2, 4-D in non-sterilized samples treated with double and half doses were recorded by 61.42, 44.19%, respectively in inoculated compared to non-inoculated samples at 30th DAA. The highest reductions of 2, 4-D residues in sterilized soil samples were recorded by 26.15, 20.88 and 14.33% in inoculated samples treated with half, full and double doses at 30th, 60th and 45th DAA, respectively compared to non-inoculated samples (Table 5.5).

Table 5.3. Residues of paraquat in soil (µg/g±SD)

T	Non-sterilized soil					Sterilized soil				
	Days after application					Days after application				
	0	**15**	**30**	**45**	**60**	**0**	**15**	**30**	**45**	**60**
NI+1/2X	0.094±0	0.07±0.003	0.054±0.003	0.049±0.003	0.045±0.003	0.091±0.003	0.082±0.003	0.079±0	0.077±0.003	0.077±0.003
NI+X	0.192±0.003	0.135±0.003	0.124±0.003	0.117±0.003	0.115±0	0.187±0.003	0.175±0.003	0.175±0.003	0.171±0.003	0.171±0.003
NI+2X	0.385±0.003	0.364±0.003	0.339±0.003	0.325±0	0.313±0.003	0.381±0.003	0.372±0	0.367±0	0.367±0	0.362±0
I+1/2X	0.091±0.003	0.049±0.003	0.04±0.003	0.035±0.003	0.033±0.003	0.089±0	0.077±0.003	0.073±0	0.073±0	0.072±0.003
I+X	0.191±0.003	0.166±0.003	0.149±0.003	0.124±0.003	0.107±0.003	0.187±0.003	0.177±0.003	0.173±0	0.171±0.003	0.171±0.003
I+2X	0.383±0	0.353±0.003	0.322±0.003	0.297±0.003	0.294±0.003	0.379±0.003	0.378±0	0.369±0.003	0.369±0.003	0.358±0.003

NI: Non-inoculated, I: Inoculated; 1/2X: Half dose, X: Full dose, 2X: Double dose

Table 5.4. Residues of pretilachlor in soil (µg/g±SD)

T	Non-sterilized soil					Sterilized soil				
	Days after application					Days after application				
	0	**15**	**30**	**45**	**60**	**0**	**15**	**30**	**45**	**60**
NI+1/2X	0.101±0.001	0.091±0.001	0.022±0.001	BDL	BDL	0.099±0.001	0.086±0.001	0.068±0.001	0.046±0.001	0.042±0.001
NI+X	0.191±0.001	0.141±0.001	0.086±0.001	0.043±0	0.031±0	0.188±0.001	0.148±0.001	0.099±0.001	0.091±0.001	0.091±0.001
NI+2X	0.29±0.001	0.238±0.001	0.186±0.001	0.129±0.001	0.063±0.001	0.287±0	0.231±0.001	0.212±0.001	0.203±0.001	0.181±0.001
I+1/2X	0.1±0.001	0.023±0.001	BDL	BDL	BDL	0.097±0.001	0.07±0.001	0.044±0.001	0.032±0.001	BDL
I+X	0.19±0.001	0.057±0.001	0.038±0	BDL	BDL	0.187±0	0.157±0.001	0.117±0.001	0.086±0.001	0.066±0.001
I+2X	0.29±0.001	0.198±0	0.103±0.001	0.042±0.001	BDL	0.286±0.001	0.26±0.001	0.229±0	0.188±0.001	0.138±0

NI: Non-inoculated, I: Inoculated; 1/2X: Half dose, X: Full dose, 2X: Double dose;

Table 5.5. Residues of 2, 4-D in soil (µg/g±SD)

T	Non-sterilized soil					Sterilized soil				
	Days after application					Days after application				
	0	**15**	**30**	**45**	**60**	**0**	**15**	**30**	**45**	**60**
NI+1/2X	0.105±0.004	0.041±0.004	BDL	BDL	BDL	0.098±0.004	0.081±0	0.065±0.004	0.048±0.004	0.041±0.004
NI+X	0.188±0	0.124±0	0.043±0.004	BDL	BDL	0.179±0.004	0.155±0.004	0.133±0.004	0.107±0.004	0.091±0.004
NI+2X	0.39±0.004	0.319±0.004	0.197±0.004	0.076±0.004	BDL	0.383±0.004	0.361±0.004	0.335±0.004	0.3±0.004	0.235±0.004
I+1/2X	0.103±0	0.024±0	BDL	BDL	BDL	0.095±0	0.072±0.004	0.048±0.004	0.038±0	0.031±0
I+X	0.186±0.004	0.072±0.004	0.024±0	BDL	BDL	0.176±0.004	0.155±0.004	0.133±0.004	0.107±0.004	0.072±0.004
I+2X	0.387±0	0.266±0	0.076±0.004	BDL	BDL	0.38±0	0.357±0.004	0.321±0.004	0.257±0.004	0.207±0.004

NI: Non-inoculated, I: Inoculated; 1/2X: Half dose, X: Full dose, 2X: Double dose

5.3.7 Degradation Study

Results of half life studies in sterilized and non-sterilized soil samples indicated the longer half lives in sterilized compared to non-sterilized treatments, irrespective of herbicides dosages and bacterial inoculation. The longest half lives of herbicides were obtained in samples treated with double dose, followed by full and half doses. In general, half lives were shorter in samples inoculated with Sb16 compared to non-inoculated samples. The longest half life in non-sterilized soil samples was recorded by 198.03 in non-inoculated samples treated with double dose of paraquat. However, in inoculated sample treated with half dose of pretilachlor and 2, 4-D and in non-inoculated sample treated with half dose of 2, 4-D, half life of <15 days was obtained (Tale 5.6).

The longest and shortest half lives in sterilized samples were recorded by 866.38 and 27.4 in non-inoculated samples treated with double dose of paraquat and in inoculated samples treated with half dose of pretilachlor, respectively (Table 5.7).

The dissipation rates of paraquat, pretilachlor and 2, 4-D in non-sterilized and sterilized soil samples showed a linear relationship between logarithmic values of herbicides concentrations against time (Figure 5.3). There was a downward trend in dissipation rate of herbicides for all treatments in both soil conditions, which follows up the first order kinetic rates.

Table 5.6. Half lives, linear regression (Ln), and regression coefficient (R^2) of paraquat, pretilachlor and 2, 4-D in non-sterilized soil

Herbicides	Treatments	Half lives (Days)	Regression Equations	R^2
Paraquat	NI+1/2X	57.28	Ln (c_t/c_0)=-0.0121x-0.154	0.924
	NI+X	90.01	Ln (c_t/c_0)=-0.0077x+0.515	0.763
	NI+2X	198.03	Ln (c_t/c_0)=-0.0035x+1.33	0.987
	I+1/2X	44.15	Ln (c_t/c_0)=-0.0157x-0.317	0.829
	I+X	71.45	Ln (c_t/c_0)=-0.0097x+0.645	0.994
	I+2X	147.47	Ln (c_t/c_0)=-0.0047x+1.318	0.956
Pretilachlor	NI+1/2X	13.51	Ln (c_t/c_0)=-0.0513x+0.2203	0.803
	NI+X	21.59	Ln (c_t/c_0)=-0.0321x+0.717	0.981
	NI+2X	28.41	Ln (c_t/c_0)=-0.0244x+1.19	0.929
	I+1/2X	-	Ln (c_t/c_0)=-0.0971x-0.015	NA
	I+X	12.93	Ln (c_t/c_0)=-0.0536x+0.5	0.926
	I+2X	16.08	Ln (c_t/c_0)=-0.0431+1.186	0.968
2, 4-D	NI+1/2X	-	Ln (c_t/c_0)=-0.0629+0.0369	NA
	NI+X	14.14	Ln (c_t/c_0)=-0.049+0.7264	0.941
	NI+2X	19.36	Ln (c_t/c_0)=-0.0358+1.5268	0.91
	I+1/2X	-	Ln (c_t/c_0)=-0.0962+0.0137	NA
	I+X	10.21	Ln (c_t/c_0)=-0.0679+0.63	0.999
	I+2X	12.81	Ln (c_t/c_0)=-0.0541+1.489	0.912

NI: Non-inoculated; I: Inoculated; 1/2X: Half dose; X: Full dose; 2X: Double dose

Table 5.7. Half lives, linear regression (Ln), and regression coefficient (R^2) of paraquat, pretilachlor and 2, 4-D in sterilized soil

Herbicides	Treatments	Half lives (days)	Regression Equations	R^2
Paraquat	NI+1/2X	256.7	Ln (c_t/c_0)= -0.0027x-0.1434	0.811
	NI+X	533.15	Ln (c_t/c_0)= -0.0013x+0.592	0.732
	NI+2X	866.38	Ln (c_t/c_0)= -0.0008x+1.3205	0.907
	I+1/2X	216.59	Ln (c_t/c_0)= -0.0032x-0.1803	0.754
	I+X	495.07	Ln (c_t/c_0)= -0.0014x+0.5938	0.776
	I+2X	770.11	Ln (c_t/c_0)= -0.0009x+1.3263	0.912
Pretilachlor	NI+1/2X	44.43	Ln (c_t/c_0)= -0.0156x+0.018	0.967
	NI+X	53.32	Ln (c_t/c_0)= -0.013x+0.5405	0.866
	NI+2X	99.01	Ln (c_t/c_0)= -0.007x+0.9886	0.927
	I+1/2X	27.4	Ln (c_t/c_0)= -0.0253x-0.0274	0.994
	I+X	39.38	Ln (c_t/c_0)= -0.0179x+0.6609	0.99
	I+2X	58.74	Ln (c_t/c_0)= -0.0118x+1.1021	0.945
2, 4-D	NI+1/2X	45.6	Ln (c_t/c_0)= -0.0152x-0.0159	0.992
	NI+X	60.27	Ln (c_t/c_0)= -0.0115x+0.5887	0.994
	NI+2X	90.01	Ln (c_t/c_0)= -0.0077x+1.3788	0.912
	I+1/2X	36.48	Ln (c_t/c_0)= -0.019x-0.0873	0.984
	I+X	48.13	Ln (c_t/c_0)= -0.0144x+0.6278	0.939
	I+2X	67.29	Ln (c_t/c_0)= -0.0103x+1.3867	0.947

NI: Non-inoculated; I: Inoculated; 1/2X: Half dose; X: Full dose; 2X: Double dose

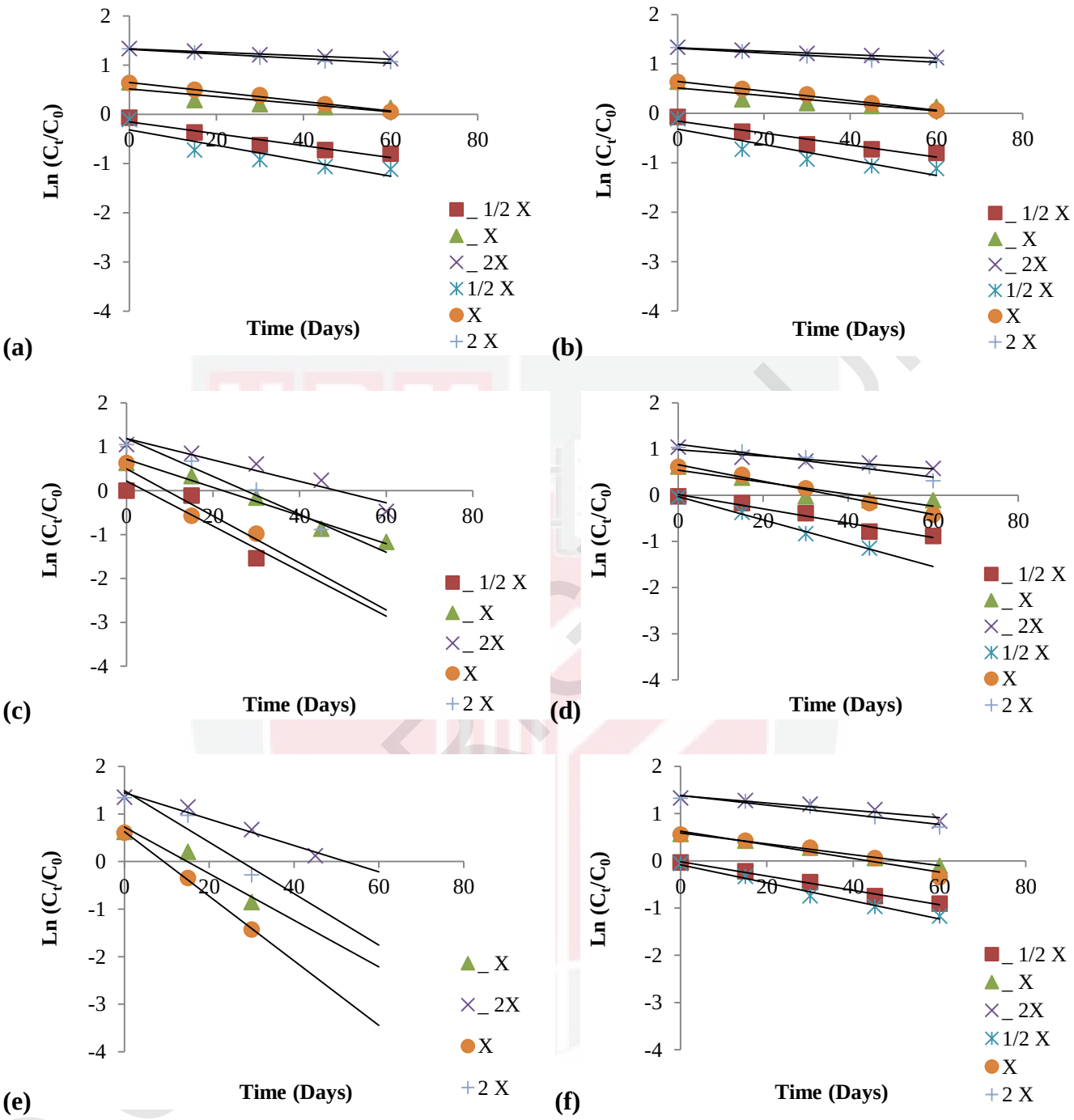


Figure 5.3. Degradation kinetics of herbicides; (a) paraquat in non-sterilized soil, (b) paraquat in sterilized soil, (c) pretilachlor in non-sterilized soil, (d) pretilachlor in sterilized soil, (e) 2, 4-D in non-sterilized soil, (f) 2, 4-D in sterilized soil; -1/2X:non-inoculated+half dose, -X:non-inoculated+full dose, -2X:non-inoculated+double dose, +1/2X:inoculated+half dose, +X: inoculated+full dose, +2X: inoculated+double dose

5.4 Discussion

The shorter persistence of herbicides in non-sterilized compared to sterilized soil indicates the prominent role of soil microorganisms in dissipation mechanisms and degradation of herbicides in natural soil conditions. This result is in line with the results made by Ismail *et al*. (2011) who reported the higher degradation of ^{14}C paraquat and 2, 4-D in non-sterilized compared to sterilized soil under laboratory conditions. The availability of organic matter, soil pH and nutrients contents in non-sterilized soil could also be the reasons of higher degradation of herbicides. The shorter half lives of herbicides in inoculated compared to non-inoculated soil in natural soil conditions could be due to the ability of Sb16 strain in degradation of the herbicides at their recommended dosages in contribution with indigenous soil microorganisms.

The higher dissipation rates of herbicides in samples treated with lower concentrations could be due to the fact that soil microbes could utilize the lower concentrations as carbon or energy sources. The dissipation of higher doses of herbicides at last sampling dates indicates that Sb16 and indigenous microbes were not capable to degrade the higher concentrations as rapid as the lower concentrations.

Soil properties play a vital role in performance of herbicides in soil, as the herbicides are directly applied to the soil (Ashton and Monaco., 1991). The soil texture in this study was sandy clay loam soil, a medium-textured soil which is normally less likely to absorb herbicides molecules. The shorter persistence of herbicide was related to high soil organic matter (Fajardo *et al*., 2000). The increase in soil organic matter leads to an enhancement of microbial population and activity, thus higher degradation of herbicides would occur.

The longest persistence of paraquat in soil compared to pretilachlor and 2, 4-D might be due to the bounding of paraquat to soil particles, which makes it unavailable to microbial and chemical degradation. Many field and laboratory studies have reported the persistent nature of paraquat. The pretilachlor residues persisted until 45^{th} DAA in non-inoculated samples treated with half dose; however they were disappeared at 30^{th} DAA in inoculated samples. The obtained results favor the observations made by Dharumarajan *et al*. (2011) who found that utilization of green leaf manure together with pretilachlor led to the disappearance of pretilachlor to below detectable level (BDL) in soil after 30^{th} days of application, whereas its application at 0.75 $kg.ha^{-1}$, 1.5 $kg.ha^{-1}$ and mixture of gypsum+pretilachlor at 1.5 $kg.ha^{-1}$ resulted in its persistence up to 45 days. The longer half life of pretilachlor in present study compared to earlier reports can be due to the low organic matter of soil, which causes lower population and activity of microorganisms responsible for pretilachlor degradation. Under lower pH of soil in this study, pretilachlor was more likely to adsorb to soil particles, thus less available in soil solution for degradation.

The 2, 4-D formulation used in this study was amine salt which normally has half life up to 10 days. However, the half life of 11.02 days was observed in non-sterilized samples treated with half dose, which is quite higher than the normal rate. This can be due to the slightly acidic pH and low organic matter of soil, which made 2, 4-D persist longer in soil. Moreover, the main metabolites of 2, 4-D (2, 4-dichlorophenol) might be more tightly adsorbed to soils and the negligible amount of 2, 4-D would be degraded (Soulas and Fournier., 1981). Abiotic and biotic factors together affect the degradation of 2, 4-D immediately after its application to soil. The microbial biomass size or total bacterial number and organic matter content of soil have been correlated positively with 2, 4-D mineralization rate (Voos and Groffman., 1997).

5.5 Conclusion

Herbicides dissipation followed the first order kinetics. Herbicides irrespective of dosages persisted longer in sterilized compared to non-sterilized soil treatments. Paraquat had the highest persistence in soil, followed by 2, 4-D and pretilachlor. Application of herbicides at double dose resulted in highest persistence, followed by full and half doses. Soils inoculated with Sb16 had shorter half lives compared to non-inoculated soils. Sb16 strain can assist in degradation of paraquat, pretilachlor and 2, 4-D at their recommended dosages in aerobic rice cropping soil in contribution with indigenous soil microbes under natural field soil conditions.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

In order to improve the rice production, a well-structured approach for nitrogen management is required. Bio-fertilizer, a probable alternative for chemical fertilizer, has gain a huge interest in sustainable agriculture. The efficacy of N_2 fixing bacteria as bio-fertilizer in agricultural system has already been confirmed in many cases. Therefore, specific diazotrophic species should be found, isolated and tested for their potential as bio-fertilizer. Aerobic rice, a water efficient strategy in rice cultivation has come up as an alternative for paddy rice. Herbicides have been proved to be the best chemical weed management approach in aerobic rice cultivation system. When herbicides are applied to soil, they might come in contact with soil indigenous microflora, as well as the introduced microbe. The interaction of herbicides with microbes can lead to either the suppression of microbial community and plant growth or microbial multiplication and plant growth improvement. Microbes are able to reduce the probable detrimental effects of herbicides to the plant through the degradation process.

The first study was conducted to observe the effects of three common rice herbicides on growth and nitrogenase activity of diazotrophic Sb16 *in-vitro* conditions. The inhibition of growth and nitrogenase activity of Sb16 strain in Jensen N-free medium culture amended with paraquat, pretilachlor and 2, 4-D at different concentrations was recovered at last incubation days. There was stimulation in population and nitrogenase activity of Sb16 in presence of half dose of herbicides, with the highest nitrogenase activity in 2, 4-D treated samples. This indicates the utilization of lowest concentration of 2, 4-D as carbon or energy sources by Sb16 strain.

The second study was carried out to determine the effects of Sb16 bacterial inoculation and application of paraquat, pretilachlor and 2, 4-D on growth of aerobic rice and the chemical properties and microbial populations of sterilized and non-sterilized soil in pot experiment under greenhouse conditions. The results indicated that the plant growth and soil chemical and microbial properties showed better performance in non-sterilized compared to sterilized soil condition. Samples inoculated with Sb16 strain and treated with herbicides had higher values of growth parameters of plant and soil chemical properties and microbial populations compared to non-inoculated samples treated with herbicides. Stimulation the population and N_2 fixing activity of Sb16 in Jensen N-free broth treated with half dose of herbicides can justify the insignificant effect of half dose of herbicides on some studied parameters. Moreover, an increase in microbial degraders of these herbicides following the application of herbicides to the soil could lead to an overall enhancement of the studied parameters. There have been some inconsistencies in studies on the effects of bacterial inoculation and herbicides application on plant growth and soil properties, which could be due to the fact that several factors including herbicides

types, dosages, bacterial species, soil chemical, physical and microbial properties and environmental factors perform simultaneously under natural soil conditions.

The results from degradation study showed the ability of Sb16 strain to assist in degradation of lower dosages of herbicides in contribution with soil indigenous microbes during plant growth under natural soil condition. Previous studies also showed faster degradation of herbicides in non-sterilized compared to sterilized soil, which was attributed to the presence of soil microbes in non-sterilized soil. Species of the genera *Bacillus*, *Arthrobacter* and *pseudomonos* could be able to degrade high percentages (82.2%-95.6) of Oxyfluorfen. Thus, these microorganisms were recommended to use in decontamination of polluted sites and agricultural fields (Mohamed *et al*., 2011).

There was highly positive correlation between overall growth and root morphological parameters of aerobic rice and between microbial populations with chemical properties and nutrients contents of soil. The correlation of plant N content with the growth parameters indicates the remarkable role of nitrogen for overall plant growth development. Enhancement in microbial population and plant growth was highly related to the increase in nutrients contents and chemical properties of soil.

In conclusion, the present study indicates that Sb16 strain could utilize the lower concentration of herbicides as carbon or energy sources for its growth and activity. Sb16 is recommended for use as a bio-fertilizer in aerobic rice cropping. Additionally, Sb16 can be useful in degradation of pretilachlor and 2, 4-D at normal recommended dose in aerobic rice cultivation under natural conditions. However, the rate of degradation is greatly affected by soil types and properties and environmental conditions. Sb16 is required to be tested under natural field conditions where multiple biotic and abiotic factors affect the interaction of soil-plant-bacteria-herbicide.

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APPENDICES

Appendix A

a)

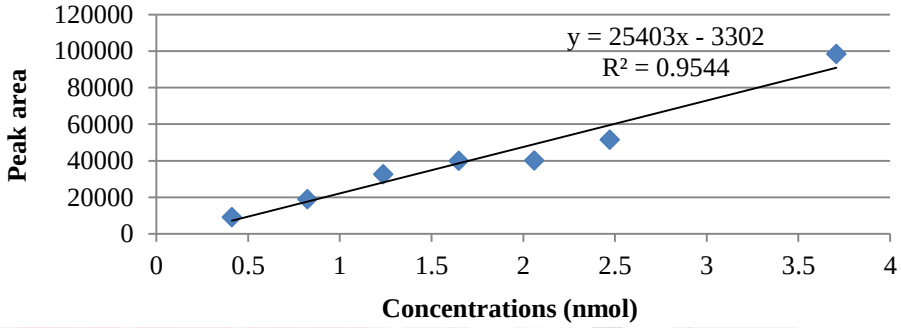
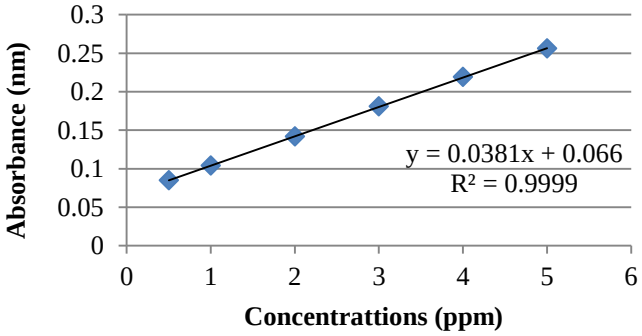


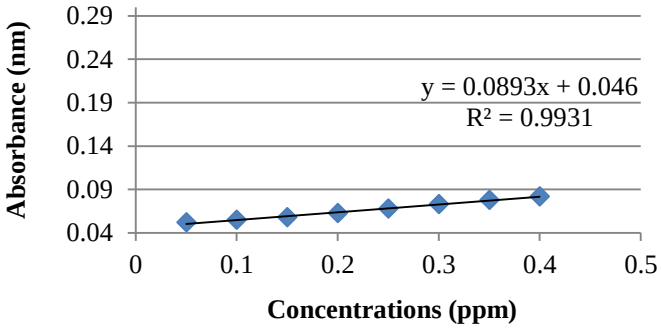
Figure A.1. Standard curve of the peak area against ethylene concentrations (nmol) using Gas Chromatography

b)

(a) Paraquat



(b) Pretilachlor



(c) 2, 4-D

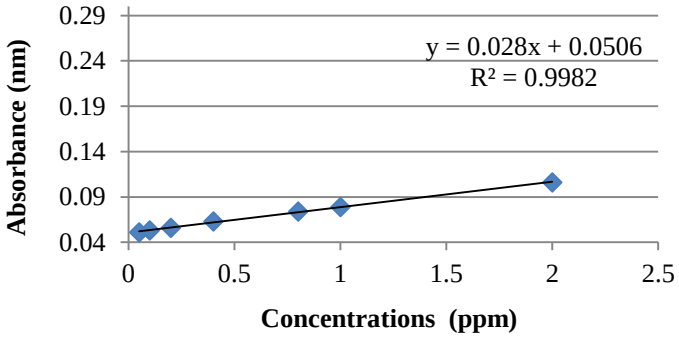


Figure A.2.Calibration curve of concentrations of (a) paraquat (b) pretilachlor (c) 2, 4-D (ppm)

Appendix B

a) ANOVA Complete Randomized Design (CRD) on population and nitrogenase activity of diazotrophic Sb16 and pH of Jensen broth

Source of Variance (S.O.V)	d.f	Mean Squares		
		Log cfu.ml^{-1}	ARA (nmol C_2H_4/mL/hour)	pH
Herbicides (Herb)	2	0.012**	27.667**	0.002**
Concentrations(Con)	3	0.456**	29.599**	0.05**
Incubation time (time)	6	16.696**	5.064**	1.521**
Herb × Con	6	0.005**	22.544**	0.001**
Herb × Time	12	0.006**	0.468*	0.001**
Con × Time	18	0.013**	0.8**	0.008**
Herb × Con × Time	36	0.002**	0.619**	0.0009**
Error	249	0.0002	0.21	0.0002

*: significant at ($P \leq 0.05$) **:significant at ($P \leq 0.01$)

b) ANOVA Randomized Complete Block Design (RCBD) on population of total bacteria, diazotrophs and fungi in soil inoculated with diazotrophic Sb16 and treated with herbicides paraquat, pretilachlor and 2, 4-D

Source of Variance (S.O.V)	d.f	Mean Squares		
		Total bacterial population (Log cfu.gr^{-1})	Diazotrophs population (Log cfu.gr^{-1})	Fungal population (Log cfu.gr^{-1})
Block	2	0.034**	0.039**	0.041**
Inoculation (Inoc)	1	6.381**	32.43**	0.463**
Herbicides (Herb)	2	0.01**	0.034**	0.137**
Concentrations (Con)	3	0.231**	0.462**	0.78**
Sampling time (ST)	4	15.347**	2.431**	7.541**
Inoc × Herb	2	0.012**	0.002**	0.005^{ns}
Inoc × Con	3	0.002**	0.062**	0.002^{ns}
Inoc × ST	4	0.345**	0.135**	0.089**
Herb × Con	6	0.003**	0.007**	0.072**
Herb × ST	8	0.004**	0.007**	0.024**
Con × ST	12	0.013**	0.015**	0.051**
Inoc × Herb × Con	6	0.002**	0.004**	0.004^{ns}
Inoc × Heb × ST	8	0.002**	0.003**	0.005*
Inoc × Con × ST	12	0.002**	0.006**	0.003^{ns}
Heb × Con × ST	24	0.0009**	0.002**	0.015**
Inoc × Heb × Con × ST	24	0.0009**	0.001**	0.001^{ns}
Error	238	0.0001	0.00009	0.002

*:Significant at ($P \leq 0.05$) **:significant at ($P \leq 0.01$) ns:not significant

c) ANOVA factorial based on Randomized Complete Block Design (RCBD) on growth parameters of aerobic rice in non-sterilized soil inoculated with diazotrophic Sb16 and treated with herbicides paraquat, pretilachlor and 2, 4-D

Source of Variance (S.O.V)	d.f	Mean Squares				
		Plant hieght (cm)	Leaf area per plant (cm^3)	Chlorophyll content (SPAD Units)	Plant N content (%)	Shoot dry weight diameter (mm)
Block	2	0.264^{*}	119.439^{*}	0.123^{ns}	0.013^{ns}	0.00002^{ns}
Inoculation	1	0.823^{**}	8328.887^{**}	2.101^{**}	1.434^{**}	0.000005^{ns}
Herbicides	2	0.109^{ns}	17.581^{ns}	0.161^{*}	0.02^{ns}	0.00008^{ns}
Concentrations	3	2.167^{**}	674.386^{**}	2.542^{**}	0.274^{**}	0.001^{**}
Inoc × Herb	2	0.254^{*}	35.914^{ns}	0.125^{ns}	0.004^{ns}	0.00003^{ns}
Inoc × Con	3	0.032^{ns}	257.251^{**}	0.081^{ns}	0.007^{ns}	0.000004^{ns}
Heb × Con	6	0.163^{*}	53.674^{ns}	0.071^{ns}	0.008^{ns}	0.00003^{ns}
Inoc × Heb × Con	6	0.099^{ns}	40.919^{ns}	0.026^{ns}	0.008^{ns}	0.00002^{ns}
Error	46	0.052	26.294	0.048	0.007	0.00003

*:Significant at ($P \leq 0.05$) **:significant at ($P \leq 0.01$) ns:not significant

d) ANOVA factorial based on Randomized Complete Block Design (RCBD) on growth parameters of aerobic rice in sterilized soil inoculated with diazotrophic Sb16 and treated with herbicides paraquat, pretilachlor and 2, 4-D

Source of Variance (S.O.V)	d.f	Mean Squares				
		Plant hieght (cm)	Leaf area per plant (cm^3)	Chlorophyll content (SPAD Units)	Plant N content (%)	Shoot dry weight diameter (mm)
Block	2	0.056^{ns}	27.754^{ns}	0.561^{**}	0.005^{ns}	0.00004^{*}
Inoculation	1	0.08^{ns}	554.9^{**}	2.42^{**}	0.089^{**}	0.000005^{ns}
Herbicides	2	0.235^{ns}	85.396^{*}	1.157^{**}	0.023^{*}	0.000005^{ns}
Concentrations	3	5.06^{**}	958.511^{**}	3.536^{**}	0.382^{**}	0.002^{**}
Inoc × Herb	2	0.049^{ns}	55.182^{ns}	0.407^{**}	0.01^{ns}	0.00009^{**}
Inoc × Con	3	0.081^{ns}	173.288^{**}	0.127^{ns}	0.003^{ns}	0.00001^{ns}
Heb × Con	6	0.024^{ns}	30.09^{ns}	0.041^{ns}	0.01^{ns}	0.000005^{ns}
Inoc × Heb × Con	6	0.026^{ns}	1.941^{ns}	0.025^{ns}	0.009^{ns}	0.00003^{ns}
Error	46	0.118	18.025	0.075	0.006	0.00001

*:Significant at ($P \leq 0.05$) **:significant at ($P \leq 0.01$) ns:not significant

e) ANOVA factorial based on Randomized Complete Block Design (RCBD) on root parameters of aerobic rice in non-sterilized soil inoculated with diazotrophic Sb16 and treated with herbicides paraquat, pretilachlor and 2, 4-D

Source of Variance (S.O.V)	d.f	Mean Squares				
		Root dry weight (g/plant)	Root length (cm)	Root surface area(cm^2)	Root volume (cm^3)	Root avergae diameter (mm)
Block	2	0.000008	3007.573^{*}	892.026^{ns}	0.771^{ns}	0.0005^{ns}
Inoculation	1	0.0002^{**}	15267.849^{**}	3.495^{ns}	5.01^{*}	0.031^{**}
Herbicides	2	0.000002	3088.998^{*}	203.513^{ns}	0.227^{ns}	0.005^{**}
Concentrations	3	0.0003^{**}	26070.471^{**}	15471.168^{**}	22.443^{**}	0.026^{**}
Inoc × Herb	2	0.00002^{*}	422.79^{ns}	2432.506^{**}	2.497^{ns}	0.003^{**}
Inoc × Con	3	0.000005	1004.964^{ns}	291.121^{ns}	2.189^{ns}	0.001^{ns}
Heb × Con	6	0.000004	1103.236^{ns}	96.33^{ns}	0.13^{ns}	0.0009^{ns}
Inoc × Heb × Con	6	0.000009	550.332^{ns}	277.381^{ns}	1.457^{ns}	0.0003^{ns}
Error	46	0.000005	634.246	439.545	1.013	0.0006

*:Significant at ($P \leq 0.05$) **:significant at ($P \leq 0.01$) ns:not significant

f) ANOVA factorial based on Randomized Complete Block Design (RCBD) on root parameters of aerobic rice in sterilized soil inoculated with diazotrophic Sb16 and treated with herbicides paraquat, pretilachlor and 2, 4-D

Source of Variance (S.O.V)	d.f	Mean Squares				
		Root dry weight (g/plant)	Root length (cm)	Root surface area(cm^2)	Root volume (cm^3)	Root avergae diameter (mm)
Block	2	0.0002^{*}	106.026^{ns}	361.439^{ns}	1.151^{ns}	0.008^{*}
Inoculation	1	0.0005^{**}	3943.697^{**}	795.36^{ns}	1.413^{ns}	0.003^{ns}
Herbicides	2	0.0002^{*}	11.17^{ns}	762.299^{ns}	5.075^{**}	0.002^{ns}
Concentrations	3	0.001^{**}	35970.028^{**}	40343.191^{**}	31.891^{**}	0.049^{**}
Inoc × Herb	2	0.00004^{ns}	1798.93^{**}	1130.132^{*}	2.468^{*}	0.003^{ns}
Inoc × Con	3	0.0003^{**}	1653.328^{**}	344.674^{ns}	1.153^{ns}	0.002^{ns}
Heb × Con	6	0.00005^{ns}	851.971^{*}	364.932^{ns}	0.318^{ns}	0.003^{ns}
Inoc × Heb × Con	6	0.00006^{ns}	522.431^{ns}	354.368^{ns}	0.467^{ns}	0.0008^{ns}
Error	46	0.00004	321.44	256.095	0.697	0.002

*:Significant at ($P \leq 0.05$) **:significant at ($P \leq 0.01$) ns:not significant

g) ANOVA factorial based on Randomized Complete Block Design (RCBD) on soil chemical properties of non-sterilized soil inoculated with diazotrophic Sb16 and treated with herbicides paraquat, pretilachlor and 2, 4-D

Source of Variance (S.O.V)	d.f	Mean Squares		
		pH	Organic matter (OM) (%)	Cation Exchange Capacity (CEC) (cmol (+) kg^{-1})
Block	2	0.002^{*}	0.008^{**}	0.036^{ns}
Inoculation (Inoc)	1	0.0006^{ns}	1.077^{**}	10.761^{**}
Herbicides (Herb)	2	0.003^{**}	0.017^{**}	10.561^{**}
Concentrations (Con)	3	0.041^{**}	1.165^{**}	79.029^{**}
Sampling time (ST)	4	0.051^{**}	8.956^{**}	239.742^{**}
Inoc × Herb	2	0.001^{ns}	0.012^{**}	0.287^{ns}
Inoc × Con	3	0.0008^{ns}	0.041^{**}	1.107^{**}
Inoc × ST	4	0.006^{**}	0.337^{**}	1.254^{**}
Herb × Con	6	0.0003^{ns}	0.009^{**}	0.809^{**}
Herb × ST	8	0.002^{**}	0.002^{**}	0.723^{**}
Con × ST	12	0.001^{**}	0.086^{**}	5.087^{**}
Inoc × Herb × Con	6	0.0002^{ns}	0.002^{**}	1.27^{**}
Inoc × Heb × ST	8	0.0009^{*}	0.005^{**}	0.504^{**}
Inoc × Con × ST	12	0.0003^{ns}	0.012^{**}	0.399^{**}
Heb × Con × ST	24	0.0002^{ns}	0.002^{**}	0.252^{**}
Inoc × Heb × Con × ST	24	0.0001^{ns}	0.002^{**}	0.22^{**}
Error	238	0.0004	0.0003	0.106

*:Significant at ($P \leq 0.05$) **:significant at ($P \leq 0.01$) ns:not significant

h) ANOVA factorial based on Randomized Complete Block Design (RCBD) on soil chemical properties of sterilized soil inoculated with diazotrophic Sb16 and treated with herbicides paraquat, pretilachlor and 2, 4-D

Source of Variance (S.O.V)	**d.f**	**Mean Squares**		
		pH	**Organic matter (OM) (%)**	**Cation Exchange Capacity (CEC) (cmol (+) kg^{-1})**
Block	2	0.014^{**}	0.014^{**}	0.229^{ns}
Inoculation (Inoc)	1	0.0005^{ns}	0.112^{**}	9.552^{**}
Herbicides (Herb)	2	0.003^{**}	0.097^{**}	3.843^{**}
Concentrations (Con)	3	0.304^{**}	2.711^{**}	156.115^{**}
Sampling time (ST)	4	0.773^{**}	4.834^{**}	36.963^{**}
Inoc × Herb	2	0.012^{**}	0.025^{**}	3.423^{**}
Inoc × Con	3	0.0008^{*}	0.009^{**}	0.348^{ns}
Inoc × ST	4	0.005^{**}	0.014^{**}	0.299^{ns}
Herb × Con	6	0.002^{**}	0.031^{**}	4.374^{**}
Herb × ST	8	0.003^{**}	0.029^{**}	3.785^{**}
Con × ST	12	0.009^{**}	0.261^{**}	10.692^{**}
Inoc × Herb × Con	6	0.002^{**}	0.005^{**}	1.481^{**}
Inoc × Heb × ST	8	0.002^{**}	0.008^{**}	1.244^{*}
Inoc × Con × ST	12	0.001^{**}	0.009^{**}	0.653^{ns}
Heb × Con × ST	24	0.001^{**}	0.009^{**}	1.09^{**}
Inoc × Heb × Con × ST	24	0.0009^{**}	0.007^{**}	0.792^{*}
Error	238	0.0003	0.0002	0.481

*:Significant at ($P \leq 0.05$) **:significant at ($P \leq 0.01$) ns:not significant

i) ANOVA factorial based on Randomized Complete Block Design (RCBD) on macro nutrients of soil inoculated with diazotrophic Sb16 and treated with herbicides paraquat, pretilachlor and 2, 4-D

Source of Variance (S.O.V)	d.f	Mean Squares		
		Soil total N (%)	Soil Available P ($mg.kg^{-1}$)	Soil Exchangeable K ($mg.kg^{-1}$)
Block	2	0.0002^{**}	1.691^{**}	0.001^{**}
Inoculation (Inoc)	1	0.014^{**}	4.273^{**}	0.002^{**}
Herbicides (Herb)	2	0.0001^{**}	9.116^{**}	0.0007^{**}
Concentrations (Con)	3	0.012^{**}	101.478^{**}	0.035^{**}
Sampling time (ST)	4	0.087^{**}	491.238^{**}	0.53^{**}
Inoc × Herb	2	0.0002^{**}	1.953^{**}	0.0004^{**}
Inoc × Con	3	0.0003^{*}	0.441^{ns}	0.0006^{**}
Inoc × ST	4	0.002^{**}	4.534^{**}	0.0003^{**}
Herb × Con	6	0.0001^{**}	1.463^{**}	0.0003^{**}
Herb × ST	8	0.0004^{**}	1.487^{**}	0.0004^{**}
Con × ST	12	0.0007^{**}	5.448^{**}	0.003^{**}
Inoc × Herb × Con	6	0.00007^{**}	0.236^{ns}	0.0004^{**}
Inoc × Heb × ST	8	0.0001^{**}	1.148^{**}	0.001^{**}
Inoc × Con × ST	12	0.0001^{**}	0.674^{*}	0.0001^{ns}
Heb × Con × ST	24	0.0001^{**}	0.736^{**}	0.0005^{**}
Inoc × Heb × Con × ST	24	0.00007^{**}	0.346^{ns}	0.0004^{**}
Error	238	0.000004	0.344	0.00007

*:Significant at ($P \leq 0.05$) **:significant at ($P \leq 0.01$) ns:not significant

j) ANOVA factorial based on Randomized Complete Block Design (RCBD) on persistence of herbicides paraquat, pretilachlor and 2, 4-D in non-sterilized and sterilized soil inoculated with diazotrophic Sb16

Source of Variance (S.O.V)	df	Mean Squares	
		Residue in non-sterilized soil (µg/g)	Residue in sterilized soil (µg/g)
Block	2	0.0003^{ns}	0.0006^{ns}
Inoculation (Inoc)	1	0.459^{**}	0.05^{**}
Herbicides (Herb)	2	1.323^{**}	0.254^{**}
Concentrations (Con)	2	1.768^{**}	1.618^{**}
Sampling time (ST)	4	1.032^{**}	0.128^{**}
Inoc × Herb	2	0.209^{**}	0.031^{**}
Inoc × Con	2	0.008^{*}	0.036^{**}
Inoc × ST	4	0.049^{**}	0.039^{**}
Herb × Con	4	0.044^{**}	0.026^{**}
Herb × ST	8	0.184^{**}	0.045^{**}
Con × ST	8	0.056^{**}	0.021^{**}
Inoc × Herb × Con	4	0.021^{**}	0.035^{**}
Inoc × Heb × ST	8	0.039^{**}	0.035^{**}
Inoc × Con × ST	8	0.056^{**}	0.028^{**}
Heb × Con × ST	16	0.037^{**}	0.031^{**}
Inoc × Heb × Con × ST	16	0.053^{**}	0.029^{**}
Error	178	0.002	0.0007

*:Significant at ($P \leq 0.05$) **:significant at ($P \leq 0.01$) ns:not significant

BIODATA OF STUDENT

Armita Nahi was born in Tehran, Iran on the 17th of September 1989. Following her high school, she obtained her Bachelor degree in agricultural engineering, soil science from Karaj Islamic Azad University. After finishing B.S. she registered for a master of science (soil science) at Universiti Putra Malaysia in 2012. Her research interests are mainly in the areas of soil microbiology and pesticides science.