MICROBIAL DIVERSITY IN SOIL COOLING FOR THE GROWTH OF LOOSE LEAF LETTUCE IN MALAYSIA

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requirements for the award of the degree of
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MARCH 2019

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Specially dedicated to my supportive supervisors, beloved family and friends

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ABSTRACT

The growing of temperate crops in Malaysia has been intensively practiced on mountain regions of Cameron Highlands due to its favorable cool climate. Temperate crops are dependent on low soil temperature for production of high quality and optimum yields. However, there are limited studies on the importance of low soil temperature in relation to soil nutrients and soil microbial diversity for growing temperate crops in greenhouses in Malaysia. Therefore, this study aims to identify soil microbial diversity in soil cooling for the growth of temperate crops in a greenhouse. The experiment was conducted using a greenhouse to emulate the temperate soil temperature (mean 20.9°C) of Cameron Highlands. Temperate agricultural soils from MARDI, Cameron Highlands were cooled using soil cooling system. A plot of loose-leaf lettuce, a temperate crop, was used for the study. The temperature for the growth of the lettuce under soil cooling were between 14.6°C and 20.1°C. The lettuce was grown for 3-growth cycles without fertilizers, and was evaluated for its growth performance in terms of weight, height, root length, number and area of leaves. The soil chemical properties were analyzed to observe the nutrient trends in soil cooling during the growth of the lettuce. The soil microbial diversity was determined by performing sequencing on target V4 region of bacterial 16S ribosomal ribonucleic acid (rRNA), and first region of fungal internal transcribed spacer (ITS-1). The findings showed that the lettuce had higher average weight at 12.44±2.46 g, 3.68±0.89 g and 2.42±0.42 g for the 1st, 2nd and 3rd growth than uncooled soils. Also, soil cooling showed changes in the availability and uptake of macro and micronutrients during the growth of the lettuce for the 3-growth cycles. In addition, taxonomic classification of 16S bacterial sequencing reported *Arthrobacter psychrolactophilus*, *Serratia marcescens,* and *Solitalea koreensis* as dominant species in uncooled soils, while *Brevundimonas diminuta*, *Flavobacterium succinicans*, *Pseudomonas umsongensis,* and *Pseudomonas viridiflava* in cooled soils. ITS fungal sequencing reported *Mortierella sp VN2-2-5* as dominant species in uncooled soils, *Pyrenochaeta lycopersici* in soils before growth, while *Mortierella oligospora*, *Pseudalueria sp*, *Eocronartium sp,* and *Trichosporon sp* were abundant in cooled soils. The results render insights into the microbial diversity in soil cooling. Understanding the functional role of each microorganism allows effective monitoring of soil health to improve the yield of high quality temperate crops under soil-cooling system in greenhouses in Malaysia.

ABSTRAK

Pertumbuhan tanaman iklim sederhana di Malaysia telah dijalankan secara intensif di kawasan pergunungan Cameron Highlands berikutan iklimnya yang sejuk. Tanaman iklim sederhana bergantung kepada suhu tanah rendah untuk menghasilkan hasil yang berkualiti tinggi dan optimum. Walau bagaimanapun, terdapat kajian yang terhad mengenai kepentingan suhu tanah rendah berhubung dengan nutrien tanah dan kepelbagaian mikrob tanah untuk tanaman beriklim sederhana di rumah hijau di Malaysia. Oleh itu, kajian ini bertujuan untuk mengenal pasti kepelbagaian mikrob tanah dalam penyejukan tanah untuk pertumbuhan tanaman iklim sederhana di rumah hijau. Kajian ini dijalankan dengan menggunakan rumah hijau untuk menghasilkan suhu tanah iklim sederhana (min 20.9°C) di Cameron Highlands. Tanah pertanian dari MARDI, Cameron Highlands didinginkan dengan menggunakan sistem penyejukan tanah. Satu plot daun salad mewakili tanaman iklim sederhana telah digunakan untuk kajian ini. Suhu untuk pertumbuhan salad di bawah penyejukan tanah adalah antara 14.6°C dan 20.1°C. Salad ditanam untuk 3-kitaran pertumbuhan tanpa baja, dan dinilai untuk prestasi pertumbuhannya dari segi berat, ketinggian, panjang akar, jumlah dan luas daun. Sifat kimia tanah dianalisis untuk melihat trend nutrien dalam penyejukan tanah semasa pertumbuhan salad. Kepelbagaian mikrob tanah ditentukan dengan melakukan penjujukan pada rantau sasaran V4 16S *acid ribonukleik ribosom* (rRNA), dan ruang tertranskripsi dalaman pertama (ITS-1) kulat. Dapatan menunjukkan bahawa salad mempunyai berat purata yang lebih tinggi pada 12.44 ± 2.46 g, 3.68 ± 0.89 g dan 2.42 ± 0.42 g untuk pertumbuhan 1, 2 dan 3 daripada tanah yang tidak disejukkan. Selain itu, penyejukan tanah menunjukkan perubahan ketersediaan dan pengambilan makro dan mikronutrien semasa pertumbuhan salad untuk 3-kitaran pertumbuhan. Klasifikasi taksonomi bagi 16S bakteria melaporkan *Arthrobacter psychrolactophilus*, *Serratia marcescens*, dan *Solitalea koreensis* sebagai spesies yang dominan di tanah yang tidak disejukkan, sementara *Brevundimonas diminuta*, *Flavobacterium succinicans*, *Pseudomonas umsongensis*, dan *Pseudomonas viridiflava* dalam tanah yang disejukkan. Penjujukan ITS kulat melaporkan *Mortierella sp VN2-2-5* sebagai spesis dominan di tanah yang tidak disejukkan, *Pyrenochaeta lycopersici* dalam tanah sebelum pertumbuhan, sementara *Mortierella oligospora*, *Pseudalueria sp*, *Eocronartium sp*, dan *Trichosporon sp* banyak terdapat dalam tanah yang disejukkan. Hasil daripada kajian ini memberi gambaran tentang kepelbagaian mikrob dalam penyejukan tanah. Memahami peranan fungsi setiap mikroorganisma membolehkan pemantauan kesihatan tanah yang berkesan untuk meningkatkan hasil tanaman beriklim sederhana yang berkualiti tinggi di bawah sistem penyejukan tanah di rumah hijau di Malaysia.

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LIST OF ABBREVIATIONS

AAS	-	Atomic absorption spectrophotometer
ARDRA	-	Amplified ribosomal DNA restriction analysis
BLAST		Basic Local Alignment Search Tool
DGGE/TTGE		Denaturing- or temperature-gradient gel electrophoresis
DNA	-	Deoxyribonucleic acid
DOA	-	Department of Agriculture
EBT	-	Eriochrome black
EDTA	-	Ethylenediaminetetraacetic acid
FAME	-	Fatty acids methyl ester
FAO	-	Food and Agriculture Organization of the United Nations
FISH	-	Fluorescence *in situ* hybridization
FLASH	-	Fast Length Adjustment of Short
G+C	-	Guanine-plus-cytosine
ITS	-	Internal transcribed spacer
LH-PCR	-	Length heterogeneity PCR
NGS	-	Next-generation sequencing
OTEC	-	Ocean Thermal Energy Conversion
PCR	-	Polymerase Chain Reaction
Q-PCR	-	Qualitative PCR
RAPD	-	Random amplified polymorphic DNA
RDP	-	Ribosomal Database Project

RISA	-	Ribosomal intergenic space analysis
rRNA	-	Ribosomal RNA
SSCP	-	Single-strand conformation polymorphism
SSU	-	Small subunit
T-RFLP	-	Terminal restriction fragment length polymorphism
UV	-	Ultraviolet
V	-	Hypervariable region
VBNC	-	Viable but nonculturable bacteria

LIST OF SYMBOLS

%	-	Percentage
° C	-	Celcius
° F	-	Fahrenheit
16S rRNA	-	16 Svedberg ribosomal DNA
18S rRNA		18 Svedberg ribosomal DNA
α	-	Alpha
β	-	Beta
δ	-	Delta
ε	-	Epsilon
bp	-	Basepair
C	-	Carbon
Ca	-	Calcium
cells/ml	-	Cells/millilitre
cm	-	Centimetre
F	-	Forward (primer)
Fe	-	Iron
g	-	Gram
h	-	Hour
Ha	-	Hectare
K	-	Potassium
kg	-	Kilogram
km	-	Kilometre
m	-	Metre
Mg	-	Magnesium

Mn	-	Manganese
ml	-	Millilitre
mm	-	Millimetre
mg/l	-	Milligram per litre
mg/kg	-	Milligram per kilogram
N	-	Nitrogen
n	-	Sample number
nm	-	Nanometre
OTU	-	Operational Taxonomic Unit
P	-	Phosphorus
R	-	Reverse (primer)
S	-	Sulphur
SD	-	Standard deviation
µl	-	Microliter
x *g*	-	Times gravity
Zn	-	Zinc

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CHAPTER 1

INTRODUCTION

During the last decade, agricultural ecosystem has encountered various environmental changes that affect crop production, which have serious impact on food security. Food and Agricultural Organization (FAO) of United Nation defined food security as i) food available in sufficient quantities of appropriate quality through domestic production or imports; ii) accessible by individuals for acquiring appropriate foods for nutritious diet; iii) utilization of foods through adequate diet to meet standard health requirements, and iv) stability in which all the population, household or individual have access to adequate food supply (Wheeler *et al.*, 2013). Therefore, to ensure continuous food security, understanding of the factors that affect temperate agriculture such as soil temperature, soil and plant nutrients as well as soil microbial diversity are essential to provide an overview on the management of agricultural ecosystem under controlled environment to increase the yield of crops of appropriate quality and nutritional values.

1.1 Background of Research

The growth of temperate crops is highly productive in temperate regions including Southern Hemisphere countries such as Chile, New Zealand and Argentina which produces grapes, apples and to a lesser extent, pears (Huang, 2004; Retamales, 2011). In Asian, China and Japan are the major producers of temperate fruits and vegetables. Considering China's low costs particularly in labour, various private firms have set up greenhouse/packing facilities to ship spinach, lettuce and celery to Japan and Singapore (Shields *et al.*, 2001; Dias *et al.*, 2011). China is a major grower for apples, mandarins, pears, garlic, onion, beetroot and radish (Huang, 2004). Likewise, Japan produces large volumes of apples, pears, citrus fruits, strawberries, tomatoes, sweet peppers, cucumbers, eggplants and lettuces (Huang, 2004). The production of temperate fruits and vegetables are highly productive in temperate regions with some exception of the crops grown on the tropical countries particularly on mountain regions such as Bandung in Indonesia, Baguio in Philippines, Nuwara Eliya in Sri Lanka, Dalat in Vietnam, and Cameron Highlands in Malaysia (Midmore *et al.*, 1996). However, due to increasing market demands, farmers tend to open new land areas inevitably with steeper slopes to accommodate a larger volume production of temperate fruits and vegetables. Therefore, the growth of temperate fruits and vegetables needs to be shifted to lowland areas to prevent further damaging activities of illegal land clearing by highland farmers (Rezuwan, 1999).

Conventional farming on the open field encounters high risk to be damaged by extreme solar radiation, high rainfall, temperature, humidity, wind speeds and infestations of insects and diseases (Kamaruddin *et al.*, 2001). Global average temperature is projected to increase from 0.3°C to 0.7°C for 2016 to 2035 in corresponds to previous reference period, 1986 to 2005 (Kirtman *et al.*, 2013; Vincent *et al.*, 2016). Future temperature increase in the tropics even at relatively small magnitude has been expected to cause more deleterious effects on the crop production (Deutsch *et al.*, 2008). This is further supported by previous studies which demonstrated decrease in wheat and rice yield by 6% to 20%, and approximately 90%, respectively upon increase in the temperature (Tao *et al.*, 2008a,

b; You *et al.*, 2009). In addition, study performed by Vincent et al. has also reported negative sensitivity of crop yields to extreme daytime temperature ranges between 30°C and 34°C depending on the crop and region (Vincent *et al.*, 2016). Such occurrence thus encourages most of the farmers to grow temperate crops under controlled environmental conditions.

Agricultural system is a managed ecosystem that changes predominantly with environmental factors, such as moisture and temperature which act either synergistically or antagonistically with other factors thus affecting the production of temperate crops. Growth conditions could be controlled through modification of aerial and soil environments to increase the crop yield and allow crop to grow throughout the year. All factors including air and soil temperatures, light, water, humidity, carbon dioxide and plant nutrition for growing temperate crops under natural environment are able to be modified or controlled in an enclosed greenhouse or hydroponics techniques for maximum crop production and high economic return (Jensen 1997, 2001). Previous study by Ahmad et al. has reported cultivation of tomato in greenhouse hydroponics in Pakistan with daily temperature maintained between 18°C and 27°C using cooling pad wall and exhaust fans (Ahmad *et al.*, 2015). Cultivation of temperate crops, thyme in greenhouse under temperature controlled also demonstrated the use of cooling system for ventilation of the greenhouse when air temperature exceeded 26°C (Al-Tawaha *et al.*, 2016). In addition, growth of temperate crops under controlled air temperature at 23.9°C in greenhouse hydroponic was also reported for lettuce cultivation (Barbosa *et al.*, 2015), which allow less temperature fluctuation during the crop production. However, controlling environment including cooling of air in a greenhouse under tropical climates requires considerable energy and is not feasible, as compared to cooling of the soils (Jensen, 2001; Zhao *et al.*, 2014; Barbosa *et al.*, 2015). Recently, cold deep seawater from Ocean Thermal Energy Conversion (OTEC) system has been used in Hawaii (Yu, 2015) and Kume Island of Okinawa Prefecture, Japan (Martin, 2017) to cool the soils and allow production of temperate crops throughout the year. However, the use of cold deep sea is limited within reasonable distance from the sea which led to the development of soil cooling system for growing of temperate crops under tropical climate.

Soil temperature has been closely correlated with the development of temperate fruits and vegetables as compared to air temperature (Watts, 1972; Scaife, 1973; Law *et al.*, 1976; Wurr *et al.*, 1981). Soil temperature showed adverse effects on various plant processes including photosynthesis, respiration, water relation, membrane stability, plant hormones levels, primary and secondary metabolites. In addition, changes in soil temperature also affect the initiation, branching, and orientation of root growth (Kaspar *et al.*, 1992). At low soil temperature, berries reported increase in the aromatic compound, while tomatoes showed higher sucrose content of the leaves and maximum fruits production (Went, 1953). Soil temperature also influenced the susceptibility of crops to disease. Studies on the effect of soil temperature on infection of sugar beet by *Polymyxa betae* has reported to be absent and delayed at soil temperature of 10°C and 15°C, respectively (Blunt *et al.*, 1991). Various diseases including brown stripe downy mildew (maize), charcoal rot (soybean), cylindrocladium black rot (peanut) and others has shown to be more susceptible to the microbes at higher soil temperature between 25°C and 37°C (Nyvall, 2013).

The growth of temperate crops is dependent on low soil temperature which also affects the abundance and diversity of soil microbial communities. Soil microbes play diverse and significant roles in nutrient cycling of all major element including carbon, nitrogen and phosphorus, which subsequently affect the structure and functions of soils that maintain the growth of temperate crops (Berg, 2009; Hayat *et al.*, 2010; Aislabie *et al.*, 2013). Soil microbes interact with one another and plants in various growth processes. Plant-microbial interactions can be considered negative when pathogenic, symbiotic mutualists and decomposers reduce the plant performance, but positive when soil microbial community supported or maintained the plant growth and development (Classen *et al.*, 2015). Beneficial microbes such as *Bacillus*, *Pseudomonas*, *Microbacterium*, *Arthrobacter* and *Lysobacter* have been identified from potatoes and lettuces (Kim *et al.*, 2011a; Sati *et al.*, 2013). Cold-tolerant species of *Bacillus* and *Pseudomonas* demonstrated high abundances attributed to the spore forming ability to resist environmental stresses (Mongkolthanaruk, 2012), and growth promotion activities in reference to phosphate solubilization (Mishra *et al.*, 2008; Selvakumar *et al.*, 2009) and biocontrol abilities

(Pandey *et al.*, 2001), respectively. The specific interactions between soil microbial communities and ecosystems in agricultural purposes are important to ensure food security of World's population.

1.2 Problem of Statement

The need to understand the importance of low soil temperature for growing temperate crops in Malaysia drives the conduct of this study. With the global climate change, the effect of soil temperature on mountain agriculture have increased the awareness on the environmental problems and food security in the world. However, the study on soil temperature in relation to soil microbial diversity for the production of temperate crops in Malaysia has received little attention from the researches.

In Malaysia, Cameron Highlands is one of the main agricultural producers of vegetables, tea, flowers and fruits due to its favorable climate. These crops are planted on platforms or terraces form on the hilltops or steep slopes as well as valley floors. The lands of Cameron Highlands was unsystematically leveled for intensive agriculture. Although the overall agricultural activities are relatively small, the steep slopes, the rainfall and high uses of fertilizer and pesticides contribute to soil erosion and environmental pollution in the region. Therefore, discovering the sustainable alternative of growing temperate crops on lowland of Malaysia besides than the mountain region of Cameron Highlands will have an impact on the use and conservation of land resources. This is crucial as the the main challenge is to imitate the cold climate and soil environment of the mountain region of Cameron Highlands for the production of temperate crops in lowland or greenhouses in Malaysia.

In Cameron Highlands, the agricultural activities are intensive due to the favorable cold climate that is conducive for the production of temperate crops. The

production of temperate crops is closely correlated with low soil temperature for optimum crop yield. However, no clear explanation on the importance of low soil temperature in relation to soil microbial diversity for the growth of temperate crops. Until now, majority of the studies investigated the single effects of abiotic soil factor on plant growth, and only a few that study the effect of abiotic soil factor on plants via soil biota (Heinze *et al*., 2016). Soil nutrients were mainly investigated for the abiotic soil effect on plant growth via soil microorganisms (Manning *et al*., 2008; Lee and Kim, 2011; Wei *et al*., 2013; Leff *et al*., 2015; Chen *et al*., 2018), whereas the influence of soil temperature on plants through soil microorganisms are scarce (Li *et al*., 2013; Thakur *et al*., 2016). Moreover, identification of soil bacteria and fungi in temperate agriculture of Malaysia have never been reported in the scientific study.

1.3 Objective of Research

The aim of this study is to identify the soil microbial diversity in soil cooling for the growth of loose-leaf lettuce as the model temperate crops in lowland Malaysia. The objectives of this study are:

i) To evaluate the growth performance of loose-leaf lettuce under soil cooling.

ii) To understand the effect of soil cooling on soil chemical properties during the growth of loose-leaf lettuce.

iii) To evaluate the abundance of soil bacteria and fungi community in soil cooling during the growth of loose-leaf lettuce.

iv) To identify the bacteria and fungi species from soils of loose-leaf lettuce grown under soil cooling.

1.4 Scope of Research

In this study, loose-leaf lettuce was selected as a model temperate crop due to its high productivity in limited space, easy to germinate indoor or sown directly on soils, and have short maturing time between 45 to 55 days as compared to other temperate crop varieties. Lettuces can be grown hydroponically or on soils. To study the soil bacterial and fungal community in soil cooling for the growth of temperate crops, lettuces were grown on soils. Considering the growth requirement of temperate crops is mainly associated with low soil temperature, only the factor of soil temperature is taken into account in this study. Therefore, during the lettuce growth, no fertilizer was added to eliminate the factor of soil nutrients. To identify the soil microbial community of loose-leaf lettuce grown under soil cooling, bacteria and fungi sequencing targeting the region of V4 of 16S rRNA and ITS-1 region, respectively was conducted using Illumina MiSeq sequencing platform. The diversity and taxonomic composition of bacteria and fungi communities is then discussed based on its functional role in the growth of loose-leaf lettuce under soil cooling.

1.5 Significance of Research

Identification of bacteria and fungi diversity from soils of temperate agriculture under soil cooling in Malaysia contribute to the understanding on the growth of temperate crops which is only possible at low soil temperature. This study provides a general overview on the microbial population that was present and its functional role that maintains the growth of temperate crops, which is so far less studied.

In addition, the use of soil cooling system for the production of temperate crops in Malaysia has served as a potential platform for growing temperate crops at

low land areas. This helps to solve several environmental problems including illegal land clearing and deforestation, soil erosion, pollution and land slide as a result of opening new farming areas by highland farmers. In addition, the use of soil cooling also allows growing of various temperate fruits and vegetables including root crops throughout the year, which reduces the transportation cost of temperate agricultural products and securing the job opportunity for lowland farmers. The findings of this study also provides potential guidelines on the growth condition of temperate produce under soil cooling for future food security and production in Malaysia or other tropical countries.

CHAPTER 2

LITERATURE REVIEW

2.1 Temperate agriculture

Temperate agriculture is practised in temperate zones which lie between the polar circles and tropics characterized by mild climate ranging from warm to cool. In temperate zones, four annual seasons; winter, spring, summer and autumn occurs in the South and North Temperate Zone. The South Temperate Zone comprise of southern South America, southern Australia, New Zealand, and South Africa whilst the North Temperate Zone consists of northern Asia, North America, northern Mexico, Great Britain and Europe (Nagarajan, 2010).

The most prevalent group of crops grown across the world are cereals including wheat, maize, rice and sorghum which dominate more than two thirds of the world croplands. Wheat is cultivated intensively in temperate latitudes of both hemispheres which most prevalently grown in North and South America particularly Argentina, Chile, Europe, Nigeria, Algeria, Sudan and South Africa, as well as small parts of China and India (Ray *et al.*, 2013; FAO, 2018). Maize production is mostly concentrated on Europe, North, Central and South America including Uruguay, Argentina, Chile, Brazil, as well as Asia, while South America, India, China and

Indonesia contributes to the production of rice (Ray *et al.*, 2013). As for sorghum, the cultivation area is mostly concentrated in United States of America, Nigeria, Sudan, Mexico, Ethiopia and India (FAO, 2018). Among the respective cereals, wheat dominates the world cultivation area at 221 million Ha, followed by maize, rice and sorghum which covers 188, 160 and 64 million Ha, respectively (FAO, 2018). The production of rice, wheat and maize account for 84%, 56% and 71% of global production, respectively as staple food or livestock feed (Grassini *et al*, 2013). However, study has reported abrupt decrease in the rate of yield gain, including rice in eastern Asia and wheat in northwest Europe which account for 31% of global rice, wheat and maize production (Grassini *et al*, 2013).

Next to cereals, major crops that dominate the temperate region are roots and tubers such as potatoes and sugar beet, pulses including green peas and lentils, and oil-bearing crops: soybeans, canola and sunflower which together cover approximately 188 million Ha of the world's croplands (FAO, 2018). This is followed by temperate fruits and vegetables which also dominate the world's cultivation area by approximately 32 million Ha (FAO, 2018). China has become one of the primary producers for the respective crops: apple, grape, pear, peach, kiwifruit, plum, strawberry, tomato, cabbage and other brassica, carrots and turnips, cauliflower and broccoli, eggplant, lettuce, potato, onion and spinach (Gilmour and Phillips, 2012; Khoury *et al.*, 2016; FAO, 2018), while India rank as the second largest producer in apple, tomato, cabbage and brassica, cauliflower and broccoli, eggplant, lettuce and chicory, onion, potato, pulses and rapeseed (Dastagiri *et al.*, 2013; Negi and Anand, 2015; Khoury *et al.*, 2016; FAO, 2018). The cultivation of these crops also concentrated in United States of America, Turkey, Spain and Italy (Negi and Anand, 2015; Khoury *et al.*, 2016; FAO, 2018). In 2016, the global productions of temperate fruits and vegetables led by sugar beet, tomatoes, apples, cucumber, grapes, cabbage and other brassicas and pears with the yield of 277, 177, 89, 81, 77, 71 and 27 million metric tons, respectively (FAO, 2018).

Temperate agriculture is also practised on the highland regions of Southeast Asia including Vietnam, Thailand, Indonesia and Malaysia (Efendi, 2013; Ariffin *et*

al., 2014; Choenkwan *et al.*, 2014; Wang *et al.*, 2014). In north-western tip of Pahang, Malaysia, Cameron Highlands is one of the famous tourist attraction and largest agricultural based highland resort area (Chan *et al.*, 2006; Leh *et al.*, 2017). Cameron Highlands is situated on the Banjaran Titiwangsa at 1,000 m (3,300 ft) above the sea level. It has an average temperature between 17°C and 20°C all year round (Razali *et al.*, 2018), mean soil temperature of 20.9°C as shown in Figure 2.1 and yearly precipitation of more than 2,700 mm (WMO, 2014). Agro-tourism has become one of the key economic contributors to Cameron Highlands due to the cool climate, farms and natural environments (Leh *et al.*, 2017). The cool climate make Cameron Highlands as one of the intensive agricultural areas in Malaysia for the cultivation of various subtropical and temperate crops including ornamental plants, fruits, vegetables and tea . According to Department of Agriculture (DOA), Malaysia has about 63,600 Ha of vegetables cultivation area yielding 1,195,600 metric tons, and 195,000 Ha of fruits producing 1,664,800 million tons in 2016 (DOA, 2016a, 2016b). Table 2.1 showes the Cameron Highlands' cultivation area and production of temperate fruits and vegetables (DOA, 2016). In 2016, tomato, leaf mustard, cabbage and lettuce dominated the cultivation and production of temperate produce in Cameron Highlands.

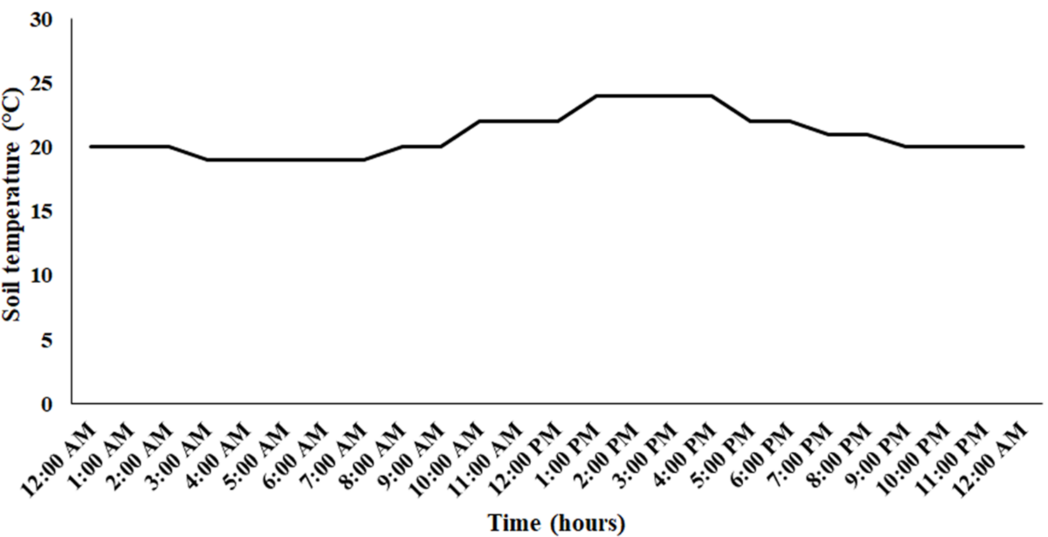


Figure 2.1 Soil temperature of Cameron Highlands, Malaysia.

Table 2.1: Cameron Highlands' agriculture area and production of temperate produce.

Temperate Produce	Area (Ha)	Harvested Area (Ha)	Production (metric tons)
Tomato	1,600	1,600	140,400
Leaf Mustard	4,000	4,000	70,500
Cabbage	2,700	2,400	66,700
Lettuce	2,300	2,300	53,300
Cucumber	300	300	18,000
Celery	570	570	16,100
Egg plant	350	350	12,900
Chinese cabbage	180	160	6,700
Chilli	170	170	6,000
Sweet pepper	49	49	4,000
French Bean	290	260	3,700
Radish	145	130	3,600
Zucchini	47	42	2,100
Spring Onion	130	130	2,000
Cauliflower	130	130	1,900
Carrot	120	110	1,800
Chinese Kale	130	130	1,400
Strawberry	47	47	1,256
Maize	180	160	1,200
Snow Pea	97	97	1,170
Broccoli	62	62	630
Beetroot	14	13	420
Water-cress	46	46	420
Sweet potatoes	27	27	290
Baby Lettuce	13	12	210
Bottle Gourd	7	7	121
Asparagus	5	5	60
Grape	0.1	0.1	1

Temperate crops have specific optimum temperature ranges (temperature threshold) that differ by crop variety and stages of development, for example, vegetative and reproductive stages (Hatfield *et al.*, 2008; Luo, 2011). Exposure to higher temperatures during reproductive stages showes to be the most detrimental as it affects the pollen viability, fertilization and grain or fruit formation (Hatfield *et al.*, 2008). Summer cereal crops including maize, rice and sorghum have a higher optimum temperature for crop development as compared to winter cereal crop such as wheat (Luo, 2011). Maize reported optimum temperature at 31.1°C for leaf and shoot growth, and 26.3°C for root growth (Sánchez *et al.*, 2014). Phenological phases in maize development including phase from emergence to the end of tassel initiation requires temperature at 28.3°C to define the potential number of kernels that subsequently contribute to the maize yield (Sánchez *et al.*, 2014). The cardinal temperature for whole rice development ranges from 13.5°C to 35.4°C with 27.6°C as optimum growth temperature (Sánchez *et al.*, 2014). As for sorghum, the optimum temperature ranges for vegetative and reproductive development is 26/34°C and 25/28°C, while grain yield and production of dry matter have optimum temperature of 27°C and 22°C, respectively (Luo, 2011). Wheat reported lethal limits of -17.2°C and 47.5°C as minimum and maximum temperature with 21°C and 20.7°C for anthesis and grain filling, respectively (Luo, 2011; Sánchez *et al.*, 2014).

Several fruits including pomes (apple and pear) and stone fruits (peach, plum, apricot and cherry) require exposure to chilling conditions in the winter to break dormancy (Gariglio *et al.*, 2012; Luedeling et al., 2012; Atkinson *et al.*, 2013; Basannagari *et al.* 2013). Chilling hours in winter is essential to avoid delay in pollination and foliation, and to improve the fruit yield and quality. Apple and pear require at least 1200 to 1500 of chilling hours, while stone fruits including peach, plum, apricot and cherry require chilling hours that ranges from 650 to 1300 hours depending on the cultivar type (Pathak *et al.*, 2018). Exposure to temperature higher than 22°C during the reproduction of apples increase the fruit size but decreases the firmness of the fruit (Hatfield and Prueger, 2015). As for cherries, increase in temperature by 3°C above the optimum temperature, 15°C cause decrease in the fruit set, while temperature greater than 30°C during the citrus fruit development decrease the content of the sugar and acid as well as the fruit size (Hatfield and Prueger,

2015). Temperate vegetables can be divided into warm and cool season crops. The warm season crops including pepper and tomatoes grow optimally at a warmer climate, while cabbage, broccoli, carrot, spinach and lettuce prefer cool temperature. Table 2.2 outlines the temperature threshold for several vegetable crops grown in temperate climates (Pathak *et al.*, 2018). Tomatoes demonstrated favourable yield during warm April and June months at temperature ranges between 20°C and 25°C (Zdorovtsov, 2012; Pathak *et al.*, 2018). Cool crops such as carrots grow and develop optimally at temperature between 15.6°C and 21.1°C (Kabir *et al.*, 2013). The exposure of crops to high temperature than the optimum will reduce the ability of the crops to produce grain and fruits due to disruption of pollination process, although the impact varies among the crop species. Therefore, temperature affects the growth and development of temperate crop variety.

Table 2.2: Temperature threshold for selected vegetable crops.

Climatic classification	Crop	Acceptable Temperature for Germination (°C)	Optimal Temperature for Yield (°C)	Acceptable Temperature Growth Range (°C)
Hot	Melon	21-32	25-27	18-35
	Sweet potato	21-32		
Warm	Cucumber	16-35	20-25	12-30
	Pepper	16-35		
	Sweet corn	16-35		
	Snap beans	16-30		
	Tomato	16-30		
Cool-Warm	Onion	10-30	20-25	7-30
	Garlic	7-25		
	Pea	10-30	18-25	5-25
Cool	Potato	7-26	16-25	5-25
	Lettuce	5-26		
	Cabbage	10-30	16-18	5-25
	Broccoli	10-30		
	Spinach	4-16		

2.1.1 Environmental problems addressed in temperate agriculture

Temperate agriculture encounters various environmental variabilities including climate change. Climate has adversely changed over the past decades, and is projected to continue increasing from 1.8°C to 4.0°C at the end of this century (IPCC, 2001). The effect of climate change to food availability has been reported in temperate zones such as European Union for wheat and United States of America for maize (Müller *et al.*, 2015). Both crops were negatively affected owing to the reduced water availability during growing season, frequent incidents of heat events damage the flowering stages, and accelerate phenology which results on decrease in biomass production (Müller and Elliott, 2015; FAO, 2016). Climate change has demonstrated negative impacts on other crops such as grapes and apples that demonstrated early flowering and maturity, although it is less reported as compared to major staple crops (Porter *et al.*, 2014). In addition, climate change is also expected to cause potential losses and damages to vegetables that are adapted to lower temperature such as carrots, tomatoes, spinach, broccoli, lettuce and other *Brassica* species (Karl *et al.*, 2009; Ayyogari *et al.*, 2014).

The current climate change also causes temperature, rainfall variation and carbon fertilization effects in temperate agriculture of Cameron Highlands which affects the cultivation of temperate fruits and vegetables (Hamdan *et al.*, 2014). Although Cameron Highlands situated in the mountain range, due to the current climate change, the mean temperature is projected to increase from 0.4°C in 2005 to 2.8°C in 2095 (MOSTI, 2009). Sudy conducted by National Hydraulic Research Institute of Malaysia projected that by 2050, Pahang will experience changes in the monthly precipitation of up to 51% (NAHRIM, 2006). Besides, the lowest minimum and highest maximum temperature in Cameron Highlands between January and April 2016 has also been reported to increase by 1.3°C and 0.8°C, respectively than the previous year, 2015 (Leo, 2016), thus adversely affecting the physical appearance and productivity of highland crops. According to farmers in Cameron Highlands, highland crops such as cabbage, tomatoes and strawberries require temperatures

between 17°C and 20°C for the whole day to grow but once the weather changes, the crops will experience lack of nutrients, shortage of fertilizer, extended growing season, pests and disease invasion such as leaf spot diseases and others (Hamdan *et al.*, 2014).

Agriculture in Malaysia is always associated with crops cultivated on fertile lowland areas. The cultivation of the crop has extended to highland areas due to the growing population and increased economic activities (Barrow *et al.*, 2010; Razali *et al.*, 2018). However the expansion of agricultural activities often occurs on the slope of highland areas. As in peninsular Malaysia, the slopes of Cameron Highlands have been developed extensively for cultivation of temperate vegetables, fruits, flowers and tea. Despite consistent attempts to grow temperate crops at lowland areas under tropical climates, large proportions of the temperate vegetables are still continuously being produced under favourable temperate climate of Cameron Highlands and are unlikely to shift to the lowland (Barrow *et al.*, 2010; Razali *et al.*, 2018). This led to opening of new farming areas by highland farmers on the slope of Cameron Highlands to accommodate the increasing demands for high quality vegetables regardless of the government restriction. In 2006 to 2009, heavy rainfalls have caused several landslides and mudflows along the highways to east coast of Malaysia, Cameron Highlands, Sabah and Penang (Pradhan *et al.*, 2010). According to local newspaper report in 2014, uncontrolled land clearing has resulted to landslides and mud floods which affect Kampung Raja, Ringlet town and Bertam Valley causing 5 deaths and destruction of homes (Bernama, 16 December 2014; Palansamy, 26 November 2014). The same landslide incidence occurred in May 2016 which hit Taman Mawar, Kuala Terla caused evacuation of 25 occupants of four houses (Bernama, 23 May 2016; Yeap, 23 May 2016). The continuous incidence of massive mud floods since 2014 due to uncontrolled deforestation and illegal land clearing for agricultural purposes has subsequently submerged several parts of Cameron Highlands (Jalil, 12 April 2016). Therefore, if these environmental problems continue to arise, it will be impossible for Cameron Highlands to accommodate production of temperate crops in the future.

2.2 Ocean Thermal Energy Conversion (OTEC)

Cooling of soils using the principle of Ocean Thermal Energy Conversion (OTEC) has served as a potential platform for growing temperate crops on tropical lowland areas.

2.2.1 Principle of OTEC

Ocean constitute more than 70% of Earth's surface allowing collection of thermal energy from the sun's heat and mechanical energy from tides and waves (Jadhav and Kale, 2005). Ocean Thermal Energy Conversion (OTEC) utilizes temperature differences between warm surface seawater of tropical oceans and cold deep seawater to produce electricity (Heydt, 1993; Masutani *et al.*, 1999). To generate a net output of energy, minimum temperature difference of about 20°C at the depth of 1000 m between warm surface water and cold deep seawater is necessary (Masutani and Takahashi, 1999). There are 3 types of OTEC's system including closed-cycle, open-cycle and hybrid cycle which require a working fluid, condenser and evaporator within all three systems. These three systems apply the thermodynamics of a working heat exchanger and temperature differences that naturally occur in the ocean as a driving force (Finney, 2008).

The closed-cycle of OTEC's system as shown in **Figure 2.2 (a)** uses a working fluid with low-boiling point such as ammonia to the turbine (Masutani and Takahashi, 1999). The warm sea water is first pumped into the evaporator where liquid ammonia is pressurized. The pressure and warm temperature causes ammonia to boil and produce steam that drive the turbine and generates electricity. The steam is then condensed back into a liquid in a condenser (heat exchanger) by cold deep sea water and recirculates back into the system (Jadhav and Kale, 2005; Finney, 2008).

Open-cycle of OTEC's system as shown in **Figure 2.2 (b)** is similar to the closed-cycle system except that the open-cycle is using steam from the sea water directly to drive the turbine (Jadhav and Kale, 2005). The warm sea water is pumped into evaporator chamber and converted into a low-pressure vapour that turns the turbine as in the closed-cycle system. After the steam has passed through a condenser, the vapour is condensed back into liquid and vented from the system either to the ocean or an isolated storage tank. Hybrid-cycle of the OTEC's system applies the principle of both closed and open-cycle to achieve maximum efficiency. Both seawater and another working fluid, usually ammonia is used in this system. Similar to closed-cycle, the fresh water is converted into steam in a vacuum vessel. In the same vessel, ammonia is evaporated through heat exchanger with the warm seawater in two-phase, two-substance mixture. The ammonia is then evaporated and separated from the steam and re-condensed back into the closed loop cycle. The change of water and ammonia through different phase turns the turbine producing energy (Finney, 2008).

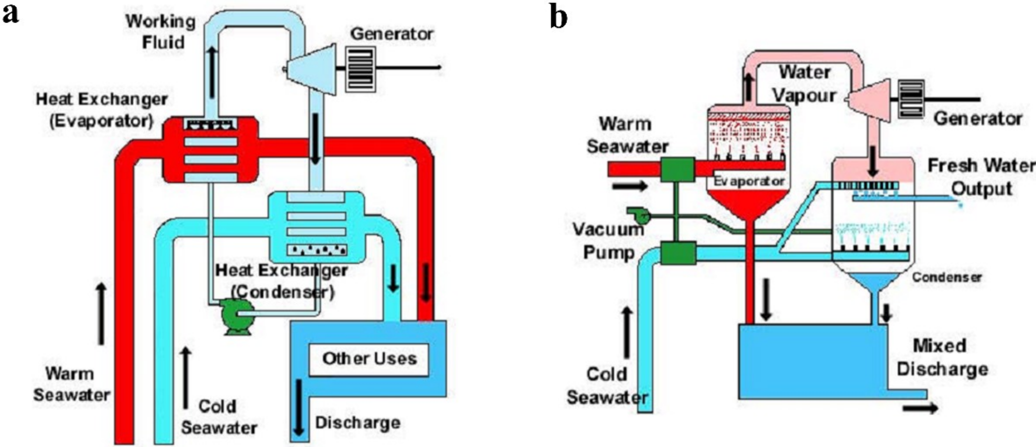


Figure 2.2 OTEC's system including closed-cycle (a), open-cycle (b) and hybrid cycle which is the combination of closed- and open-cycle.

2.2.2 Application of OTEC in agriculture

Besides producing electricity, OTEC's system can also be used in the production of fresh water, air conditioning and refrigeration, mariculture and agriculture (Masutani and Takahashi, 1999; Jadhav and Kale, 2005; Finney, 2008). Briefly, the condensate of open- and hybrid cycle of OTEC's system is desalinated water that can be consumed as drinking water and used for irrigation of the crops (Masutani and Takahashi, 1999). As for the cold deep seawater, it can be used to retain a cold storage spaces, providing air conditioning to homes and offices, and cultivating medium for marine organisms such as oysters, sea urchins, lobsters, nori and others (Masutani and Takahashi, 1999).

As OTEC's system is strategically located at the coastal area of tropical regions, the use of cold deep sea water for growing temperate crops in the tropics throughout the year has become possible. The cold deep seawater is pumped through an array of underground pipes that chills the surrounding soils providing a cool and conducive growth condition for temperate crops such as strawberries and others (Masutani and Takahashi, 1999; Jadhav and Kale, 2005; Finney, 2008). Besides cooling of the soil, condensation of cold deep seawater on above-ground hoses could also be used for irrigation of the crops (Masutani and Takahashi, 1999).

These cold water agriculture or soil cooling systems have been implemented in Hawaii and Kume Island of Okinawa Prefecture, Japan. In Kume Island, cold deep seawater is pumped through an array of pipes that are embedded within the soils. The cold deep seawater cools the surrounding soils and ensures crops such as spinach to thrive. Although the greenhouse can get very hot during the day, the roots of the spinach remains cool (Martin, 2017). Likewise, Hawaii uses cold deep seawater running in closed hoses underneath the grapes and chills the soil (Yu, 2015). The cold deep seawater also runs through hoses placed above the grapes for irrigation by pulling off moisture from the air which then drop one at a time and irrigate the crops.

2.3 Factors affecting plant growth

The nature of soil which is affected by its physical, chemical and biological properties plays significant role in determining the growth, development and reproductive success of individual plant (Van der Putten *et al.*, 2013). Most of the physical factor have significant effect on the chemical and biological properties and processes which subsequently influence the plant growth. Soil is a physical system that can be described in terms of size, density, porosity, moisture content, and temperature. The growth of temperate crops is highly dependent on the factor of soil temperature which affects various physiological processes such as photosynthesis and respiration (Agalave, 2017). Physical factors including soil temperature also indirectly affect other chemical and biological factor such as nutrient supply, pH, soil micro and macroorganisms (Hillel, 2008; Conniff, 2013).

2.3.1 Soil temperature

Soil temperature is defined as a function of heat flux in soil and heat exchange between soil and atmosphere (Elias *et al.*, 2004). Soil temperature varies daily and seasonally due to change in radiant energy and energy transfer through soil surfaces (Chiemeka, 2010). Soil temperature alters the soil physical, chemical and biological properties that subsequently affect the growth, development and productivity of the plants. Soil physical properties that are related to soil organic matter including aggregate stability, soil structure and pore spaces are mostly affected by high soil temperature. Soil temperature higher than 30°C leads to increase in aggregate stability resulting from thermal transformation of iron and aluminium oxide that acts as cementing agents for clay particles (Fox *et al.*, 2007; Terefe *et al.*, 2008). Increase in soil temperature also affects the soil structure by inducing dehydration of clay minerals and heat-induced cracks in sand-sized particles which eventually lowers the clay sand contents and increase the silt content

(Arocena and Opio, 2003; Assaf *et al.*, 2004; Pardini *et al.*, 2004). Increase in soil temperature resulted to decrease in water viscosity and soil moisture content that allows more water to percolate through the soil profiles (Kolay, 2008; Broadbent, 2015; Kidron and Kronenfeld, 2016). High soil temperature was also reported to maximize the evaporation rates that restrict the movement of water in soils. Increase in soil temperature demonstrated change in the pore spaces due to increase in the content of carbon dioxide through soil microbial activity (Allison, 2005).

High soil temperature affects the soil chemical properties including pH, cation exchange capacity and soil nutrients. Soil temperature ranges between 25°C and 39°C lead to increase in the soil pH as a result of organic acid denaturation (Mensies and Gillman, 2003). Soil temperature also affects the cation exchange capacity in which at high soil temperature, the cation exchange capacity decreases due to decline in the organic matter composition and clay size fraction (Certini, 2005). Soil nutrients are commonly cycled in the environment through processes of rock weathering and biological decomposition, and slowly release for the uptake of plants and soil microorganisms. However, at high soil temperature, nutrients respond differently according to its inherent temperature threshold. Soil nutrients that have low temperature threshold are easily loss through volatilization. These thresholds are divided into three categories: sensitive, moderately sensitive and insensitive. Based on the threshold, nitrogen and sulphur are categorized under sensitive, potassium and phosphorus under moderately sensitive while calcium, magnesium and manganese as insensitive (Peerzada and Chauhan, 2018). Despite this, high soil temperature also alters the nutrient availability to plants by in situ changes or translocation of organic substances such as nitrogen down the soils (DeBano, 1991).

Soil temperature affects soil biological properties including structure and diversity of microorganisms, microbial activity, decomposition and mineralization of organic matter and nutrient availability. Previous study by Feng and Simpson has reported changes in the diversity of soil microorganisms at soil temperature of 10°C to 20°C (Feng and Simpson, 2009). This is further supported by Borowik and Wyszkowska which reported 15°C as the optimal soil temperature for the

development of heterotrophic bacteria, *Azotobacter*, actinomycetes and fungi (Borowik and Wyszkowska, 2016). Soil temperature influences soil biota activity through direct change in physiological activities involving enzyme, or indirectly alters the physiochemical properties including diffusion and solubility of nutrients, mineral weathering and rate of evaporation (Paul, 2006; A'Bear *et al.*, 2014. Soil microorganisms were reported to perform optimally at soil temperature of 10°C to 28°C (Conant *et al.*, 2008; Allison *et al.*, 2010; Frey *et al.*, 2013; Borowik and Wyszkowska, 2016), while soil temperature ranges between 10°C and 24°C resulted to increase in the metabolic activity of soil macroorganism such as earthworms and arthropods (Conant *et al.*, 2008). Soil temperature that ranges from 10°C to 28°C reported changes in soil respiration. This is due to increase in extracellular enzyme activity that contribute to the degradation of polymeric organic matter and increase in microbial soluble substance retake (Frey *et al.*, 2013; Zhang *et al.*, 2015; Borowik and Wyszkowska, 2016; Tang *et al.*, 2018). Soil temperature between 21°C and 28°C has been reported to increase the decomposition rate of soil organic matter due to changes in diffusion of soluble substrates and increase in soil microbial activity (Broadbent, 2015). Increase in decomposition rate of soil organic matter and soil microbial activity at optimum soil temperature have been reported to improve the rate of nitrogen mineralization thus contribute to soil nitrogen supply (Gavito *et al.*, 2001; Conant *et al.*, 2008; Allison *et al.*, 2010; Wallenstein *et al.*, 2010; Yan and Hangwen, 2014). A study performed by Johnston et al. has reported a significant effect of soil temperature on phosphorus bioavailability (Johnstone *et al.*, 2005). Soil phosphorus demonstrated increase at about 40% upon 10°C increment in soil temperature from 5°C to 25°C during lettuce production (Johnstone *et al.*, 2005). Previous study by Yilvainio and Pettovuori have also reported increase in water soluble phosphorus as a result of diffusion at soil temperature of 5°C and 25°C, while lower soil temperature was reported to hinder the releasing of phosphorus from soil organic matter (Yilvainio and Pettovuori, 2012).

Soil temperature has significant effects on the growth and development of plants by affecting the water and nutrient uptake as well as root and shoots growth. Previous study has reported decrease in water uptake at lower soil temperature (<10°C) due to increase in water viscosity and decrease in the rate of water

absorption. Soil temperature lower than 10°C has also demonstrated decrease in root growth, rate of nitrogen uptake, assimilation of carbon dioxide and stomata conductance as compared to plants subjected to high soil temperature (Toselli *et al.*, 1999; Lahti *et al.*, 2002). Soil temperature has demonstrated influence on various aspects of seed biology and reproductive development including seed viability, germination, and dormancy as well as pollen viability, floral development, fertilization and postfertilization process (Porter, 2005; Ribeiro *et al.*, 2013). Increase in soil temperature has been reported to inhibit the respiration and photosynthetic activity, influence membrane permeability, synthesis of sucrose and starch as well as leaf water potential and leaf area that leads to premature leaf senescence, shortened life cycle, reduce dry matter production and plant productivity (Peerzada and Chauhan, 2018). Increase in soil temperature showed positive effect on plant nutrient uptake and plant size during vegetative growth, but a latter effect on the shoot and root phenology which only visible at the end of vegetative growth (Gavito *et al.*, 2001). Previous literature have demonstrated that soil temperature may drive the crop maturation for over 25% during cool-temperate climate. Increase in soil temperature from 18°C to 25°C in cool-temperate season has resulted to increase in biomass and grain yield (Stone *et al.*, 1999). Warm season crop such as chilli grow optimally at a soil temperature of 17°C and poorly at soil temperature below 10°C (Basu and De, 2003). As for the cool-season crop including lettuce, high soil temperature of 25°C and 32°C was reported to inhibit the germination of the seeds, depending on lettuce varieties. This soil temperature also resulted to stunted growth, bitterness and poor quality of the lettuce leaves (Coons *et al.*, 1990). Study by Stone et al. concluded that the effect of soil temperature on crop yield vary with sowing time and latitude of growth location (Stone *et al.*, 1999). At a certain growth location, for example the northern part of Nigeria, an increase in soil temperature causes a heat flux that increase the moisture evaporation from the soils. These then limit the mobilization of the soil nutrients that are essential for the crop optimal performance (Ogbodo *et al.*, 2010).

2.3.2 Soil nutrients

Soil nutrients refer to the requirement of basic chemical elements in the crops. There are 16 basic elements which can be divided into macronutrients and micronutrients. Carbon, oxygen and hydrogen can be derived from the atmosphere, soil and water. Macronutrients such as nitrogen, phosphorus, potassium, magnesium, sulphur and calcium can also be found in soils and are absorbed in a large amount (Uchida, 2000; Leye Samuel *et al.*, 2014). As for the micronutrients such as manganese (Mn), iron (Fe), zinc (Zn), boron (B), copper (Cu), chlorine (Cl) and molybdenum (Mo) are absorbed in a small amount (Call, 1999). The plant nutrients uptake depends on its optimum nutrient range and minimum requirement. Too little nutrient uptake will lead to nutrient deficiency, while excessive nutrients uptake will cause poor growth due to toxicity (Uchida, 2000).

Among these nutrients, nitrogen (N) is the most important element in plant growth and development followed by phosphorus (P) and potassium (K) (Leye Samuel and Omotayo Ebenezer, 2014). Nitrogen (N) is available to plants as nitrate and ammonium ions. It helps in the building blocks of protein, and is involved in all enzymatic reactions of a plant and a major part of chlorophyll molecule which is necessary for photosynthesis reaction (Uchida, 2000). The lack of nitrogen uptake by the crops will lead to general yellowing or light greening of the leaves and stunted growth (Hosier *et al.*, 1999; Wong, 2005). Phosphorus (P) is involved in plant processes including photosynthesis, respiration, energy reservoir and transfer, as well as cell division and enlargement. These elements constitute a major component of genetic information and aids in the flower initiation, seed, flower and fruit development (Uchida, 2000). Deficiency of phosphorus will cause the leaf tips to look burnt and the older leaves to change to dark-green or reddish purple leaves (Hosier and Bradley, 1999; Wong, 2005). The other macronutrients that is essential for the crop development is potassium (K). Potassium does not become the part of crop chemical structure but acts as an enzyme catalyst for chemical reactions, water and nutrient transport through opening and closing of stomata, photosynthesis, transport of sugar, synthesis of protein and starch (Prajapati *et al.*, 2012). In addition,

potassium also improves the crop quality in term of physical appearance, disease resistance and storage conditions (Uchida, 2000; Prajapati and Modi, 2012). Without adequate potassium, the margin of the leaves may wilt, turn chlorotic and necrotic. Besides that, the stem is also weak and undergoes a slow and stunted growth (Uchida, 2000; Wong, 2005). Secondary macronutrients such as sulphur (S), calcium (Ca) and magnesium (Mg) play a major role in the formation of plant proteins, cell wall membrane and transport of sugar within the crops (Uchida, 2000). Without these nutrients, the leaves will form uniform chlorosis, change to light green colour, become distorted or irregular in shape, undergo thickening and shortening of the roots and cause interveinal chlorosis on the older leaves (Hosier and Bradley, 1999; Wong, 2005).

Micronutrients including manganese (Mn), iron (Fe), zinc (Zn), boron (B), copper (Cu), chlorine (Cl) and molybdenum (Mo) are needed in a small amount and only perform its function in plant nutrition when other essential elements are available in a balanced ratio (Tavakoli *et al.*, 2014). Manganase (Mn) plays vital role in the oxidation and reduction process as electron transfer in photosynthesis, lipids metabolism, nitrogen metabolisms and assimilation, cell division and plant growth as well as activator for decarboxylase, dehydrogenase and oxidase enzymes (Malvi, 2011; Tavakoli *et al.*, 2014). Manganese is important for chlorophyll production and essential in Photosystem II that involved in the photolysis reaction (Uchida, 2000; Tavakoli *et al.*, 2014). Manganese deficiency resulted to interveinal chlorosis of young leaves, interveinal white streaks (wheat), grey specks (oats), interveinal brown spots and streaks (barley) (Lohry, 2007). In monocot plants, manganese deficiency appears as grey-green spots on the base of leaves, while deficiency in dicot plants was demonstrated by small yellow spots on the leaves (Uchida, 2000; Tavakoli *et al.*, 2014). The major symptom of manganese deficiency was reported to reduce the production of dry matter and crop yield due to decrease in the efficiency of photosynthesis (Tavakoli *et al.*, 2014). As for iron (Fe), these elements are important for heme enzyme system in plant metabolism including nitrogen fixation, nitrate and sulphate reduction, synthesis of chlorophyll and maintaining of the chloroplasts structure as well as component of plant defence enzyme such as catalase and peroxidase (Uchida, 2000; Malvi, 2011; Tavakoli *et al.*, 2014). Iron deficiency are

often observed as interveinal chlorosis on young leaves and twig dieback in corn, sorghum, turf, several soybean varieties, tree cops and ornamentals (Wong, 2005; Tavakoli *et al.*, 2014).

Zinc (Zn) is an essential component of proteinase, dehydrogenase and peptidase enzyme (Malvi, 2011). Zinc plays vital role in building and activation of plant enzyme as well as RNA and protein synthesis (Uchida, 2000). Zinc stimulates growth hormones, starch formation, seed maturation and pollen formation (Tavakoli *et al.*, 2014). The symptoms of zinc deficiency include interveinal chlorosis of younger leaves, whirling, short internodes and small leaves (Wong, 2005). In monocot particularly corn, deficiency appear as band on the main nervure of both sides of the leaves, while dicot plants showed decrease in internode distance and leaf size (Tavakoli *et al.*, 2014). As for citrus crop, deficiency of these element lead to formation of irregular interveinal chlorosis with mottled, small and pointed leaves as well as decline in the fruit formation (Uchida, 2000). Boron (B) posses synergistic relationship with potassium that exhibit overlapping physiological roles including cell division, pollen germination, flowering and fruiting, nitrogen and carbohydrate metabolism, active salt absorption, hormone action and movement, water relations and metabolism in plants (Malvi, 2011). In addition, boron also acts as a buffer that is essential for regulatory effect on other nutrients and maintenance of conducting plant tissues (Malvi, 2011). Generally, lack of boron resulted to stunted and small plant with thick, brittle and misshapen leaves while symptoms such as pith in hollow stem, crooked stems and black hearts appear to be specific in cabbage, celery and beets, respectively (Uchida, 2000, Tavakoli *et al.*, 2014). Copper (Cu) involve in several enzyme systems in plant, electron transport and energy capture by oxidative enzymes and proteins, cell wall formation, photosynthesis, carbohydrate and protein metabolism (Lohry, 2007; Malvi, 2011; Tavakoli *et al.*, 2014). Copper deficiency is likely to appear first in wheat, barley, oats and canary seeds which is highly sensitive to the level of copper in soils. Copper deficiency lead to stunted growth, leaves with chlorotic or deep blue-green with rolled up margins, increase in the incidence of ergot, root and stem rot (Uchida, 2000; Lohry, 2007; Malvi, 2011; Tavakoli *et al.*, 2014). Chlorine (Cl) involve in photosynthesis process, osmoregulation, charge compensation and fungal disease control (Uchida, 2000; Lohry, 2007). Deficiency of

these element includes wilting of plant, chlorosis of younger leaves and excessive branching of lateral roots (Uchida, 2000; Lohry, 2007). As for molybdenum (Mo), this element is essential for nitrate reductase and nitrogenase that involved in N assimilation and upon deficiency, the activity of nitrate reductase decrease thus inhibit the ability of the plant to synthesize proteins (Uchida, 2000; Lohry, 2007). Moreover, symptoms such as interveinal chlorosis, stunting, lack of vigor, scorching of marginal and rolling of leaves were also reported in plant with molybdenum deficiency (Uchida, 2000; Lohry, 2007).

2.3.3 Soil organisms

Soil organisms are very diverse and exceed, by far, the eukaryotic organisms. There are about 8 to 15 tons of bacteria, Actinomycetes, fungi, protozoa, nematodes, earthworms and arthropods in the soils (Hoorman *et al.*, 2010). Generally, one gram of soil consists of up to 10 billion microorganisms of bacteria (10^8-10^9/ gram soil), Actinomycetes (10^7-10^8/ gram soil), fungi (10^5-10^6/ gram soil), algae (10^4-10^5/ gram soil), protozoa (10^3-10^4/ gram soil) and nematodes (10^2-10^3/ gram soil) (Hoorman and Islam, 2010). Soil organisms serve as primary regulator of nutrient cycling and soil organic matter, soil carbon sequestration, soil structure and water process, enhancement and suppression of plant growth, suppression of disease, parasites and pests (FAO, 2015). Soil organisms are divided based on their size or functional roles (Havlicek and Mitchell, 2014). Soil microflora such as bacteria, actinomycetes, fungi and algae play key role in decomposing or metabolizing plant-derived organic substances and mineralizing nutrients. Soil fauna is categorized into microfauna (nematodes and protozoans), mesofauna (microarthropods including mites and springtails) and macrofauna (earthworms and termites) (Lartey, 2006; Hillel, 2008; Havlicek and Mitchell, 2014).

Bacteria are unicellular organisms that range from 0.2 to 2 μm in width, and 1 to 10 μm in length (Hillel, 2008; Hoorman, 2016). Bacteria are highly diverse in soil environments and belong to three domain shapes which is rod (known as bacilli), spherical (cocci) and spiral (spirilla) (Hoorman, 2016). These microorganisms are categorized into four functional group including decomposers, mutualists, pathogens and lithotrophs that perform essential soil processes related to nutrient cycling, water dynamics and disease control (Hillel, 2008; Hoorman, 2016). Decomposer bacteria breakdown soil organic matter into simpler forms useful for other soil organisms and immobilize nutrients within the cells to prevent nutrient loss from the root zone, while mutualist form symbiotic association with the roots of legumes (Hillel, 2008; Hoorman, 2016). Bacteria are also pathogenic to the plants and lithotrophic by converting N, S, Fe and other nutrients for energy and essential for nitrogen cycling and degradation of pollutants (Hillel, 2008; Hoorman, 2016). In addition, bacteria improve soil structure by producing glycoprotein that binds soil particles into stable microaggregates (Hoorman, 2016). These microorganisms also show the capability to grow and adapt to various environmental conditions (Hoorman, 2016). Dominant members of the soil bacterial phyla in soils are summarized in Table 2.3 in reference to the previous literature (Janssen, 2006; Aislabie *et al.*, 2013). According to the table, bacterial phyla Acidobacteria, Actinobacteria and Proteobacteria are abundant in soils whereas, members of phyla Verrucomicrobia, Bacteroidetes, Firmicutes, Chloroflexi, Planctomycetes and Gemmatimonadetes are generally less prevalent.

Actinomyctes are large group of bacteria that exhibit similar form and function as fungi. These microorganisms (1 to 2 μm) are Gram-positive slow growing, aerobic and heterotrophic microorganisms. Actinomycetes are commonly discover within the genera of *Streptomyces*, *Micromonospora* or *Nocardia* of Order Actinomycetaceae with *Streptomyces* as the dominant species in soil (Hillel, 2008). Actinomycetes form branch filamentous networks and capable of decomposing recalcitrant compounds including chitin, lignin, keratin, animal polymers and cellulose fungal (Lartey, 2006; Hillel, 2008; Hoorman, 2016). Actinomycetes are essential in producing stable humus that improves the soil structure, storage of soil nutrients and soil water retention (Hoorman, 2016).

Table 2.3: Dominant bacterial phyla in soils.

Phyla/Subphyla	**Average contribution (%)**	**Range (%)**	**Examples**
α-Proteobacteria	19	2-43	*Sphingomonas, Rhizobium, Mesorhizobium, Bradyrhizobium, Methylobacter, Methylophilus, Nitrospira, Nitrobacter, Rhodobacter*
β-Proteobacteria	10	2-31	*Burkholderia, Alcaligenes, Acidovorax, Collimonas, Nitrosospira, Thiobacillus, Rhodocyclus, Methylomonas*
γ-Proteobacteria	8	1-34	*Pseudomonas, Xanthamonas, Azotobacter, Thiocapsa, Chromattium*
δ-Proteobacteria	2	0-10	*Desulfovibrio, Bdellovibrio*
ε-Proteobacteria	<1	0-1	*Helicobacter, Campylobacter*
Acidobacteria	20	0-35	*Acidobacterium*
Actinobacteria	13	0-25	*Arthhrobacter, Rhodococcus, Streptomyces, Mycobacterium, Rubrobacter, Terrabacter, Acidimicrobium*
Verrucomicrobia	7	0-21	*Chthoniobacter, Opitutus*
Bacteroidetes	5	0-16	*Chittinophaga*
Firmicutes	2	0-7	*Clostridium, Bacillus, Lactobacillus*
Chloroflexi	3	0-16	
Planctomycetes	2	0-8	
Gemmatimonadetes	2	0-4	*Gemmatimonas*
Other groups	5	2-10	
Unknown	2	0-13	

Fungi are eukaryotic microorganism that grow as hyphae forming filamentous networks through interaction with soil particles, roots and rocks. These networks produce a wide variety of extracellular enzymes that break down various organic matter, decompose soil components and regulate the nutrient cycling of carbon and nutrients (Žifčáková *et al.*, 2016; Frąc *et al.*, 2018). Fungi also serve as biological controllers that aid in the soil structure formation and ecosystem regulators that control diseases, pests and growth of other microorganisms (Hoorman, 2011a). The filamentous hyphae of fungi bridge the gaps between pockets of soil moisture and nutrients, penetrate substrate including decaying wood and redistribute the soil carbon, water and minerals through the soils (Taylor and Sinsabaugh, 2015). Fungi also serve as important symbionts that associated with major groups of organisms, bacteria, plants and green algae as well as animals including insects. Mycorrhizal fungi are divided into arbuscular mycorrhizae (AM), ectomycorrhizae (EM), orchid mycorrhiza and ericoid mycorrhiza (Van Der Heijden *et al.*, 2015). AM fungal reported 74% association with all plant species, 2% for EM fungal, 9% for orchid mycorrhizas and 1% for ericoid mycorrhiza (Van Der Heijden *et al.*, 2015). These mycorrhizal fungi connect the above and belowground soil via hyphal networks that allow translocation and movement of soil nutrients among coexisting plants. Mycorrhizal fungi grow within the cortex, surface or around the epidermal cells of the roots. The fungi also grow out from the roots into the soils to enhance the acquisition of water and nutrients that are limiting to plant growth particularly nitrates and phosphates in exchange for carbohydrates produced by the plant hosts (Hoorman, 2011a; Taylor and Sinsabaugh, 2015; Van Der Heijden *et al.*, 2015).

Soil fungal are first classified into four phyla including Ascomycota (cup fungi), Basidiomycota (club fungi), Chytridiomycota (water molds) and Zygomycota (bread molds) (Taylor and Sinsabaugh, 2015). Several new phyla, subphyla and class-level fungal classification were recently introduced including Aphelidiomycota, Basidiobolomycota, Blastocladiomycota, Cryptomycota, Calcarisporiellomycota, Entomophthoromycota, Entorrhizomycota, Glomeromycota (arbuscular mycorrhizal fungi), Kickxellomycota, Mortierellomycota, Mucoromycota and Neocalimastigomycota (former members of Chytridiomycota), Olpidiomycota and Rozellomycota (Hibbett *et al.*, 2007; Webster *et al.*, 2007; Taylor and Sinsabaugh,

2015; Ruggiero *et al.*, 2015; Todersoo *et al.*, 2018). The abundance of fungal species is estimated to be millions with only less than 2% been formally reported. The abundance and diversity of soil fungi are summarized in Table 2.4 according to the previous literature reported by Tedersoo and colleagues (Todersoo *et al.*, 2018). The most dominant fungal taxa were reported to be Basidiomycota (55.7%) and Ascomycota (31.3%) (Todersoo *et al.*, 2018). Basidiomycota comprised of three major subphyla: Pucciniomycotina, Ustilaginomycota and Agaricomycotina (Hibbett *et al.*, 2007; Aislabie *et al.*, 2013). Members of the first two subphyla groups (Pucciniomycotina and Ustilaginomycota) includes rust and smut fungi that are pathogenic to beans, coffee, apple, oats, wheat, maize, and sugarcane, while members of the Agaricomycotina cover most of the EM fungal taxa that form association with the woody plants of temperate forest and woodlands as well as brown and white rot fungi (Aislabie *et al.*, 2013; Taylor and Sinsabaugh, 2015). The brown and white rot fungi involved in the degradation of plant polysaccharides with brown rot fungi selectively degrades cellulose and hemicellulose in the wood, leaving recalcitrant lignin, while white rot fungi degrades cellulose, hemicellulose and lignin (Webster and Weber, 2007; Aislabie *et al.*, 2013). Pylum Ascomycota encompasses the most species-rich fungal phlum accounting for 75% of described fungal species fall within these three subphyla: Taphrinomycotina, Saccharomycotina and Pezizomycotina. Some of the members of Ascomycota are macroscopic producing distinctive reproductive structures such as truffles and morels, while most of the members are microscopic and exist as the single-celled (e.g. *Saccharomyces*) or filamentous fungi (e.g. *Aspergillus*) (Hibbett *et al.*, 2007; Aislabie *et al.*, 2013). Besides than single-celled yeast and filamentous fungi, lichen formed from the association of fungi and algae, few EM taxa, all ericoid mycorrhizal species, several pathogens and saprotrophs were also discovered within this phylum (subphylum Pezizomycotina) (Taylor and Sinsabaugh, 2015). As for Glomeromycota, the members of this phylum form arbuscular mycorrhizal (AM) association intracellularly with the plant roots. This fungal phylum consists of approximately 250 species including *Acaulospora*, *Entrophosphora*, *Glomus* and *Gigaspora* which were one of the key drivers that exert positive effects on the soil biodiversity and ensuring ecosystem multifunctionality (Hibbett *et al.*, 2007; Webster and Weber, 2007; Van Der Heijden *et al.*, 2015; Frąc *et al.*, 2018).

Table 2.4: Global diversity of soil fungal.

Phyla	Subphyla/Class	Average abundance (%)
Ascomycota	Sordariomycetes	8
	Eurotiomycetes	7.7
	Leotiomycetes	7.1
	Dothideomycetes	4.5
	Pezizomycetes	1.8
	Archaeorhizomycetes	0.4
	Geoglossomycetes	0.4
	Lecanoromycetes	0.4
	Saccharomycetes	0.4
	Archaeorhizomycetes	0.4
	Orbiliomycetes	0.2
	Others	0.4
Basidiomycota	Agaricomycetes	50
	Tremellomycetes	3.3
	Wallemiomycetes	1.1
	Microbotryomycetes	0.6
	Others	0.6
Chytridiomycota		0.9
Cryptomycota		0.7
Mortierellomycota	Mortierellomycotina	6.3
Mucoromycota	Mucoromycotina	4.4
Glomeromycota		0.2
Other and unknown		0.7

Algae are ubiquitous and ranges from 10 to 40 micrometres in diameter that exists either individually (single cells) or in clusters (colonies) (Hillel, 2008; Havlicek and Mitchell, 2014). Algae are able to associate with fungi forming symbiotic lichens that are able to survive in harsh conditions (Hillel, 2008). These soil phototrophs including cyanobacteria and eukaryotic microalgae are major

primary producers that fix inorganic carbon from atmosphere via photosynthesis and also able to fix atmospheric nitrogen (Lartey, 2006; Abdel-Raouf *et al.*, 2012). Algae are important component in soil ecosystem in which these soil phototrophs improves the availability and uptake of phosphorus through secretion of extracellular phosphatases, increase soil organic matter, stabilize the soil structure through production of extracellular polysaccharide, form biological crusts that influence soil factors including water accessibility, nutrient content and erosion susceptibility (Řeháková *et al.*, 2011; Abdel-Raouf *et al.*, 2012; Sharma *et al.*, 2012). In addition, algae also stabilize soil erosion by regulating the water flows, serve as bioremediation agents in metal polluted soil, produce and release bioactive extracellular substances including plant growth regulators, vitamins and amino acids that affects the growth and development of the plants (Řeháková *et al.*, 2011; Abdel-Raouf *et al.*, 2012; Sharma *et al.*, 2012; Al-Sherif *et al.*, 2015).

The soil fauna or soil engineer comprises of microfauna (protozoans and nematodes), mesofauna (microarthropods such as mites and springtails), and macrofauna (earthworms and termites). Protozoa ranges from 5 µm to 500 µm in diameter are single-celled organisms that feed primarily on bacteria but also consume other protozoa, fungi and soluble organic matter (Lartey, 2006; Hoorman, 2011b). Protozoa are categorized into species of Mastigophora or *flagellates*, Ciliophora or *ciliates* and Sarcodina or *amoeba* which serve as key player in mineralizing soil nutrients making it available for the uptake of plants and other soil organisms, as well as regulating the ecological balance between bacteria and algae population in the soils and helps to control disease by competing with or feed on the pathogens (Hoorman, 2011b). As for nematodes, these soil microfauna are non-segmented worms with developed central nervous system, fertility and complete digestive systems that range from 50 µm in diameter and 1 mm in length (Hoorman, 2011b). Nematodes consist of root-feeders that feed on plant roots, and free-living in soils that feed on bacteria, fungi, protozoans as well as other nematodes. Free-living nematodes contribute to nutrient cycling and release of soil nutrients for plant growth and development. In addition, nematodes also serve as indicators for soil quality and soil health due to differences in the grazing and feeding behaviours (Ito, 2015).

Mesofauna commonly acari (mites), collembola (springtails) and enchytraeids refer to soil organisms with body width between 0.1 mm and 2 mm (Culliney, 2013; Briones, 2014). Mesofauna affects the interaction between soil microbial communities by feeding on specific microorganisms. Members of Acari feed on dead plants and microflora, as well as component of micro and mesofauna for species of Prostigmata and Mesostigmata (Culliney, 2013), while Collembola (springtails) feed on fungal hyphae which changes the mycorrhizal symbiosis and influence the plant growth. In addition, the springtails's feeding behaviour on mycorrhizal fungi also supports the growth of bacterial communities by reducing the fungal biomass and increase the availability of resources to soil bacteria (Kuťáková *et al.,* 2018). In addition, microarthropods contribute to litter decomposition, SOM formation, release of phosphorus and nitrogen during litter decomposition, and formation of soil aggregates through production of organic matter such as faecal pellets (Culliney, 2013; Briones, 2014; Maaß *et al.*, 2015; Soong and Nielsen, 2016). As for enchytraeids, these microorganisms feed on partially degraded plant debris, bacteria, fungi, microalgae while several feed on rotting seaweed (thalloid algae), feces of invertebrates and animal remains (Gajda et al., 2017).

Soil macrofauna such as earthworms, molluscs, termites and ants are invertebrates that exhibited body width of more than 2 mm (Briones, 2014). These soil ecosystem engineers are vital for shaping the soil structure and balancing the fluxes of energy and nutrients across different spatial and temporal scales. Earthworm are the key players in soils that involve in pedogenesis, development of soil structure, regulation of water process, degradation of organic matter and molecules, nitrogen mineralization, climate regulation through incorporation of organic matter into soils and formation of macroaggregates through burrowing and egestion activities (Blouin *et al.*, 2013). Molluscs also influence the structure and stability of soils through production of cutaneous mucous that cement soil particles and make it less vulnerable to soil erosion (Menta, 2012). Termites are macroinvertebrates that contribute to the conversion and mineralization of large proportion of plant litter directly into biomass, and main agents for the degradation of highly recalcitrant materials (Jouquet *et al.*, 2011). Termites as other soil macrofauna influences the soil formation, bioturbation, water infiltration and the

diversity of soil microbes, animals and plants (Jouquet *et al.*, 2011). Termites mound soils are rich in N and other mineral nutrients (Ca2+, Mg2+, K+), as well as higher cationic exchange capacity (Jouquet *et al.*, 2011). In addition to termites, the nests of ants also have high level of nutrients including N and P, as well as rich in substrates for microbial degradation and mineralization of soil organic matter (Culliney, 2013; Cammeraat and Risch, 2008). Besides than nutrient cycling, ants also contribute to various ecosystem services including soil textural, hydrological and biological properties (Cammeraat and Risch, 2008). Although soil fauna serves as key players in shaping the soil structure at large spatial scales, soil microflora such as mycorrhizal fungi, phosphorus solubilizing bacteria, free-living nitrogen fixing organisms and rhizobia are presumably to exert the most significant effect on plant physiology as well as the whole ecosystem productivity (Pritchard, 2011).

2.3.3.1 Contribution of soil microbial community to nutrient cycling

Soil microorganisms play vital role in the cycling of C, N and P that affects the structure and functions of soil ecosystem (Dungait *et al.*, 2012; Aislabie *et al.*, 2013). In soil ecosystem, this bacterial and fungal community completes the C cycle through respiration and mineralization of organic material. Organic materials resulted from primary production conferred to living organisms and non-living organic material extracted from living organisms. Heterotrophic bacteria and fungi, the saprotrophs converts organic material that is formed by primary producers back to carbon dioxide through respiration process. As for the mineralization process, organic compound is degraded completely into inorganic products such as carbon dioxide, ammonia and water (Eilers *et al.*, 2010). Members of phyla especially Actinobacteria and Proteobacteria are involved in the global C cycle in which they degrade soluble organic molecules including amino acids, organic acids and sugars. Likewise, phyla Bacteroidetes, saprotrophic fungi, phyla Chytridiomycota, and phyla Ascomycota specialize in degrading recalcitrant C compounds such as cellulose, lignin and chitin.

Nitrogen is an essential element in protein and nucleic acid. Microbes carry several important processes in N cycle namely nitrogen fixation, nitrification, dissimilatory nitrate reduction to ammonium (DNRA), anammox and denitrification (Aislabie *et al.*, 2013). Nitrogen fixation (N-fixation) is the reduction process of atmospheric nitrogen gas to ammonium which is then assimilated into amino acid and subsequently polymerized into proteins. Nitrogen is fixed by free-living microbes (e.g. *Azotobacter*, *Burkholderia* and *Clostridium*), microbes that form symbiotic relationship with plant (e.g. *Rhizobium*, *Mesorhizobium*, *Frankia*) and those that form association with rhizosphere of plant. During N-fixation, ammonium is oxidised to nitrite and then to nitrate. Soil bacterial *Nitrosospira* and *Nitrosomonas* is involved in the oxidation of ammonium to nitrite, whereas oxidation of nitrite to nitrate is mediated by *Nitrobacter* and *Nitrospira*. The other alternative processes involved in nitrogen cycle are dissimilatory nitrate reduction to ammonia (DNRA) and annamox which occurs under anaerobic condition. DNRA is an eco-physiological process performed by bacteria and fungi in which nitrate was traceably removed from soils without either being assimilated into microbial biomass or loss as nitrogenous gases. This process can generate a cellular proton-motive force that conserves energy to support growth at cellular level depending on the enzymes that are activated (Stein and Klotz, 2016). As for anammox or anaerobic ammonium oxidation, this process utilizes pool of nitrite and ammonium to form nitrogen via intermediates nitric oxide and hydrazine (Stein and Klotz, 2016). DNRA and annamox are mediated by *Alcaligenes* and *Escherichia* which reduce nitrate to nitrite, and members of Planctomycetes which oxidise ammonium to nitrogen gas, respectively (Aislabie *et al.*, 2013). Denitrification is the major biological mechanisms in which fixed nitrogen is returned to the atmosphere from soil and water, thus completing the nitrogen cycle (Stein and Klotz, 2016). Denitrification involves sequential reduction from nitrate, nitrite, nitrous oxide to nitrogen gas which is mediated by members of Proteobacteria, Actinobacteria, Firmicutes and fungi (Aislabie *et al.*, 2013).

Phosphorus is not an element that is abundantly found in soil organic matter but is essential for normal growth and maturity. Phosphorus is vital for photosynthesis, respiration, storage and transfer of energy, cell division, cell

enlargement and several other processes in plants (Sims *et al.*, 2005). However, if presence, the soil microbes will transform phosphorus through two ways which involved: (1) mineralization of organic P to inorganic phosphate catalyzed by phosphatase enzyme, or (2) transformation of insoluble, immobilized P to soluble P in a process that is commonly mediated by organic acid production (Aislabie *et al.*, 2013). Upon this process, phosphorus was release for the use of the microbes as well as plants and other soil habitants.

The soil of Earth's ecosystem has serve as a natural habitat for a vast bacterial and fungal biodiversity. The estimated relative abundance of the soil microbial community varies depending on the soil type and texture, aggregate size, moisture, pH, predation and temperature (Buyer *et al.*, 2010). The identification, cultivation and screening of soil microbial community for agricultural importance have progressed further in the tropics than in temperate countries (Macrae *et al.*, 2013). A study performed by Baldani and Baldani has reported a method for identifying and employing tropical soil bacteria to improve the crop cultivation in arid and savannah condition (Baldani *et al.*, 2005). From the study, tropical soil inoculants using *Herbaspirillum seropedicae* strain has been used to promote the growth of maize, rice, sorghum and sugarcane. The specific interactions that the soil microbial community share with agricultural ecosystem are important to feed the World's population and ensure sufficient food production of high quality.

2.4 Approaches for describing diversity of soil microbial community

2.4.1 Culture dependent method

Culture dependent method including dilution plating and community-level physiological profiles has been utilized to study the soil microbial communities (Hill

et al., 2000). Dilution plating and culture methods use various growth media to recover different microbial communities from soil ecosystem. However, these methods only recover 1% of the soil microbes as no culture media and growth conditions that generally represent all the microorganisms present in the soil sample (Hill *et al.*, 2000; Lutton *et al.*, 2013; Bhatia *et al.*, 2015). For instance, aerobic microbes does not form colonies under anaerobic condition and vice versa, slow growing microbes are outgrown by fast growers which produce large colonies and strict microbes which only grow at certain pH, temperature, nutrients or other growth conditions (Lutton *et al.*, 2013; Bhatia *et al.*, 2015). In addition, it is also difficult to imitate the symbiotic, mutualistic and parasitic relationship of the microorganisms in soil ecosystem (Bhatia *et al.*, 2015). To overcome this limitation, other method such as community-level physiological profiles has been widely used to analyse the soil microbial communities using BIOLOG®, a commercial taxonomic system (Hill *et al.*, 2000). BIOLOG® system provides a rapid identification of bacterial isolates through utilization of 95 carbon sources in a 96-well plate (Stefanowicz, 2006). The isolates utilized carbon substrates producing formazan which cause colour changes in tetrazolium dye. The specific pattern of colour change on the 96-well plate enables identification of bacterial isolates through comparison with the BIOLOG databases (Preston-Mafham *et al.*, 2002; Stefanowicz, 2006). However, this method has its own drawbacks in which (i) visible changes only occurred when the total number of bacterial isolates reaches 10^8 cells/ml to utilize the substrates; (ii) identification of the microbes is based on the assumption made from the colour changes on each well corresponding to the function of microorganisms present in the sample which are able to utilize specific substrates; and (iii) the 95 sole carbon sources used in this method are most likely not representing the diversity of substrates present in the environment (Hill *et al.*, 2000).

2.4.2 Culture independent method

Due to the limitations of culture dependent method, molecular approaches have been categorized into partial and whole community analysis method based on their capability to discover the structural and functional diversity of soil microorganisms. Method of partial community analysis is based on the amplification of conserved genes such as prokaryotes 16S, eukaryotes 18S ribosomal DNA (rRNA) or internal transcribed spacer (ITS) through polymerase chain reaction (PCR), while whole community analysis method relied on the analysis of whole genomes (Rastogi *et al.*, 2011).

2.4.2.1 Partial community analysis

Partial community analysis is a polymerase chain reaction (PCR)-based method that generate PCR products reflecting various mixture of bacterial gene signatures from all organisms present in a sample, including viable but nonculturable bacteria (VBNC). Amplification of conserved genes such as 16S rRNA from an environmental sample has been extensively used to characterize soil microorganisms due to the following traits: i) ubiquitous in which the genes present in all prokaryotes; ii) conserved in terms of its structure and function; and iii) consists of variable and highly conserved regions (Hugenholtz, 2002). There has been growing number of 16S rRNA gene sequences available for comparison in the database to identify new isolates or molecule sequences based on the phylogenetic similarity observed between the reference database and the sample sequences.

PCR products of these conserved gene has been primarily analysed using clone library method, genetic fingerprinting techniques, DNA microarrays, qualitative PCR (Q-PCR), fluorescence *in situ* hybridization (FISH) and microbial

lipid analysis (Malik *et al.*, 2008; Rastogi and Sani, 2011; Gosal *et al.*, 2015). Clone library method analysed PCR products by cloning, sequencing and comparing the gene fragments to the reference sequence in the database such as GenBank, Ribosomal Database Project (RDP) and Greengenes (Rastogi and Sani, 2011; Gosal and Mehta, 2015). PCR products can also be directly analysed using genetic fingerprinting techniques based on i) different linear gradient of DNA denaturant and melting behaviour as in denaturing- or temperature-gradient gel electrophoresis (DGGE/TTGE); ii) different electrophoretic mobility of single stranded DNA in non-denaturing gel using single-strand conformation polymorphism (SSCP); ii) difference in the localization of restriction endonuclease digestion sites along the gene fragments as in random amplified polymorphic DNA (RAPD), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP); and iv) length polymorphism of entire gene fragments using length heterogeneity PCR (LH-PCR) and ribosomal intergenic space analysis (RISA) (Malik *et al.*, 2008; Rastogi and Sani, 2011; Gosal and Mehta, 2015). The other molecular approaches include DNA microarrays, Q-PCR and FISH. DNA microarrays determine the presence of target microorganisms by identifying signals from fluorescent labelled PCR products that hybridized to a known molecular probes attached to microarrays (Rastogi and Sani, 2011; Gosal and Mehta, 2015). As for the Q-PCR and FISH, these techniques determine the abundance and expression of taxonomic and functional gene marker by assessing the accumulation of amplicon in real time during each PCR cycle and phylogenetic identification of microorganisms by whole cell hybridization with oligonucleotide probes which corresponding to cellular rRNA contents and growth rates, respectively (Malik *et al.*, 2008; Rastogi and Sani, 2011; Gosal and Mehta, 2015). Besides nuclei acid, soil microbial community can also be characterized by analysing other biomolecules such as lipids (Malik *et al.*, 2008; Rastogi and Sani, 2011; Gosal and Mehta, 2015). Different taxonomic group possesses its own signature fatty acids methyl ester (FAME) which once extracted, analysed by gas chromatography and compared with the known FAME in the database will identify the unique fatty acid and the corresponding microbial signature by multivariate statistical analysis (Rastogi and Sani, 2011; Gosal and Mehta, 2015).

2.4.2.2 Whole community analysis

On the other hand, the whole genetic information present in the total DNA extracted from environmental sample offer a more comprehensive view of genetic diversity than the libraries of PCR-amplified genes. DNA reassociation is one of the techniques that denatured and incubated extracted DNA under conditions that allow the DNA to hybridize and reassociate, which correlated with the diversity of soil microbial communities (Rastogi and Sani, 2011). Whole DNA extracted from environmental sample can also be analysed by density-gradient centrifugation based on guanine-plus-cytosine (G+C) content that differ in each prokaryotic group; and whole-microbial-genome sequencing that involves (i) extraction of DNA, (ii) fragmentation of genomic DNA randomly into small fragments of approximately 2 kb, (iii) ligation and cloning of DNA fragments into plasmid vectors and (iv) sequencing of DNA fragments in bidirectional to understand the microbial ecology and functional diversity (Rastogi and Sani, 2011).

2.4.2.3 Metagenomics

Metagenomics is an emerging research field that allow study of genomes recovered from environmental samples. Metagenomics are defined as the application of modern genomics techniques to study microbial communities directly in their natural environments, without the need for isolation and laboratory cultivation of individual species (Chen *et al.*, 2005). Previous study has reported that only 0.001-0.1% of the total microbes in the sea, 0.25% in freshwater, 0.25% in sediments and 0.3% in soils that could be cultivated in *vitro* (Ghazanfar *et al.*, 2010). Metagenomics analysis relies on the efficiency of the following steps: i) isolation of DNA from environmental sample, ii) library construction and iii) analysis of metagenomics libraries using sequencing technologies to understand the biological diversity present in the water, soil and other environmental samples (Ghazanfar *et*

al., 2010). Samples for metagenomics analysis could be retrieved from any environment, soil or habitat in which DNA are isolated using various kits that are commercially available for the isolation of genomic DNA. Soil samples are particularly a complex matrix containing diverse cell wall traits that have varying lysis method and substances such as humic acid that co-extracted during DNA isolation (Ghazanfar *et al.*, 2010). Genomic DNA could be extracted through two techniques: i) direct or *in situ*, in which cells are lysed in soil sample and directly recovered; ii) indirect, in which cells are removed first from the soil then lysed for DNA recovery (Handelsman, 2004; Gutiérrez-Lucas *et al.*, 2014). Library construction for most sequencing technologies require amplification of starting material targeting specific region of small subunit (SSU) of rRNA such as hypervariable (V) region of 16S and 18S rRNA gene, or internal transcribed spacer (ITS) as to produce high nanograms or micrograms of DNA. However, due to the need for PCR amplification, various problems associated with reagent contamination, formation of chimera and sequence bias depending on the amount and type of starting material and the required amplification cycle needed to produce sufficient amounts of nuclei acid for construction of metagenomics libraries could have significant impact on the microbial community analysis (Ghazanfar *et al.*, 2010). Upon construction of metagenomics libraries, genetic materials are analysed in terms of sequence-based and function-based analysis. Sequence-based metagenomics contribute to the determination of microbial distribution in community, linkage of traits, horizontal gene transfer and genomic organization, while function-based allow identification of new enzymes, novel antibiotics or, antibiotics resistance gene (Handelsman, 2004; Ghazanfar *et al.*, 2010).

2.5 Metagenomics using next generation sequencing (NGS) technologies for analysis of microbial diversity

Development of next-generation sequencing (NGS) technologies has allowed deeper analysis of environmental samples which is vital for presenting an unbiased

view of phylogenetic composition and microbial diversity with niche function. Sequencing technologies including Roche 454 and Illumina have shed a new light into discovery of soil microorganisms in complex environmental systems (Bartram *et al.*, 2011).

2.5.1 Sequencing technologies

Next-generation sequencing (NGS) technologies are capable of parallel sequencing millions of DNA molecules with varying sequence length and output yield at high accuracy and low sequencing cost. Roche 454 pyrosequencing is the first commercial NGS technologies platform that relies on (i) immobilization of DNA fragments on DNA-capture beads in water-oil emulsion; (ii) PCR amplification of the fixed fragments; (iii) addition of one-by-one nucleotide in which pyrophosphatase is transformed into luminous signal upon DNA polymerization reaction; and (iv) detection of luminous light by machine and translation of polymerized DNA into nucleotide sequences with respected base quality value (Escobar-Zepeda *et al.*, 2015; Oulas *et al.*, 2015). This technology takes only 10 hours for sequencing start till completion and has the longest read length but at high sequencing cost (Liu *et al.*, 2012; Scholz *et al.*, 2012).

Similar to Roche 454, Ion Torrent by PGM also relies on the emulsion PCR amplification of DNA fragments captured on beads (Escobar-Zepeda *et al.*, 2015). Ion torrent releases proton upon the addition of nucleotide to sequencing reaction that can be detected by the change in pH (Liu *et al.*, 2012; Escobar-Zepeda *et al.*, 2015). Ion torrent generate a maximum yield of approximately 500 million reads of 400 base pairs (bp) but at a much lower sequencing cost (Escobar-Zepeda *et al.*, 2015).

Illumina has become one of the most commercial sequencing platforms due to its low cost and high throughput yield (Liu *et al.*, 2012; Thomas *et al.*, 2012; Escobar-Zepeda *et al.*, 2015). These sequencing technologies immobilize random DNA fragments on flow cell followed by bridge amplification to form clusters of identical DNA fragments. Prior to sequencing, DNA fragments splices into single strands with the aid of linearization enzyme followed by addition of nucleotides labelled with different cleavable fluorescent dye and removable blocking group. Incorporation of labelled nucleotide at a time to DNA template cause fluorescent molecule to be excited by a laser which then produce signal that could be captured by a charge-coupled (CCD) device (Liu *et al.*, 2012; Escobar-Zepeda *et al.*, 2015). Illumina has a broad range of instruments that covers various applications from benchtop (i Seq, MiniSeq, MiSeq, NextSeq) to production-scale sequencing systems (NextSeq, HiSeq, HiSeq X, NovaSeq). MiSeq is very convenient for amplicon and bacterial sequencing, clone checking, ChIP-Seq and small genome sequencing that can complete smaller run in a day (approximately 8 hours) for a complete run (Liu *et al.*, 2012), while HiSeq is favourable for large whole genome sequencing (Ari and Arikan, 2016). MiSeq and HiSeq2000 differs in which MiSeq generate 1.5 Gb output per day from 5 million of 150 bp paired end reads, whereas HiSeq2000 produces more than 50 Gb output per day within 10.8-day run generating 1.6 million of 100 bp paired-end reads (Caporaso *et al.*, 2012). In comparison, although HiSeq is lower in costs and allows massively parallel sequencing, MiSeq facilitates smaller sequencing project and less run time which is convenient for research purposes. In addition, although MiSeq generate smaller data output than HiSeq, this platform is preferable for de novo sequencing of small genomes due to the ability of MiSeq to produce longer read length and better assemblies at low sequencing depth (Glenn, 2011; Clooney *et al.*, 2016).

In addition to Illumina, SoLiD sequencer by Applied Biosystem has been broadly used due to its high accuracy than any other sequencing technologies. This technology is based on the ligation sequencing in which DNA libraries can be sequenced by 8 base-probe ligation containing ligation site, cleavage site and 4 different fluorescent dyes that linked to the first base, fifth base and last base, respectively (Liu *et al.*, 2012). Upon polymerization of probe to DNA template,

fluorescent signal was recorded and faded through the cleavage of probes' last 3 bases. The sequence of the DNA fragment is then determined after 5 cycle of sequencing using ladder primer sets (Liu *et al.*, 2012). A complete run could take up to 7 days with a read length of 35 bp reads but short read length and expensive computational infrastructure limits the application of SoLiD sequencer for assembly of large contigs and direct annotation of unassembled reads (Liu *et al.*, 2012; Thomas *et al.*, 2012).

2.5.2 Reconstruction of soil microbial community from NGS data

The term 'sequence-' and 'functional-based analysis' has been used to describe the methodologies in metagenomics analysis in addition with the amplification of gene of interest (e.g. V region 16S or 18S rRNA gene, or ITS region) which has been also used to characterize the soil microbial community from environmental sample. The former 'sequence-based analysis' could be referred as 'marker gene metagenomics' while 'functional-based' could be referred as 'full shotgun metagenomics'. Marker gene metagenomics offers a fast way to acquire taxonomic diversity and community distribution profile through PCR amplification and sequencing of conserved marker genes of 16S or 18S rRNA gene fragments (Escobar-Zepeda *et al.*, 2015). Sequencing of 16S rRNA has been frequently used as a target marker gene for bacterial and archae, while marker genes such as internal transcribed spacer (ITS) and 18S rRNA gene fragment, respectively are favoured for the taxonomic and phylogenetic purposes of fungi and eukaryotes. Full shotgun metagenomics analysis on the other hand has served as a potential platform for sequencing of whole genome in the environmental sample to address what is present in the environment, their functional role and how these microorganisms interacts within the ecosystem to achieve balance ecological niche (Oulas *et al.*, 2015).

2.5.2.1 Marker gene metagenomics

In marker gene metagenomics, several terminologies need to be understood for efficient analysis of metagenomics data. These include: i) Amplicon: an amplified DNA fragment of one or more variable regions of 16S or 18S rRNA gene or ITS region which are generated using standard/universal PCR primer; ii) Operational Taxonomic Unit (OTU): a species distinction in microbiology, or taxonomic unit of a bacterial/eukaryotes genus or species based on the sequence similarity threshold; and iii) Barcode: a specific, short DNA sequence that is added to each read during PCR amplification (Oulas *et al.*, 2015).

Denoising step is an important, platform-specific step to (i) filter noisy reads based on the appearance of low signal intensities; (ii) removing sequencing noise; (iii) removing PCR noise and (iv) identification and removal of chimera for each sequence in which exact pairwise alignments are performed with equal or greater abundance with set of possible parents as reference (Oulas *et al.*, 2015). Using barcode, reads attached with specific DNA fragments are allowed to be mixed (multiplexed) to lower the sequencing cost (Oulas *et al.*, 2015). During analysis, the sequences are then separated (demultiplexed) followed by clustering of OTU, a species distinction typically rRNA and percentage of similarity threshold to classify microbes (Oulas *et al.*, 2015). For bacteria/archae, a minimum of 97% sequence similarity are needed for genus classification while 99% sequence similarity for species identification (Janda *et al.*, 2007). Picking of OTU are based on various approaches which includes (i) de novo approach which is applied when the amplicon overlap and no reference sequence collection is available; (ii) closed-reference, an approach that is used when reference sequence collection are available but amplicon do not overlap and (iii) open-reference approach which clusters reads against reference dataset (Oulas *et al.*, 2015). All of above are then incorporated into software for microbial communities' analysis such as QIIME. Taxonomic assignment of OTU can be conducted through various algorithms including BLAST, RDP classifier and others to search the closest match to an OTU from which the taxonomic lineage is conjecture (Oulas *et al.*, 2015). To search for the closest match,

a reference database of marker gene such as RDP (16S) and SILVA (16S and 18S) are essential. In addition, QIIME also supported the analysis of alpha- and beta-diversity which occur within and across samples, respectively, taxonomic composition and phylogenetic analyses (Oulas *et al.*, 2015).

2.5.2.1.1 Target marker genes

A pioneer study conducted by Carl Woese and colleagues focusing on the comparative analysis of SSU rRNAs (16S and 18S rRNA) has provided an unbiased framework for determining the evolutionary relationship between microorganisms as well as 'quantifying' the diversity based on the sequence divergence on a phylogenetic tree (Hugenholtz, 2002). SSU rRNA has served as a common or 'gold standard' for estimating the species richness, diversity, composition and community structure based on the sequences attained from sufficiently V region that were typically clustered into OTU at a few distance of rRNA gene sequences (Kim *et al.*, 2011b). Sequencing of 16S rRNA gene (~1500 bp) and 18S rRNA gene (~1800 bp) is usually performed in partial sequences of 700 bp or shorter due to constrain in service cost and sequencing technology. Less than 44% of bacterial and 15.3% of archael sequences in the RDP database are longer than 1200 bp demonstrates that most of the taxonomic classification was performed using partial 16S rRNA gene sequences (Kim *et al.*, 2011b). Likewise, fungal 18S rRNA gene reported 90% of the amplicons to be within the range of 530 to 700 bp in length and 80% of the amplicons to range between 600 to 650 bp in length (Hadziavdic *et al.*, 2014).

The structure and sequence of SSU rRNA gene (16S and 18S) has been characterized and reported to contains both highly conserved regions for primer design and nine-highly variable regions, V1 to V9 to identify the phylogenetic diversity of microorganisms (Hadziavdic *et al.*, 2014; Yang *et al.*, 2016). There have been distinct differences between the V region in eukaryotes and prokaryotes for

example, V6 regions that are more conserved and often avoided in the eukaryotes (Neefs *et al.*, 1993) while in prokaryotes, this region has been considered variable and suitable for prokaryotic studies (Chakravorty *et al.*, 2007). V4 region also demonstrated difference in terms of the sequence length in which this region appear to be the largest region in eukaryotes (Hugerth *et al.*, 2014) but shorter in prokaryotes. The conserved regions of SSU rRNA allow PCR amplification of target V regions using universal primers. Amplification of target V region generated partial sequence of 16S/18S rRNA gene that display abundant sequence diversity among different bacterial/ fungal species that are subsequently used for species identification (Chakravorty *et al.*, 2007). However, the use of partial sequences generated from 16S rRNA gene could lead to uncertainty in taxonomic classification due to i) the divergence of gene sequence that is not distributed evenly throughout 16S rRNA gene but concentrated predominantly in nine-highly variable regions (Stackebrandt *et al.*, 1994); ii) certain hypervariable regions that are more variable than others (Youssef *et al.*, 2009); and iii) certain region of 16S rRNA genes that produce more valid taxonomic classification than the others (Liu *et al.*, 2008). Likewise, adoption of sequencing technologies for 18S rRNA which are slower than prokaryotes 16S rRNA could also provide uncertainty in taxonomic classification due to i) the limited study on the most suitable variable region that provide suitable representation of diversity (Hugerth *et al.*, 2014); ii) the huge differences in variation among the target region of 18S rRNA gene (Wooley *et al.*, 2010; Hugerth *et al.*, 2014; Bradley *et al.*, 2016); and iii) the limited number of sequences entries in SILVA database (Hadziavdic *et al.*, 2014). In order to allow an accurate phylogenetic analysis and taxonomic classification from V regions of 16S/18S rRNA gene, various studies have been conducted to investigate the most efficient V regions that representing the full-length of 16S/18S rRNA sequences (Youssef *et al.*, 2009; Kumar *et al.*, 2011; Dunthorn *et al.*, 2012; Guo *et al.*, 2013; Mizrahi-Man *et al.*, 2013; Hadziavdic *et al.*, 2014; Hugerth *et al.*, 2014; Yang *et al.*, 2016).

Study conducted by Yousseff and colleagues has reported that V regions of **16S rRNA gene** sequences could be divided into groups of overestimates (V1+V2 and V6 gene sequence), underestimates (V3-, V7-, and V7+V8 gene sequence) and comparable estimates (V4-, V5- V5+V6, and V6+V7 gene sequences) of OTUs and

species richness in relative to the full-length V regions (Youssef *et al.*, 2009). Among the hypervariable regions, V4, V5 and V6 are shown to have the highest sensitivity and recommended to represent the optimal sub-regions for taxonomic arrangements. As for the V3 and V7, these regions underestimate the phylogenetic studies and display moderate sensitivity, while V1+V2 and V8 region have poor phylogenetic resolution at phylum level (Youssef *et al.*, 2009). Previous study has reported that V4-V6 regions represented the closest representation of the full-length 16S rRNA sequences in phylogenetic studies especially at phylum level, while V2 and V8 reported to be the least reliable region in phylogenetic analysis (Yang *et al.*, 2016). Studies conducted by Dunthorn and colleagues has also demonstrated similarity in species-richness estimates based on V4 region to those of full-length 16S rRNA gene sequences (Dunthorn *et al.*, 2012; Burke *et al.*, 2016). Study by Mizrahi-man et al. has recommended the amplification of V3 and/or V4 to identify the bacterial communities within environmental samples (Mizrahi-Man *et al.*, 2013).

For eukaryotes **18S rRNA**, each V region demonstrated specific characteristics that serve as a potential 'tools' for assessing fungal diversity. The details of each V region are summarized as the following: i) V1: short (100 bp) and highly conserved; ii) V2: long (150-200 bp) but neither are highly variable nor highly conserved for target primer sets; iii) V3: not very long and lacked in variability; iv) V4: longest region (350 to 450 bp) and have the highest length polymorphisms; v) V5: have short variable region; vi) V6: relatively conserved and excluded; vii) V7: have highly variable region of approximately 20-25 bp; viii) V8: long (over 150 bp) with variable and conserved region interspersed across the V8 and conserved segment towards the 3' end; ix) V9: high nucleotide variability (~60 bp) in the center of the region (Hadziavdic *et al.*, 2014). Among the nine-highly variable region of 18S rRNA gene, V4 and V9 region has demonstrated the highest variability for assessing biodiversity of environmental sample (Stoeck *et al.*, 2010; Behnke *et al.*, 2011; Pawlowski *et al.*, 2011). Pyrosequencing of V4 and V9 regions from the marine anoxic water has reported to identify 38,116 V4 and 15,156 V9 unique sequences that cover wide range of taxonomic groups although the groups are more dominant to dinoflagellates and close relatives (Stoeck *et al.*, 2010). Furthermore, a study conducted by Hugerth and colleagues also demonstrated that V4 region was

able to achieve accuracy level of 30% for species level and approximately 75% for genus level (Hugerth *et al.*, 2014), although the accuracy level at approximately 75% for genus-level taxonomic classification is lower than the analysis conducted on prokaryotes 16S rRNA (97%) (Drancourt *et al.*, 2000). The disparity of accuracy level observed in 18S rRNA is likely to be due to the inherent and distinct morphology of eukaryotes which tend to be more finely classified than the prokaryotes 16S rRNA (Hugerth *et al.*, 2014). Despite the disparity observed between 18S and 16S rRNA gene, the discovery of V4 and V9 region of 18S rRNA gene has provided an insight to sequencing technologies to study eukaryotic microorganisms in a complex environments.

In addition, **internal transcribed spacer (ITS)** region are also widely used as a marker for taxonomic classification and phylogenetic analysis particularly fungi. These regions are known as an 'unofficial fungal DNA marker' for characterization of fungal communities due to high degree of variability at species or within species level, conserved primer site and high copy number of rRNA gene (Reddy, 2005; Barseghyan *et al.*, 2012; Schoch *et al.*, 2012). The rRNA gene complex consists of a section that code for ribosomal RNA (rRNA) including 16-18S, 5.5S and 23-28S rRNA, internal transcribed spacer (ITS) and two external spacer sequences (5' and 3' ETS) (Iwen *et al.*, 2002; Korabecna, 2007). However, differences exists for eukaryotes and prokaryotes in terms of the ITS region in rRNA transcript in which main eukaryotic transcripts contain two ITS regions: ITS1 (between the 18S and 5.8S) and ITS2 (between the 5.8S and 28S), whereas in prokaryotes, there is only one ITS region (between the 3'end of 16S and 5' end of 23S, homology to eukaryotic 5.8S) (Woodhams *et al.*, 2007). Nowadays, ITS region has been widely sequenced for classification of fungi with approximately 172,000 of full-length ITS sequences are available in GenBank and more than 100,000 sequences been deposited in the International Nucleotide Sequence Database (INSD) (Schoch *et al.*, 2012; Benson *et al.*, 2013). About 56% of the sequences are related with Latin binominal constitute of approximately 15,500 species and 2,500 genera obtained from the scientific studies (Schoch *et al.*, 2012). The length of ITS region for fungi varies from 527 to 700 bp with general length of ITS1 and ITS2 regions differ from 216 bp to 223 bp, and from 205 bp to 227 bp, respectively (Hřibová *et al.*, 2011; Das *et al.*, 2015). Taxonomic

resolution at species level demonstrated by ITS1 has been reported to be more variable than ITS2 in most fungal lineage (Nilsson *et al.*, 2008; Mullineux *et al.*, 2009). Study by Nilsson et al. reported that from an evaluation of 4,185 ITS reads, 66% of the higher variability was recorded from ITS1, whereas 34% variability were from ITS2 region (Nilsson *et al.*, 2008; Blaalid *et al.*, 2013). Using BLAST, rates of identification in ITS1 was higher in Ascomycota with exception of Basidiomycota which showed similar percentage value for both ITS1 and ITS2 (Wang *et al.*, 2015). ITS1 was shown to be higher in diversity and sequence number than ITS2, but considering that two ITS subloci is depending on the fungal species and diversity, and do not evolve independently of each other (Nilsson *et al.*, 2008), the datasets of ITS1 and ITS2 was revealed to be complementary indicating that both ITS1 and ITS2 were suitable as DNA metabarcoding markers (Mello *et al.*, 2011). Nevertheless, amplification of ITS1 gene has reported higher efficiencies in species discrimination in all major group including families and genera of fungi (Wang *et al.*, 2015). To allow accurate and deep-sequencing for phylogenetic analysis and taxonomic arrangement of microbial community, it is important to select the most suitable target marker gene (e.g. 16S, 18S rRNA or ITS) without introducing bias into community profile.

2.5.2.1.2 Functional genes

Functional diversity comprises of various microbial activities in the environment is an important ecological key to sustainable production. Microbial functions including photosynthesis, methane oxidation, fixation of atmospheric nitrogen, denitrification and sulphur oxidation may be performed with the use of specific target genes and established primers (Imhoff, 2016). Target gene and its sequences allow identification of organisms that involved in biogeochemical function in a niche as well as phylogenetic interaction between the communities. Table 2.5 summarized several primers that has been reported for bacteria, archae and fungi for functional gene analyses in the environment (Gaby and Buckley, 2012; Rusch, 2013;

Levy-Booth *et al.*, 2014; Hannula and van Veen, 2016; Imhoff, 2016; Reali *et al.*, 2017). Primer set (pufL67F/pufM781R), (F-Start-fmo-modif/r-889-FMO) and (bchY-fwd/bchY-rev) has been reported to specifically analyze communities of anoxygenic phototrophic bacteria by targeting functional genes *pufLM*, *fmoA* and *bchlY* (Alexander *et al.*, 2002; Tank *et al.*, 2009; Thiel *et al.*, 2010; Müller *et al.*, 2015). The *pufLM* gene encodes for the photosynthetic reaction center type II structural protein of all member in Proteobacteria including purple sulphur bacteria, purple non-sulphur bacteria, aerobic phototrophic purple bacteria producing bacteriochlorophyll and photosynthetic structure, as well as phototrophic Chloroflexi (Tank *et al.*, 2009). The sequence of *fmoA* gene covers bacteriochlorophyll-a protein specific for the green sulphur bacteria and phototrophic Chloroacidobacteria (Alexander *et al.*, 2006), while *bchY* gene target the bacteriochlorophyll-containing anoxygenic phototrophic bacteria (Yutin *et al.*, 2009). Sulphur cycle is essential for the generation of energy in microorganisms through production and oxidation of adenosine-5’-phosphosulfate (APS) as intermediate and Sox-pathway (Imhoff, 2016). Key enzymes of the sulphate-reducing bacteria include APS reductase (AprA) and dissimilatory disulphide reductase (dsrAB) by phototrophic sulfur-oxidizing gamma- and deltaproteobacteria, as well as sulphate thioesterase (SoxB) which ubiquitous among members of sulfur-oxidizing chemo- and phototrophic bacteria (Imhoff, 2016). As for nitrogen cycle, the functional role of bacteria in ammonia oxidation is assessed through amplification of *amoA* gene that catalyze membrane-bound ammonia monooxygenase encoded by the *amoCAB* operon, while gene *nifH* encode for enzyme nitrogenase that involved in nitrogen fixation (Imhoff, 2016; Reali *et al.*, 2017). Besides, denitrification pathway that involved in nitrate reduction through the action of membrane-bound nitrate reductase (*narG*), periplasmic nitrate reductase (*napA*), nitrite reductase (*nirK*, *nirS*) and nitrous oxide reductase enzyme (*nosZ*) also involved in N cycling (Levy-Booth *et al.*, 2014; Imhoff, 2016). The community of methanotrophic that oxidize and assimilate methane has been reported through the amplification of *pmoA* gene that encode for methane monooxygenase (Imhoff, 2016). Likewise, amplification of archael genes in nitrogen metabolism and methane oxidation has also been reported using various primers set (Rusch, 2013).

Fungi contribute to ecosystem functioning through carbon sequestration and nutrient cycling. Several primers have been reported to identify genes that involved in the breakdown and conversion of lignin or in oxidation of aromatic derivatives (Kellner and Vandenbol, 2010; Hannula and van Veen, 2016). Fungi involve in the production of ligninolytic enzymes such as manganese peroxidase particularly in basidiomycetal fungi, laccases in asco-, basidiomycota and several bacteria, as well as cellobiose dehydrogenases which also vital for cellulose degradation in ascomycota (Hannula and van Veen, 2016). Oxalate decarboxylase enzyme amplified by primer set (OxDC_190F/ OxDC_340R) also involve in the lignin degradation through regulation of oxalic acid as chelator of manganese (III) ions, while heme-thiolate peroxidase involved in the breakdown of aromatic compounds including phenolics (Kellner and Vandenbol, 2010; Hannula and van Veen, 2016). Fungi serve as a primary decomposer for cellulose and hemicellulose through production of glycosyl hydrolases (GH) which also involve in the degradation of xylan backbone, mannan and hydrolizing of starch as well as xylan sidechains (arabinan or glucuronic acid) (Kellner and Vandenbol, 2010). Functional role of fungi in chitin degradation and mannose, glucose and xylose oxidation could be assessed through amplification of target gene GH8 and glucose oxidase by using primer set (GH18Fa,b/GH18R) and (Glu-Ox1F1/Glu-Ox1R1,2,3), respectively (Hannula and van Veen, 2016). In addition, fungi also contribute to N cycling through production of nitrate reductase gene that could be amplified by using the following primer sets: niaD01F, niaD04R, niaD15F, niaD12R and niaD13R (Hannula and van Veen, 2016).

Table 2.5: Primers for functional gene analyses in environment (Table caption continued on next page).

Function	Target gene	Primers	Sequences (5'-3')
Bacteria			
Photosynthesis	*pufLM*	pufL67F	TTCGACTTYTGGRTNGGNCC
		pufM781R	CCAKSGTCCAGCGCCAGAANA
	fmoA	F-Start-fmo-modif	ATT ATG GCT CTN TTC GGC
		r-889-FMO	CCGACCATNCCGTGRTG
	bchlY	bchY-fwd	CCNCARACNATGTGYCCNGCNTTYGG
		bchY-rev	GGRTCNRCNGGRAANATYTCNCCC
Sulphur oxidation	*soxB*	soxB432F	GAYGGNGGNGAYACNTGG
		soxB1446B	CATGTCNCCNCCRTGYTG
Sulphur oxidation Sulphur reduction	*aprA*	AprA-1-FW	TGGCAGATCATGATYMATGG
		AprA-5-rv	GCGCCAACYGGRCCTTA
	dsrAB	rDSR1Fa	AARGGNTAYTGGAARG
		rDSR1Fb	TTYGGNTAYTGGAARG
		rDSR1Fc	ATGGGNTAYTGGAARG
		rDSR4Ra	CCRAARCAIGCNCCRCA
		rDSR4Rb	GGRWARCAIGCNCCRCA

Table 2.5: (Table caption continued from previous page) Primers for functional gene analyses in environment

		amoA-1F	GGGGTTTCTACTGGTGGT
Ammonia oxidation	*amoA*	amoA-2R	CCCCTCKGSAAAGCCTTCTTC
		amoC58f	CTAYGACATGTCRCTGTGG
	amoCAB	amoB1179r	CCAAARCGRCTTTCCGG
		FGPH19	TAC GGC AAR GGT GGN ATH G
Nitrogen fixation		PolR	ATS GCC ATC ATY TCR CCG GA
	nifH	PolF	GAC GAT GTA GAT YTC CTG
		AQER	TGC GAY CCS AAR GCB GAC TC
		nirK1F	GGMATGGTKCCSTGGCA
		nirK5R	GCCTCGATCAGRTTRTGG
	nirK	FlaCu	ATCATGGT(C/G)CTGCCGCG
Nitrate reduction Denitrification		R3Cu	GCCTCGATCAG(A/G)TTGTGGTT
		Cunir3	CGTCTAYCAYTCCGCVCC
		Cunir4	GCCTCGATCAGRTTRTGG
	nirS	nirS1F	CCTAYTGGCCGCCRCART
		nirS6R	CGTTGAACTTRCCGGT
	nosZ	nosZ-F	CG(C/T)TGTTC(A/C)TCGACAGCCAG

Table 2.5: (Table caption continued from previous page) Primers for functional gene analyses in environment

		nosZ1622R	CGC(G/A)A(C/G)GGCAA(G/C)AAGGT(G/C)C
Nitrate reduction Denitrification	*narG*	narG-R	GAGTTGTACCAGTCRGCSGAYTCSG
		1960m2F	TAYGTSGGGCAGGARAAACTG
		2050m2R	CGTAGAAGAAGCTGGTGCTGTT
	napA	NapV16F	GCNCCNTGYMGNTTYTGYGG
		NapV17R	RTGYTGRTTRAANCCCATNGTCCA
Methane oxidation	*pmoAC*	pmoC617f	ACACCTTCTGGTTCATGG
		pmoA682r	GAASGCNGAGAAGAASGC
Archae			
Ammonia oxidation	*amoA*	Arch-amoAF	STAATGGTCTGGCTTAGACG
		Arch-amoAR	GCGGCCATCCATCTGTATGT
Nitrogen fixation	*nifH*	arc-Nif-f	TAYGGAAARGGNGGNATYGG
		arc-Nif-r	CCNCCRCAGACRACRTCNCC
	nirA	arc-NirA-F	AAYMTSCCNCGGAAGTKSAA
		arc-NirA-R	AGAACTCCBTRCCSGTRCAS
Nitrate reduction Denitrification	*nirB*	arc-NirB-F	ATGCTGAGCCATTAYATAGC
		arc-NirB-R	CCGTTGTACTCGGCRCAGTC

Table 2.5: (Table caption continued from previous page) Primers for functional gene analyses in environment

Methane oxidation	*mmoX*	886R	ACCCANGGCTCGACYTTGAA
Fungi			
Lignin oxidation and breakdown	Mn-Peroxidase	Mn-F1	GGIGGIGCIGAYGGITC
		Mn-R1	GGIGTIGARTCGAABGG
Phenolics and lignin oxidation	Laccase	Cu1Fmod1	ACGGTYCAYTGGCAYGG
		Cu2Rmod1	GRCTGTGGTACCAGAAIGTNC
Oxalic acid degradation	Oxalate decarboxylase	OxDC_190F	GGIGAYYTITGGTAYTTYCC
		OXDC_340R	GT IGG RTG CCA RTG IAR YTG
Lignin oxidation and cellobiose decomposition	Cellobiose dehydrogenase	CdbF1	YTBVTNGNNGCNTGGSCN
		CbhR1	WSNGGNATHGGNCCN
		CbhR2	GCNGGNTGYNTNYTBGGNGGN
Oxygenations	Hemethiolate peroxidase	APO_65F	AAY GCI ATG GCN AAY CAY GC
		APO_130R	GC RTC RTG YTG UAT NCC
Cellulose and xylan backbone degradation	GH3	Glc1_155F	GGIMGIAAYTGGGARGGNTT
		Glc1_235R	AYIGCRTCIGCRAANGGCCA
Mannan and cellulose degradation	GH5	GH5_130F	GIGTHTGGGGITTYAAYGA
		GH5_245R	GGYTCRTTISGIARYTCCCA

Table 2.5: (Table caption continued from previous page) Primers for functional gene analyses in environment

Cellulose degradation	GH6	GH6F	YURCCNGAYMGRGAYUGY
		GH6_400R	TCNCCNCCIGGYTTDACCCA
	GH7	fungcbhIF	ACCAAYTGCTAYACIRGYAA
		fungcbhIR	GCYTCCCAIATRTCCATC
	GH74	GH74_130F	TTYAARGTIGGIGGNAAYATG
		GH74_280R	CCRTCRTAIGGICCNGCNCC
Xylan backbone degradation	GH10	GH10_80F	CCIGARAAYWSIATGAARTGG
		GH10_190R	TARTCRTTIATRTAIARYTT
	GH11	GH11_80F	GGIAARGGNTGGAAYCCNGG
		GH11_200R	TAICCYTCIGTIGCDACDAT
Xylan sidechain (arabinan) degradation	GH51	GH51_280F	AGNTGGCARTGGAAYGCNAC
		GH51_350R	ATYTGRTCDATIGCYTGYTG
Xylan degradation	GH76	GH76F	GGNAAYGAYGAYCAR
		GH76R	GGNGGNNURMGRUGGCAR
Starch degradation	GH15	GH15F	GGMYTRGGNGARCCNAAR
		GH15R	GAYYTRTGGGARGAR
	GH31	GH31_350F	CAY CAR TGY MGI TGG GGN TA

Table 2.5: (Table caption continued from previous page) Primers for functional gene analyses in environment

Starch degradation	GH31	GH31_660R	TT RTC ICC NCC CCA RTG NCC
Chitin degradation	GH18	GH18Fa (Forward)	ATHGGNGGNTGGGGNGAY
		GH18Fb (Forward)	ATHGGNGGNTGGACNGGN
		GH18R	GAYNTNGAYTGGGARTAY
Mannose, glucose and xylose oxidation	Glucose oxidase	Glu-Ox1F1	GAYTAYRTWRTNRYNGGHGGNGGH
		Glu-Ox1R1	WCNGGNRTHGGN
		Glu-Ox1R2	GARGTNATHYTRGCNGCNGGN
		Glu-Ox1R3	GTNYTRGTNATHGAR
Nitrate reduction	Nitrate reductase	niaD01F	GTNTGYGCNGGNAA
		niaD04R	GTNGGRTGYTCRAA
		niaD15F	GGNAAYMGNMGNAARGARCARAA
		niaD12R	AACCANGGRTTRTTCATCATNCC
		niaD13R	GGTRGCGTTCCAGTACATRTC

2.5.2.2 Full shotgun metagenomics

The analysis of whole genomes presents within the sample allow selection of wide range of phylogenetic and ribosomal marker to assess taxonomic annotation as well as functional role of microbial community (Escobar-Zepeda *et al.*, 2015). The whole genomes are reconstructed through assembly of shorter reads into genomic *contigs* and orientation into scaffolds using either de novo assembly or reference-based assembly (Thomas *et al.*, 2012). De novo assembly generate genomic *contigs* using no prior reference and depending on sophisticated graph theory algorithm such as de-Bruijin graphs (Oulas *et al.*, 2015). Using de-Bruijin graph, the mixture of datasets are converted into k-mers, possible subsequence generated from the reads and assemble into *contigs* and scaffolds. These lead to a more effective grouping/assemble of *contigs* that distinguish each reads to different species. As for the reference-based assembly, the whole genome are constructed based on one or more reference genomes which serve as a ‘map’ to create *contigs* that represent the genomes or part of the genome to specific species or genus (Thomas *et al.*, 2012; Oulas *et al.*, 2015). The reads or contigs are then grouped (binning) into individual genomes and assign into specific species, subspecies or genus through compositional binning or similarity-/homology-based binning (Thomas *et al.*, 2012; Oulas *et al.*, 2015). Compositional binning sorted DNA sequences based on nucleotide composition, for example certain guanine and cytosine (GC) composition while similarity-/homology-based binning utilize alignment algorithm such as BLAST to obtain similarity information with reference database which then binned sequences into their assigned taxonomic information (Thomas *et al.*, 2012; Oulas *et al.*, 2015). Upon binning, annotation of metagenome has two general steps which are (i) identification of genes (feature prediction) and, (ii) functional annotation which is determined by homology-based search of gene of interest against reference database with known gene function (Thomas *et al.*, 2012; Oulas *et al.*, 2015).

2.5.3 Application of soil metagenomics in agriculture

Soil is the major reservoir of various microbial diversity that plays important role in the environment including transformation of carbon through decomposition of organic matter, cycling of nutrients, maintenance of soil structure and biological regulation of soil population that contributes to the yield and quality of agricultural products (Kibblewhite *et al.*, 2008; Ghazanfar *et al.*, 2010). Structural metagenomics approach allow identification of all microorganisms inhabiting soil environments to give more information on the structure, diversity, richness and dynamics of the microbial communities in response to spatiotemporal parameters (Myrold *et al.*, 2014; Esposito *et al.*, 2016). These enable deeper understanding on the relationship between individual components that form a community and decipher the biological or ecological functions among the microorganisms (Alves *et al.*, 2018). Metagenomics approach allow overall reconstruction of the community members and the metabolic network that are established within the whole microbiome. Soil metagenomics also discover alteration in the taxonomic composition and genetic redundancy upon environmental changes associated to soil and agricultural management (Esposito *et al.*, 2016). In a different manner, functional metagenomics approach determine functional genes of soil microbial community, such as genes involved in N cycling and connections between the community genes and functions (Jansson and Hofmockel, 2018). This knowledge is essential to understand the factors that affect the growth, activity and diversity of soil microbial community, their control by soil physiochemical properties and their interactions with other microorganisms in soil ecosystem.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

This chapter describes in detail the experimental works that were performed in this study. The purpose of this study is i) to evaluate the yield of loose-leaf lettuce under soil cooling; ii) to understand the effect of soil cooling on soil chemical properties, iii) to evaluate the abundance of soil microbial community in soil cooling and iv) to identify the bacteria and fungi species from soil of loose-leaf lettuce grown under soil cooling. Figure 3.1 showes the flow diagram of simplified methodology that was performed in this study. Soil was sampled from temperate agricultural site in MARDI, Cameron Highlands for soil chemical and sequencing analysis. The soil from MARDI were also transferred to soil cooling system to observe the changes of soil nutrients and soil microbial community during the growth of lettuce. The lettuce was grown for 3-growth cycle and evaluated in terms of growth performance, analysis of soil chemical properties and identification of soil microbial community.

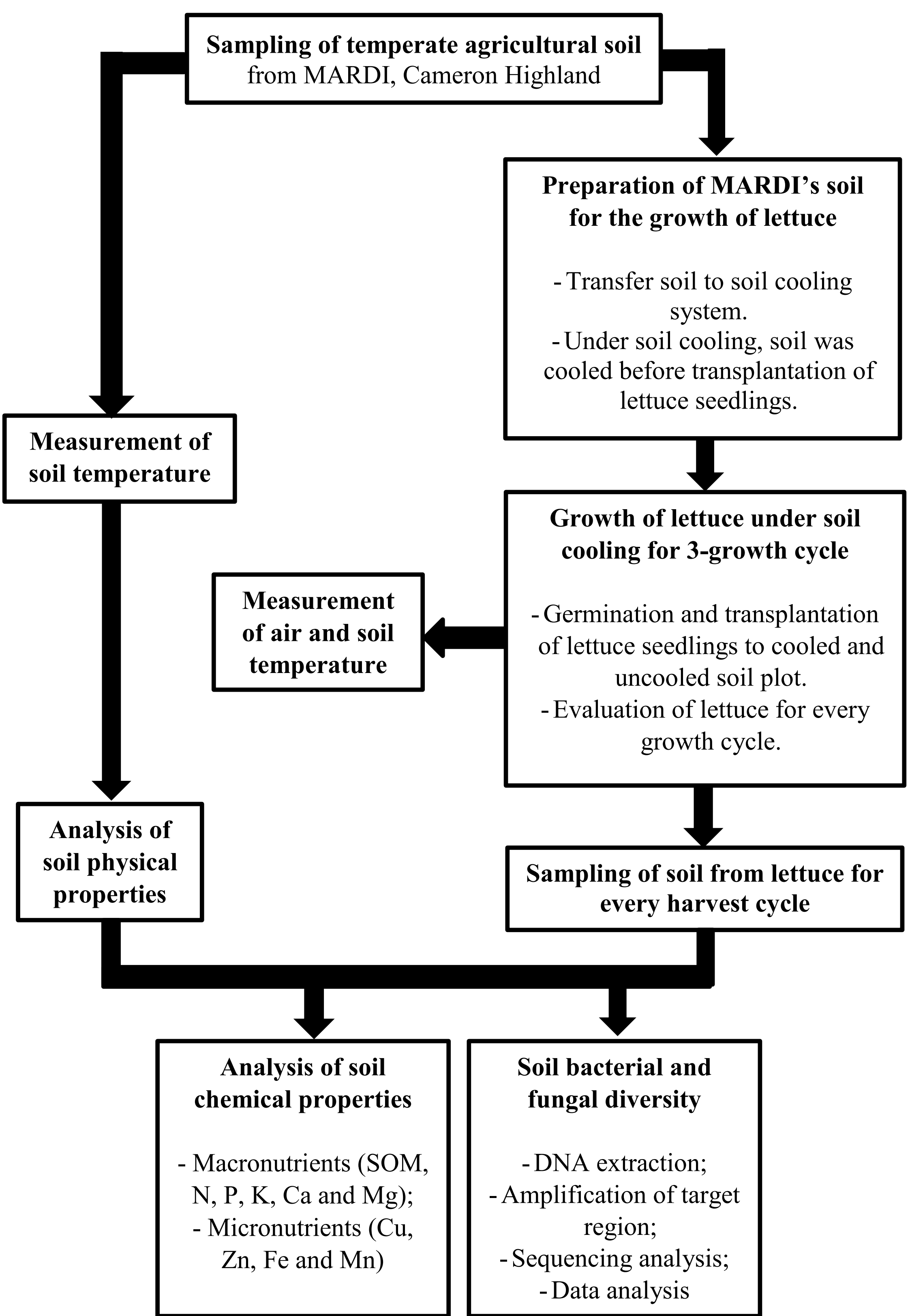


Figure 3.1 Flow diagram describing experimental framework.

3.2 Soil sampling in MARDI, Cameron Highlands

Temperate agricultural soils were collected on 24th March 2016 at MARDI agricultural site (Fig. 3.2) with geographical coordinates of 4°28' N, 101°23' E, and situated 1 km away from Tanah Rata town. The agricultural site was located next to the observation tower (Fig. 3.3a) and currently inactive from agricultural activities (Fig. 3.3b).

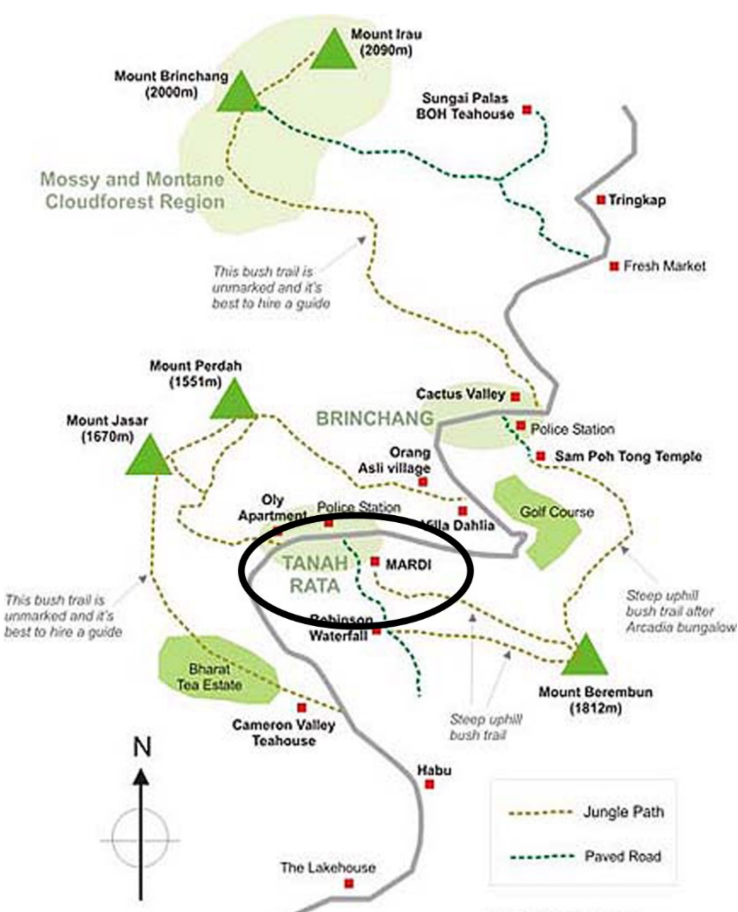


Image was retrived from http://cameronservice.blogspot.my/2015/03/19-jungle-trekking-walk-into-rainforest.html

Figure 3.2 Soil sampling was conducted at MARDI, Cameron Highlands situated 1 km away from Tanah Rata.

Image was retrived from http://agromedia.mardi.gov.my/tourismch2006/map.htm

Figure 3.3 Soil sampling at MARDI agricultural sites. Sampling was conducted at sites located next to the observation tower marked as ‘X’ (a), and were currently inactive for agricultural activities (b).

Sampling was conducted at three sampling points using soil probe at the depth of 20 cm, kept in a zip lock bag and stored in a cool box upon transferring to laboratory (Appendix A). Soil sampling was conducted at A horizon or topsoil that is rich in organic matters and minerals. This horizon is the layer where plant roots, bacteria, fungi and small organisms are primarily abundant. Most of the root biomass is located in 0-20 cm soil depth that associated with high organic matter, nutrients, aeration and water availability in top soil layer as compared to lower soil depth (Fageria and Moreira, 2011). The soil samples were then sieved using a 0.35 mm sieve to remove rocks and large organic debris or directly stored at 4°C prior to soil chemical analysis and genomic extraction. The physical properties of the soil was analysed (Appendix B) and showed in Appendix C.

Soils of MARDI in Cameron Highlands were also collected for soil cooling system (Appendix D). The soils were kept in collection bags and stored in cool box during transferring to 'OTEC' greenhouse at OTEC Centre, Universiti Teknologi Malaysia, Kuala Lumpur (UTM, KL). The collected soils were then transferred to cooled and uncooled soil plot on the same day it was collected to preserve the condition of the soil. The soils were selected from agricultural site that has been used for the cultivation of temperate vegetables including green mustard, tomato and cabbage. The soils from MARDI serve as a representative soil of temperate agricultural activities in Malaysia and reservoir of soil microorganisms for the growth of loose-leaf lettuce under the soil cooling. The soil bacteria and fungi in these soils were used as a reference microbial community in which shift in the community provide understanding on the need of soil cooling for the growth of lettuce on lowland of Malaysia. The soils were transferred to soil cooling system to observe the changes of soil microbial community during the growth of lettuce.

3.3 Preparation of soil for the growth of loose-leaf lettuce

Temperate agricultural soils from MARDI, Cameron Highlands were cooled using soil cooling system for the growth of lettuce is described as below:

3.3.1 Cooled soils

Lettuces were grown under soil cooling system established at greenhouse of Ocean Thermal Energy Conversion (OTEC) Centre, UTM KL as illustrated in Figure 3.4. The 'OTEC' greenhouse consists of a chiller (DC-300, D-D The Aquarium Solutions), custom-made chilled water storage tank, exhaust fan, circulating and embedded pipes, and soil plots. The function of equipment in soil cooling system is described in Appendix C. The soil plot with a dimension of 3 m x 2 m x 0.35 m consist of cooled and uncooled soil plots. Cooled and uncooled soil plots were filled with agricultural soils collected from MARDI, Cameron Highlands, respectively. Each cooled soil plot was installed with pipes embedded within the soils.

The details of the system were as follows. The water chiller was set to 4°C to emulate the temperature of cold deep seawater and connected to chilled water storage tank through circulating pipes. Tap water in the storage tank was cooled down by cold water from the chiller. The chilled water was pumped from the storage tank to pipes embedded within cooled soil plot to cool the soils. The water then flows back to storage tank to be cooled by cold water that is continuously pumped out from the chiller. The cold water recirculates within the system to keep soils in cooled soil plot cool for the growing of temperate crops. The temperature of the soils throughout this experiment was measured using soil thermometer during the growth of the lettuce.

3.3.2 Uncooled soils

Uncooled soil plot as shown in Figure 3.4 was located next to the cooled soil plot with no chilled embedded pipes. Under uncooled condition, soils were exposed to natural sunlight radiation for the growth of the lettuce and measurement of soil temperature.

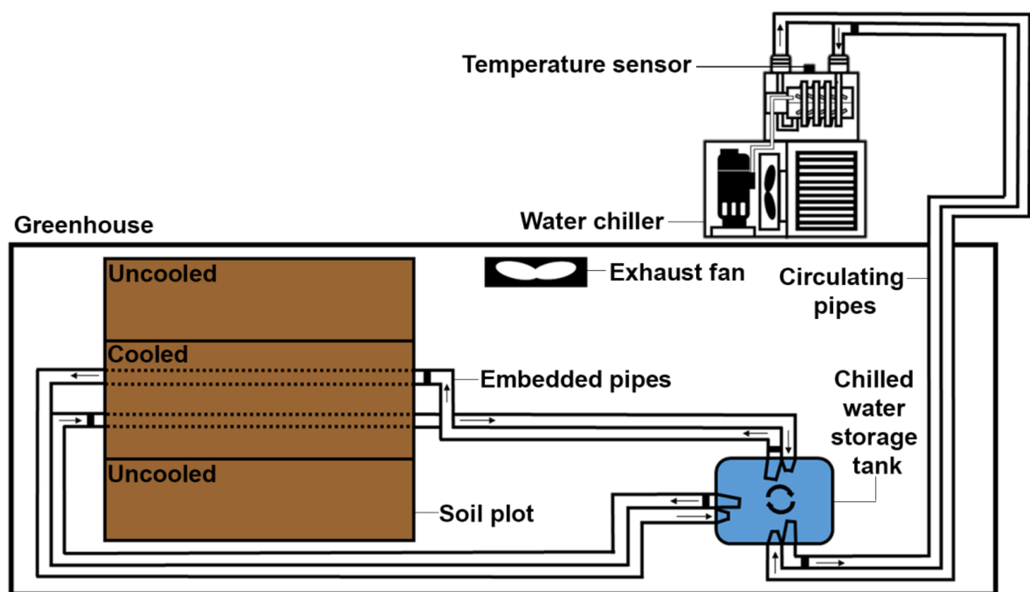


Figure 3.4 Soil cooling system for growing loose leaf lettuce in this study.

3.4 Growth of loose leaf lettuce for 3-growth cycle

Loose leaf lettuce seeds (633 Green Parade, Leckat) were germinated indoor at a temperature between 21°C and 24°C until sprouting of seeds was observed. Upon sprouting, seedlings were exposed to natural sunlight to prevent leggy seedlings for about 7 to 14 days as shown in Figure 3.5a or until the seedlings were with or more than four true leaves (Figure 3.5b) (Chamangasht *et al.*, 2012; Moreira

et al., 2014). The seedlings were then transplanted to cooled and uncooled soil plot with approximately 15 cm spacing between each lettuces (Figure 3.5c,d). For cooled soil plot, lettuce seedlings were transplanted next to the embedded pipes as shown in Figure 3.5d.

Figure 3.5 Germination of lettuce seedlings for 7 to 14 days (a) or until seedlings with four or more true leaves (b). Transplantation of the lettuce on uncooled (a) and cooled (b) soil plot.

Lettuces were grown consecutively for 3-growth cycle in which each cycle was a 7-weeks or 50-days growing period starting from transplanting of lettuce seedlings to harvesting of matured lettuce. Seedlings were transplanted and harvested upon reach maturity at the 1st-growth cycle, and continued to be transplanted and harvested until the 2nd- and 3rd-growth cycle, respectively. Seedlings for the 2nd-growth cycle were transplanted on the same growing point where harvested lettuce from the 1st-growth cycle was grown and so on. The timeline of the lettuce growth cycle was illustrated as in Figure 3.6. For every growth cycle, two batches of lettuces were germinated and transplanted on cooled and uncooled soil plot, respectively as shown in Figure 3.7. Each batch consisted of 10 lettuce seedlings (n=10) and was transplanted alternately one week with another for each growth cycle as summarized in Table 3.1. Lettuces were harvested after 50 days of growing for each growth cycle. Throughout the lettuce growth, no additional fertilizers were added to the soils. Lettuces were watered every day in the morning and late evening. The soil was watered at the top 6 inch with each watering session to ensure adequate moisture.

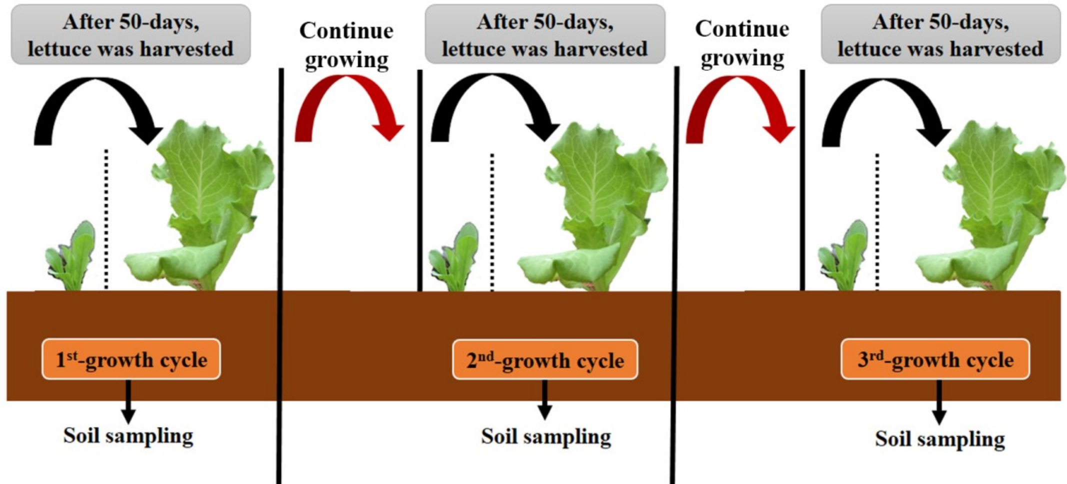
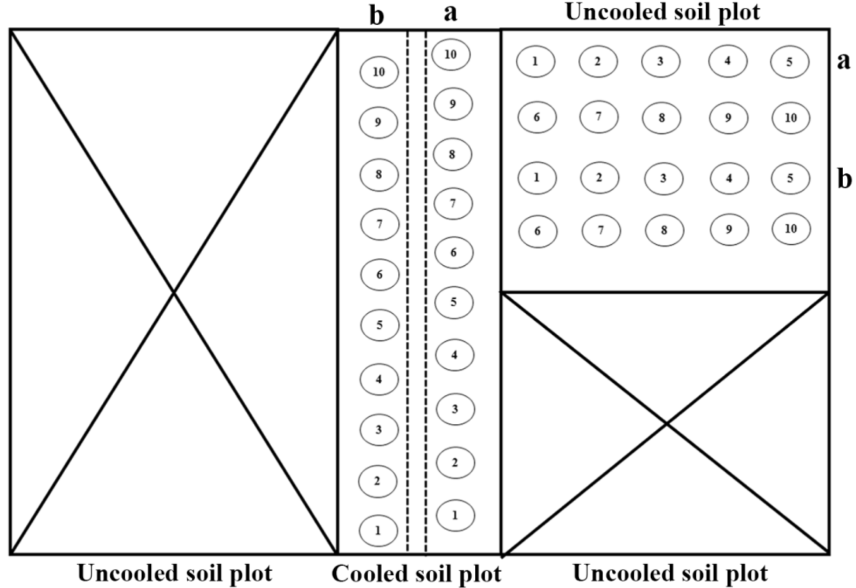


Figure 3.6 Timeline of the lettuce growth.



Note that ‘a’ refer to 1st, 3rd, 5th batch; ‘b’ refer to 2nd, 4th, 6th batch and ‘X’ refer to unused soil plot

Figure 3.7 Illustration of lettuce grown on cooled and uncooled soil plot.

Table 3.1: Details for germination and transplantation of loose leaf lettuces.

Growth cycle	1st growth		2nd growth		3rd growth	
Batch	**1st**	**2nd**	**3rd**	**4th**	**5th**	**6th**
Date of seed sowing	28-March	4-April	18-May	25-May	1-July	11-July
Date of seed sprouting	31-March	7-April	21-May	28-May	4-July	14-July
Date of transplant	17-April	24-April	5-Jun	2-Jun	18-July	25-July
Date of harvest	6-Jun	13-Jun	17-Jun	24-July	29-August	5-September

3.4.1 Measurement of air and soil temperature

During growth session, soil temperature at each growing point was recorded using soil thermometer at the depth of 20 cm (Moreira *et al.*, 2014), while air temperature inside the greenhouse is recorded at 10 cm from the soil surface using air thermometer for 24 hours. The reading was averaged and represented in mean±standard deviation (SD).

3.4.2 Sampling of soils of harvested lettuces

Prior to growth assessment, cooled and uncooled soils from representative matured lettuce were sampled using a sterilized hand shovel. Hand shovel was used to carefully lift the matured lettuce and surrounding soils without damaging the roots. Soil surrounding the lettuce roots were sampled and stored at 4°C for further analysis. Cooled and uncooled soils from each harvest cycle were analysed in terms of soil chemical properties, while only soil of the 1st- and 3rd harvest was further sequenced for identification of soil bacterial and fungal community. As each growth cycle only accounted for 50-days to reach maturity, the second cycle was omitted for sequencing analysis to observe the overall shift of soil microbial community from the 1st to 3rd growth under the soil cooling.

3.4.3 Evaluation of growth

Upon harvest of each growth-cycle, the matured lettuces were photographed and evaluated on the same day it was harvested in terms of weight, height, number

and area of leaves and root length (Moreira *et al.*, 2014). The weight of lettuces (n=3) in triplicate reading were averaged and expressed in mean±standard deviation (SD). Lettuce height was measured as a vertical distance between the soil surface and the highest part of the lettuce (Ogbodo *et al.*, 2010). Number of leaves was recorded by counting all the visible leaf on lettuce including the tips of new emerging leaves. Leaf area was measured using Image J (NIH, USA) according to the previously published literature (O'neal *et al.*, 2002). Length of root was measured by using a measuring tape to the nearest centimetre (cm) in triplicate readings and averaged for every harvest cycle (Norris, 2005). Statistical difference was performed by independent samples t-test in SPSS Statistics software (Version 21) at $p = 0.05$ as statistically significant.

3.4.4 Analysis of soil chemical properties

Chemical properties of the soils were determined in terms of pH, soil organic matter (SOM) content, nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), copper (Cu), zinc (Zn), iron (Fe) and manganese (Mn). To determine the soil pH, 10 g of sample was weighed in a 50 ml beaker, added with 20 ml of distilled water and stir to mix the sample. The suspension was stand for 1 hour prior to reading with pH meter (Leye Samuel and Omotayo Ebenezer, 2014).

Soil organic matter (SOM) content was determined using loss on ignition method based on the Malaysian Standard (MS 417: Part 8: 1917). The sample was oven-dried at 105°C overnight, cooled in a desiccator, and weighed. The crucible and its content were then combusted in a muffle furnace at 500°C to 550°C. These temperatures were maintained for at least 1 hour. After 1 hour, the crucible was cooled in a desiccator and the residue was weighed to calculate the percentage of soil organic matter (Wright *et al.*, 2008).

$$\text{Organic matter (\%)} = \frac{W_2\,(W_3 - W_1)}{\text{Mass, g}} \times 100\ ; \quad \begin{aligned} W_1 &= \text{Crucible} \\ W_2 &= \text{Sample} \\ W_3 &= \text{Crucible + Sample (dried at 550°C)} \end{aligned}$$

*Conversion factor: 1% = 10,000 mg/kg

The percentage of nitrogen (N) in soil sample was calculated using Kjeldahl method following the Malaysian Standard (MS 417: Part 3: 1994). Kjeldahl method consist of 3 steps which was carried out in the following order: (i) 0.25 g of soil samples were digested with a mixture of sodium sulphate (R & M Chemical, Malaysia), copper (II) sulphate (R & M Chemical, Malaysia), concentrated hydrochloric acid (R & M Chemical, Malaysia), concentrated sulphuric acid (Sigma Aldrich, Malaysia) and boiling chips (Sigma Aldrich, Malaysia) which aid in the conversion of organic compound to ammonium sulphate for 1 hour of digestion; (ii) digested reaction mixture was then transferred to distillation flask with 25 ml of sodium hydroxide (R & M Chemical, Malaysia) and heated to boil off ammonia gas from the digest; iii) ammonia gas produced was collected in a 50 ml of hydrochloric acid which was then titrated against sodium hydroxide (R & M Chemical, Malaysia) to determine the amount of ammonia produced, indicated by the colour change from grey-violet to green using screened methyl orange (Bremner, 1965).

$$\text{Nitrogen (\%)} = \frac{\text{(Sample titration-Blank)} \times \text{Normality} \times 14 \times \text{Dilution}}{\text{Weight, g}}$$

*Conversion factor: 1% = 10,000 mg/kg

The concentration of phosphorus (P) in soil samples was determined in accordance with the standard protocols of American Public Health Association (APHA) (APHA *et al.*, 1992). Soil sample (0.25 g) was added to 100 ml of distilled water and digested with 10 ml of concentrated hydrochloric acid (R & M Chemical, Malaysia) and 5 ml of nitric acid (R & M Chemical, Malaysia). The mixture was then boiled for 10 minutes, cooled, transferred to 250 ml volumetric flask and mark up with distilled water. The mixture was mix and filtered into 50 ml volumetric flask prior to addition of 10 ml of vanadate-molybdate reagent (Merck Millipore,

Malaysia). The blue solution was mark up with distilled water, mixed and read with ultraviolet (UV) spectrophotometer at the wavelength of 470 nm. The reading from UV spectrophotometer was referred to standard P curve (Appendix E) to determine the concentration of P in soil sample.

$$\text{Phopshorus (\%)} = \frac{\text{Concentration, mg/L x Dilution x Factor (0.229)}}{\text{Weight of sample, g}}$$

*Conversion factor: 1% = 10,000 mg/kg

The available potassium (K) that was present in soil sample was determined according to the Malaysian Standard (MS 678: Part VI to IX: 1980). Soil sample of 2 g that has been air dried and sieved was added with 20 ml of ammonium acetate (R & M Chemical, Malaysia) for 5 minutes, filtered into conical flask and analysed using atomic absorption spectrophotometer (AAS) at the wavelength of 766.5 nm. Reading from AAS was referred to standard K curve (Appendix E) which was proportional to the concentration of K present in the soil sample.

$$\text{Potassium (\%)} = \frac{\text{Concentration, mg/L x Dilution x Factor (0.1205)}}{\text{Weight of sample, g}}$$

*Conversion factor: 1% = 10,000 mg/kg

In reference to the standard method of ammonium acetate extraction, the concentration of calcium (Ca) and magnesium (Mg) was determined by adding 30 ml of ammonium acetate (R & M Chemical, Malaysia) to 4 g of soils followed by shaking for 5 minutes and filtered (Nathan *et al.*, 2006). 20 ml of the filtrate was pipetted out and transferred into conical flask prior to the addition of 2 ml of ammonium chloride-ammonium hydroxide buffer (Sigma Aldrich, Malaysia) and 2 drops of eriochrome black (EBT) (R & M Chemical, Malaysia). The mixture was then titrated with standard ethylenediaminetetraacetic acid (EDTA) (R & M Chemical, Malaysia) to permanent blue colour indicated the presence of Ca and Mg

together. The presence of Ca alone was then measured by pipetting 20 ml of the filtrate, added with 2 ml of sodium hydroxide (R & M Chemical, Malaysia) and 2 drops of EBT. The mixture was titrated with EDTA until the colour is changed from red to purple. The concentration of Mg (mg/kg) was calculated by subtracting the value of Mg and Ca together with the value of Ca alone (mg/kg).

- Calcium (mg/L) = $\frac{\text{(Sample – Blank) x standard } CaCO_3 \text{ x 1000}}{\text{sample volume, mL}}$

  $$\text{mg/kg} = \frac{\text{concentration. mg/L x markup volume, L}}{\text{weight, kg}}$$

- Magnesium (mg/kg) = Total hardness – calcium (mg/kg)

Micronutrients including copper (Cu), zinc (Zn), iron (Fe) and manganese (Mn) were analysed according to the standard protocols of American Public Health Association (APHA) (APHA *et al.*, 1992). Soils were digested with 18 mL of concentrated hydrochloric acid (R & M Chemical, Malaysia) and 6 mL of concentrated nitrc acid (R & M Chemical, Malaysia). The mixture was boiled until remaining with 5 mL solution, cooled and repeated with the addition of 18 mL of concentrated hydrochloric acid and 6 mL of concentrated nitric acid. The mixture was then filtered, markup to 50 mL of distilled water and analysed using atomic absorption spectrophotometer (AAS) at 324.7 nm for Cu, 213.9 nm for Zn, 248.3 nm for Fe and 279.5 nm for Mn. Reading from AAS was referred to standard curve (Appendix E) for the concentration of micronutrients present in soil sample. Moisture content was determined by oven-drying soil sample to constant mass at 105°C for 24 hours. The moisture content (%) was calculated from the sample weight before and after drying (O'Kelly, 2005).

$$\text{Micronutrients(mg/kg wet)} = \frac{\text{concentration, mg/L x DF x markup volume, L}}{\text{weight of sample, kg}}$$

$$\text{mg/kg dry} = \frac{\text{mg/kg wet}}{\text{(100 – moisture content, \%)}} \text{ x 100}$$

3.5 Identification and characterization of soil microbial and fungal community

In order to identify soil microbial community from soil of lettuce grown under soil cooling, genomic DNA was extracted and send to BGI Co., Ltd, Hong Kong for amplicon sequencing and bioinformatics analysis.

3.5.1 Extraction of soil genomic DNA

Genomic DNA was extracted from soil samples using Ezup Column Soil DNA Purification kit (Bio Basic Inc., USA) following the manufacturer's standard protocol. Briefly, 0.3 g of soil samples were added to 500 µl of Buffer SCL, vortex vigorously for 6 minutes and incubated for 5 minutes at 65°C. The mixture was then centrifuge at 13,000 x *g* for 3 minutes at room temperature. An equal volume of Buffer SP was added to the supernatant, mix thoroughly by inverting and kept on ice for 10 minutes prior to centrifugation. Next, add 200 µl of chloroform to the supernatant and mix through vortexing. Upon centrifugation, 1500 µl of Buffer SB was added, mix and transfer to spin column followed by spining at 12,000 x *g* for 30 second. Wash solution was added at 700 µl to the spin column and repeat spin twice to remove residual ethanol. Spin column was then transferred to new centrifuge tube, added with 50 µl to 100 µl of TE Buffer and incubated for 2 minutes at room temperature. The tubes were centrifuged at 13,000 x *g* for 1 minute to spin-off the genomic DNA followed by storage at -20°C for further analysis, or direct observation by gel electrophoresis and quantification by Qubit Fluorometer to verify the presence of genomic DNA. The presence of extracted DNA was verified on 0.8% agarose gel at 100 V for 30 minutes and stained with Diamond™ Nucleic Acid Dye (Promega, USA). The gel was then visualized under UV using Vilber Lourmat Quantum ST5 Gel Documentation System (Fisher Scientific, USA) as shown in Figure 3.8. DNA concentration was quantified using Qubit® 3.0 Fluorometer

(Thermo Fisher Scientific Inc., CA, USA) according to the manufacturer's standard protocol (Table 3.2). Briefly, prepare Qubit® working solution by diluting 1 µl Qubit® dye in 199 µl Qubit® buffer, and vortex. Then add 190 µl working solution to 10 µl Qubit® standard to appropriate tube, and mix by vortexing. Add sample at ranges between 1 µl to 20 µl to corresponding volume of Qubit® working solution from 180 to 199 µl so that thefinal volume in each tube is 200 µl, then mix by vortexing. Incubate all tubes at room temperature for 2 minutes followed by reading of the standards and samples.

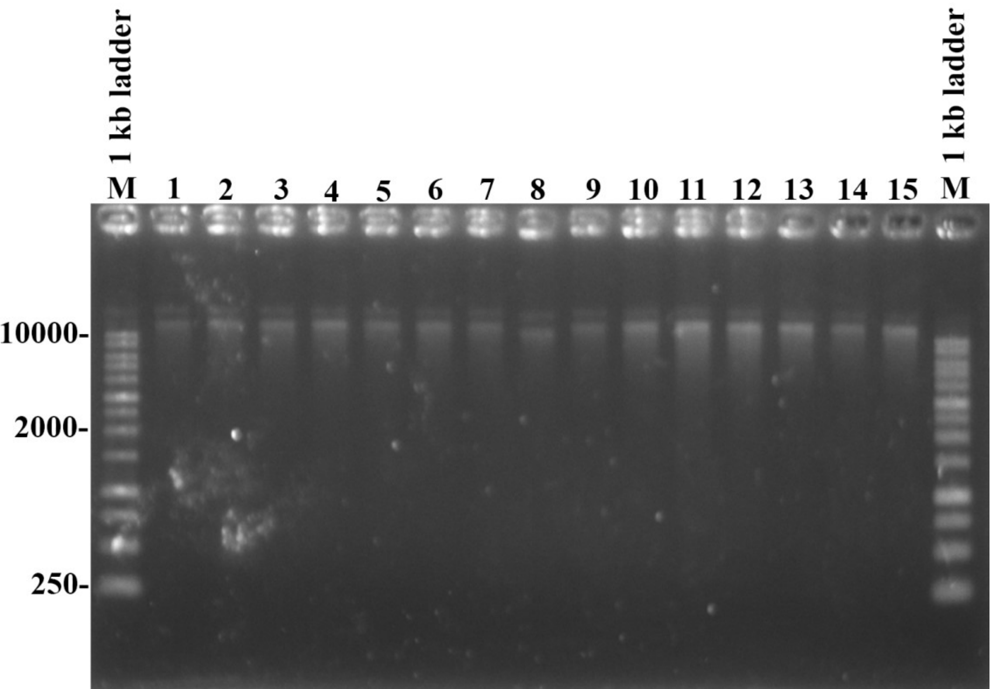


Figure 3.8 Gel electrophoresis of soil genomic DNA in triplicate. Lane 1-3 refer to soils before growth (BG); Lane 4-6 refer to 1st harvest: cooled soil (1-SC); Lane 7-9 refer to 1st harvest: uncooled soil (1-Co); Lane 10-12 refer to 3rd harvest: cooled soil (3-SC) and Lane 13-15 refer to 3rd harvest: uncooled soil (3-Co).

Table 3.2 Concentration of genomic DNA in triplicate.

Sample	Concentration (ng/µl)	Sample	Concentration (ng/µl)	Sample	Concentration (ng/µl)
BG-1	0.26	1-SC-1	0.486	3-SC-1	0.876
BG-2	0.28	1-SC-2	0.722	3-SC-2	0.14
BG-3	0.24	1-SC-3	0.288	3-SC-3	0.746
		1-Co-1	0.344	3-Co-1	1.02
		1-Co-2	0.246	3-Co-2	0.68
		1-Co-3	0.38	3-Co-3	0.275

BG: Before Growth; **1-SC**: 1st harvest-cooled soil; **1-Co**: 1st harvest-uncooled soil; **3-SC**: 3rd harvest-cooled soil; **3-Co**: 3rd harvest-uncooled soil.

3.5.2 Ilumina library preparation and amplicon sequencing

Genomic DNA was sent to BGI Co., Ltd, Hong Kong for amplicon sequencing. Upon DNA arrival, the sample was quantified to ensure sufficient DNA quantity and quality for sequencing analysis. High-quality genomic DNA was then used for the preparation of 16S and ITS library according to Illumina standard protocol. The hypervariable, V4 region of bacterial 16S rRNA and fungal ITS-1 genes were amplified using composite primer specifics to each domain (Table 3.3). A reaction mixture of 2.5 µl microbial DNA (5 ng/µl), 5 µl of forward primer (1µM), 5 µl of reverse primer (1µM) and 12.5 µl of 2x KAPA HiFi HotStart Ready Mix (Kapa Biosystems, USA) were used in PCR with the following protocol: 95°C for 3 minutes, 25 cycles of: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes. The amplicon PCR was then purified from free primers and primer dimer by adding 20 µl of AMPure XP beads (Beckman Coulter, USA). The beads and PCR product was gently mixed by pipetting up and down for at least 10 times until the beads are evenly dispersed prior to incubation at room temperature for 5 minutes. The mixture was placed on magnetic stand for 2 minutes or until the supernatant has cleared. The supernatant was then carefully removed

without disturbing the pellet. The beads were washed with 200 µl of 80% freshly prepared ethanol, incubate for 30 seconds and the supernatant was carefully discarded without removing plate from magnetic stand. The second ethanol wash was repeated and the supernatant was pipetted off as much as possible to remove the excess ethanol. Without removing the plate from magnetic stand, air-dry the beads for 5 minutes. The plate was then removed from the magnet and the beads were added with 51 µl of 10 mM Tris (pH 8.5), mixed by pipetting and incubated at room temperature for 2 minutes. The plate was placed on magnetic stand for 2 minutes or until the supernatant has cleared. Fifty (50) µl of the supernatant was transferred to new plate and stored at -20°C or proceeded to Index PCR to attach the dual indices and sequencing adapters by using Nextera XT Index Kit (Illumina, USA).

Table 3.3: Primers used in this study.

Primer	Sequence (5'-3')"	Reference	Targeted region
515F	GTGCCAGCMGCCGCGGTAA	Caporaso et al. (Caporaso *et al.*, 2011)	Bacterial V4
806R	GGACTACHVGGGTWTCTAAT	Caporaso et al. (Caporaso *et al.*, 2011)	
ITS-F	TTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (Gardes and Bruns, 1993)	Fungal ITS-1
ITS-R	GCTGCGTTCTTCATCGATGC	White et al. (White *et al.*, 1990)	

For index PCR, 5 µl of the resuspended purified PCR product was transferred to new 96-well plate and placed in TruSeq Index Plate Fixture. The following reaction mixture: 5 µl of Nextera XT Index Primer 1, 5 µl of Nextera XT Index Primer 2, 25 µl of 2x KAPA HiFi Hotstart Ready Mix and 10 µl of PCR grade water was mixed, pipetted up and down, and covered with microseal. PCR was performed with the following protocol: 95°C for 3 minutes, 8 cycles of: 95°C for 30

seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 30 seconds. The PCR products were then purified using 56 µl of AMPure XP beads similar to previous purification protocols. Upon air dried, the beads were eluted with 26 µl of Tris buffer, mixed by pipetting followed by incubation at room temperature for 5 minutes. The well plate was placed on magnetic stand and 25 µl of the supernatant was transferred to a new well plate. The size of final library was verified using Bioanalyzer DNA 1000 chip. Amplicon sequencing was then conducted using Illumina® MiSeq platform (Illumina Inc. San Diego, CA) at BGI Co., Ltd (Hong Kong). In preparation for cluster generation and sequencing, libraries were quantified using Qubit assay and diluted to 4 nM. Five (5) µl of diluted DNA were aliquoted from each libraries and mixed. Pooled libraries were then denatured with 5 µl of 0.2 N NaOH, diluted with hybridization buffer to 4 ρM and added with PhiX control (4 ρM). For each sequencing run, a minimum of 5% PhiX was required as an internal control to increase the diversity of low nucleotide pool and to assess sequencing error rates. The mixture of amplicon libraries and PhiX control were heat denatured prior loading onto MiSeq v3 reagent catridge and onto the MiSeq system for automated cluster generation and paired-end sequencing. Subsequently, result from sequencing analysis was deposited in NCBIs – Sequence Read Archieve (SRA) database (https://submit.ncbi.nlm.nih.gov/) with temporary submission ID (SUB3258057) (Appendix F).

3.5.3 Bioinformatics analysis

3.5.3.1 DNA sequence analysis

Raw sequencing data was filtered by in-house method to obtain clean data (Appendix G) by removing reads contaminated by adapter, ambiguous bases (Ns), reads with low complexity of 10 consecutive similar bases, truncation of sequence reads that having less than 75% from the original length and average quality of 20

sliding window of 30 bp. As for the ITS sequencing, removal of paired end reads without overlap was followed by removal of primer sequences. The forward and reverse amplification primer sequences were retained from the reads if 4 consecutive bases at the 3'-end of primer can match completely with the tags with mismatch bases less than 2. The overlapped sequences of paired end reads were then generated using FLASH (Fast Length Adjustment of Short reads, v1.2.11) (http://ccb.jhu.edu/software/FLASH/) by allowing minimal overlapping with length of 15 bp, and mismatching ratio <=0.1 for the overlapped region (Appendix H). The high-quality paired-end reads for 16S sequencing was then combined to tags based on the overlap and generated 3,782,738 tags with average length of 253 bp, while ITS sequencing generated 936,942 tags with average length of 238 bp (Appendix I).

3.5.3.2 Community patterns and diversity analysis

The tags were clustered into OTU by using the UPARSE-OTU algorithm (v7.0.1090) (https://www.drive5.com/usearch/) (Appendix J). Set of quality filtered unique sequences were clustered at 97% similarity by aligning the sequence to each other to determine the representative of OTU sequence. The sequence was screened and filtered for chimera with UCHIME (v4.2.40) by using gold database (v20110519) and UNITE (v20140703) as a reference template for 16S rDNA and ITS sequences, respectively. The sequence were aligned and mapped to each OTU representative sequence using USEARCH GLOBAL and summarized into OTU abundance. All three steps of the above process were repeated until all of the sequence was successfully classified.

The taxonomic unit was classified with the RDP (Ribosomal Database Project) (http://rdp.cme.msu.edu/classifier/classifier.jsp) based on Bergey's taxonomy by using a Bayesian assignment calculation to calculate the probability of each sequence being assigned to the rank on the genus level. One representative sequence with the highest OTU abundance was automatically distinguished by the RDP classifier to categorize the species, with the default value of taxonomy

threshold of 0.6 for bacterial 16S and 0.8 for fungal ITS. Species annotation for bacterial and fungal community were determined using Greengene (default) V201305 and UNITE (default) Version 6 20140910, respectively. Species with abundance less than 0.5% in all samples were classified into 'other' ranks.

A cluster of multiple sequences based on the distance between sequence, OTU classifications, and the similarity of the sequence threshold value was determined using Mothur v1.31.2 (https://www.mothur.org/) (Appendix K). Subsequently, all sequence clusters were calculated based on the alpha (α)-diversity index parameter including observed species, Chao1, ACE and Shannon index. The rarefaction curve value and graph were generated based on 97% of sequence similarity threshold of every species, genus, and family level. The corresponding rarefaction curve was generated using software R (v3.1.1) (https://www.r-project.org/).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Growth of loose-leaf lettuce under soil cooling

4.1.1 Measurement of air and soil temperature during the growth of loose-leaf lettuce

A greenhouse with soil cooling system was used to grow loose-leaf lettuce under tropical climate. Air temperature of the greenhouse was recorded at a minimum and maximum temperature of 26.0°C and 36.0°C, respectively. Figure 4.1 demonstrated the soil temperature profile for soil cooling system throughout the lettuce growth cycle. Under soil cooling, cooled soils reported 14.6°C as the minimum and 20.1°C as the maximum soil temperature. In contrast, uncooled soils reported soil temperature ranges from 26.4°C to 30.9°C.

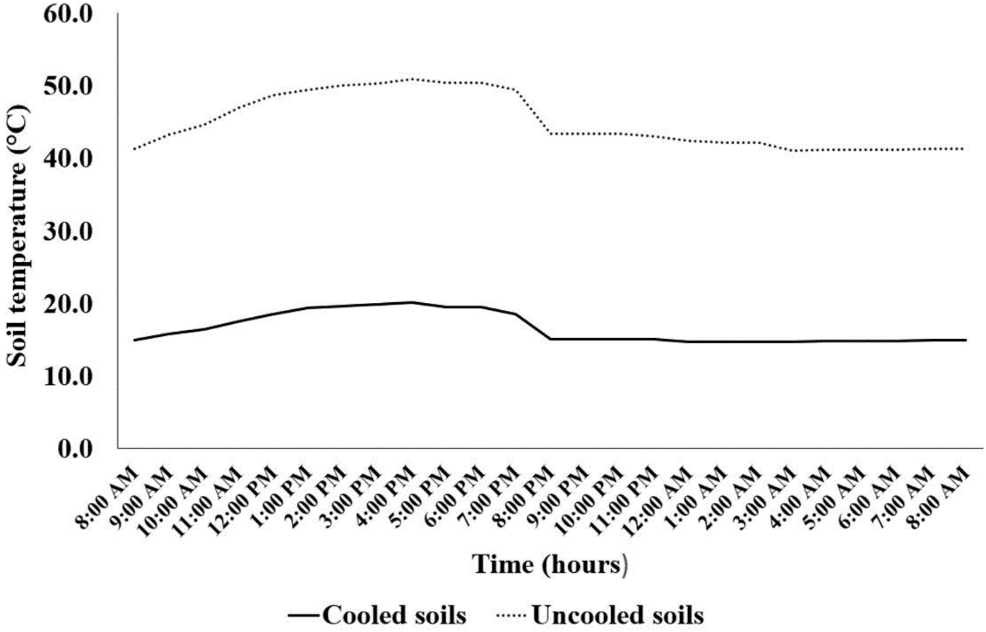


Figure 4.1 Soil temperature profile for cooled and uncooled soil.

4.1.2 Growth evaluation of loose-leaf lettuce under soil cooling

Lettuces were grown consecutively for 3-growth cycle on cooled and uncooled soils. Upon maturity, lettuces were evaluated in terms of weight, height, number and area of leaves and root length. Figure 4.2 showed the average weight of lettuces grown on cooled and uncooled soils for 3-growth cycle. Upon the 1st harvest, lettuces showed higher in average weight between cooled (12.44±2.46 g) and uncooled soils (2.24±0.91 g). The average weight decreases during the 2nd (3.68±0.89 g) and 3rd harvest (2.42±0.42 g) for lettuces grown on cooled soils. Likewise, lettuces that were grown on uncooled soils also demonstrated decreases in average weight from 2nd (1.02±0.10 g) to the 3rd harvest (1.01±0.13 g). From the 1st to 3rd growth, lettuces demonstrated decrease in average weight for both soils but as compared to uncooled soils, cooled soils demonstrated higher yield throughout the 3-growth cycle.

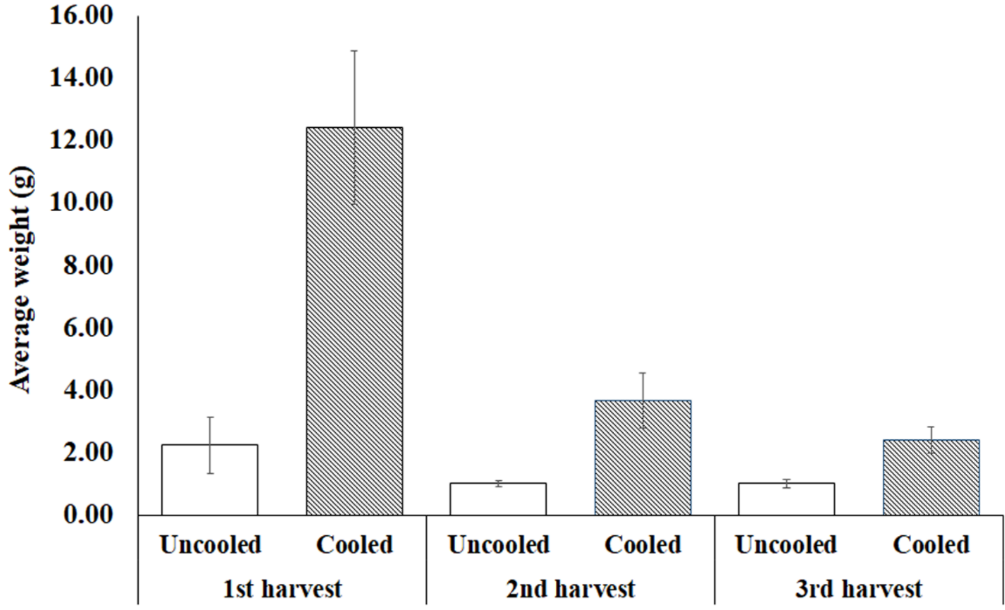


Figure 4.2 Average weight of loose-leaf lettuce for 3-growth cycle.

Table 4.1 showed the height, number and area of leaf, as well as length of roots for 3-growth cycle. Lettuces grown on cooled soils showed slight difference in height after the 1st harvest (16.2±0.4) as compared to uncooled soils (12.0±2.0). Leaf area is the highest for lettuces grown on cooled soils after the 1st harvest (87.07±11.79) as compared to uncooled soils (61.19±7.06). Following the 1st harvest, cooled and uncooled soils demonstrated continuous decrease in the lettuce height and leaf area. Likewise, number of leaves and length of roots also reported decrease upon the 1st harvest but showed a higher number of leaves and root length for lettuces grown on cooled soils. Upon every harvest cycle, lettuces grown on cooled soils demonstrated longer root length than uncooled soils. Figure 4.3 showed the representative of lettuce harvested from cooled and uncooled soils for 3-growth cycle. For every harvest cycle, lettuces grown on cooled soil demonstrated a high-quality and marketable produce as compared to uncooled soils.

Table 4.1: The height, number and area of leaves, and length of roots for lettuce for 3-growth cycle.

	Soil cooling	Height (cm)	Number of leaves	Leaf area (cm^2)	Length of roots (cm)
1st harvest	Uncooled	12.0±2.0	7	61.19±7.06	7.3±0.1
	Cooled	16.2±0.4	12	87.07±11.79	8.8±0.3
2nd harvest	Uncooled	11.7±1.8	5	53.51±15.82	5.4±0.2
	Cooled	13.0±2.1	8	68.51±4.97	7.2±0.5
3rd harvest	Uncooled	10.9±1.7	6	39.36±1.77	2.9±0.3
	Cooled	11.7±1.0	8	54.03±4.73	6.9±0.7

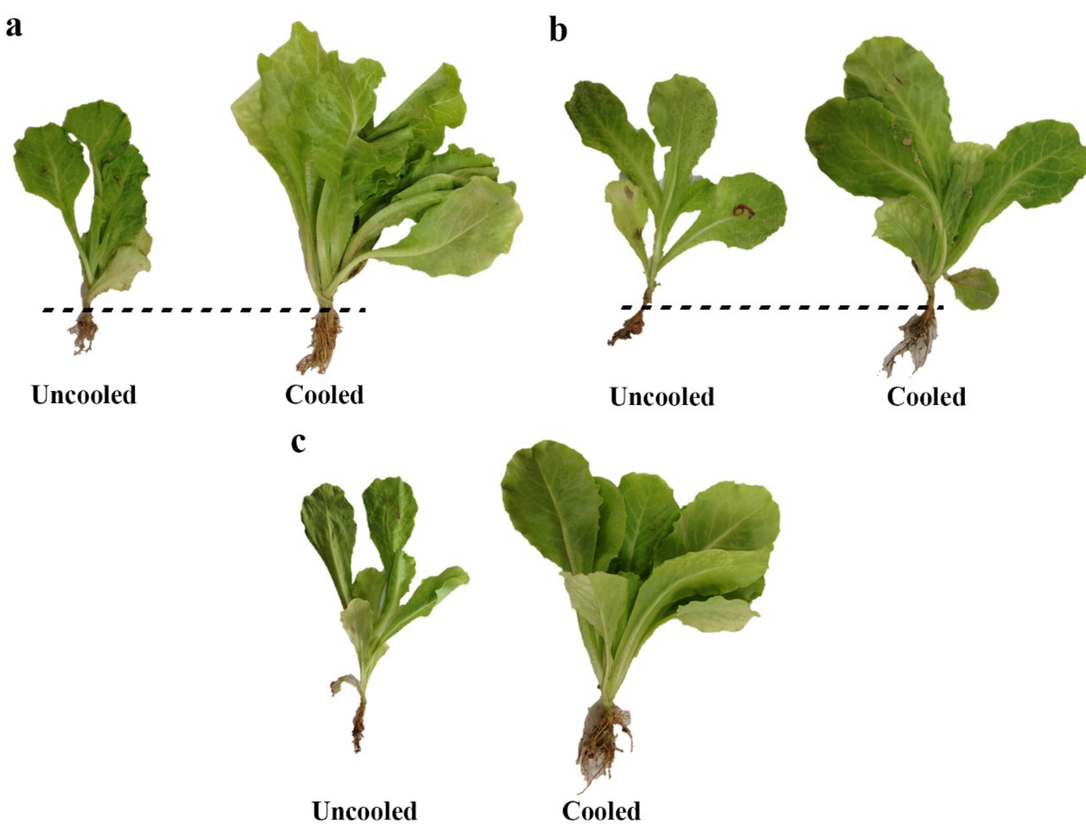


Figure 4.3 Representative of lettuces grown under soil cooling after the 1st (a), 2nd (b), and 3rd harvest (c).

4.2 Soil chemical properties

The chemical properties of cooled and uncooled soil were evaluated for each growth cycle (Table 4.2). pH value showed increase during the 1st and 2nd growth cycle, while cooled and uncooled soil of the 3rd growth demonstrated decrease in pH value. SOM decrease from soil before growth to the 1st and 2nd growth for cooled and uncooled soils in the range of 0.002 mg/kg to 0.003 mg/kg. As for the 3rd growth, SOM increased to 270,400 mg/kg and 206,000 mg/kg for cooled and uncooled soil, respectively. Macro and micronutrients reported rapid and unconsistent changes during the 3-growth cycle under the soil cooling. Total N showed decrease from soils before growth to the 1st and 2nd growth followed by increase in total N during the 3rd growth. Cooled soils reported higher value in total N at 451,800 mg/kg than uncooled soils after the 3rd harvest. Likewise, total P also demonstrated decrease from 4,700 mg/kg to 2,400 mg/kg and 1,600 mg/kg for uncooled soil of the 1st and 2nd harvest. In contrast, cooled soils showed increase in P during the 2nd and 3rd harvest. Total P was the highest in uncooled soil of the 3rd growth at 35,100 mg/kg. Soils of the 2nd and 3rd growth showed rapid changes in available K in which the value is thousandfold than soils before the lettuce growth. Available K after the 3rd harvest reported decrease to 23.73 mg/kg and 63.40 mg/kg for cooled and uncooled soils, respectively. Calcium in cooled soil decreased during the 1st growth but increased to 282.48 mg/kg and 644.38 mg/kg after the 2nd and 3rd harvest, while uncooled soils showed continuous decrease in available Ca until the 3rd growth. As for Mg, soils during the 2nd growth demonstrated sudden increase then decrease at the 3rd harvest. Micronutrients including Cu and Zn demonstrated rapid decrease and increase during the 1st and 2nd growth cycle. During the 3rd growth, Cu in cooled soils showed decreased to 16.46 mg/kg, while uncooled soils reported increased to 17.89 mg/kg. In contrast, Zn in cooled and uncooled soil decreased during the 3rd growth. Fe and Mn in cooled soils showed continue decrease throughout the growth, while uncooled soil demonstrated increased from 1st to 2nd harvest then decrease during the 3rd growth.

Table 4.2: Chemical properties during the lettuce growth

Chemical properties		1st harvest		2nd harvest		3rd harvest	
	Before	**Cooled**	**Uncooled**	**Cooled**	**Uncooled**	**Cooled**	**Uncooled**
pH	6.66	7.69	7.36	7.95	7.57	7.30	7.25
OM (mg/kg)	227,000	0.003	0.002	0.003	0.003	270,400	206,000
N (mg/kg)	457,100	4,200	4,900	3,800	4,000	451,800	3,100
P (mg/kg)	4,700	2,100	2,400	2,400	1,600	19,700	35,100
K (mg/kg)	6.11	2,798.77	1,098.79	2,725.62	965.84	23.73	63.40
Ca (mg/kg)	420.72	95.80	92.93	282.48	45.52	644.38	25.20
Mg (mg/kg)	176.70	67.16	24.69	115.88	51.87	111.19	7.36
Cu (mg/kg)	18.35	11.05	9.07	30.07	11.02	16.46	17.89
Zn (mg/kg)	14.35	14.21	14.23	17.95	18.01	12.47	15.35
Fe (mg/kg)	7,329.94	5,288.15	5,500.90	5,158.09	6,673.79	5,019.46	6,322.23
Mn (mg/kg)	19.20	15.59	16.27	15.32	19.37	14.83	17.99

4.3 Sequencing reads and diversity analysis

4.3.1 Soil bacterial community

Sequencing of 16S bacterial communities generated 3,860,411 paired raw reads with read length of 250 bp. After quality filtering process and removal of chimeric sequences, 686,149 reads from highland soils before the growth, 833,656 reads from cooled and 773,407 reads from uncooled soils of the 1st harvest, as well as 774,097 reads from cooled and 784,091 reads from uncooled soils of the 3rd harvest were obtained for downstream analysis (Table 4.3). At 97% similarity, the number of OTUs for soil bacterial communities recovered from highland soils before the growth, for cooled and uncooled soils after the 1st harvest was 1146, 1117, and 980, while cooled and uncooled soils from the 3rd harvest reported OTUs of 1096 and 1123, respectively.

Diversity of soil bacteria was evaluated through several indices including Chao1 and ACE value reflecting the species richness of community, while Simpson and Shannon index representing the species diversity and is dependening on species abundance and evenness. Based on Table 4.3, uncooled soils from the 1st harvest was reported to have the lowest number of observed OTUs, species richness Chao1 and ACE index, cooled soils from the 3rd harvest showed the lowest Shannon diversity index, while highland soils before growth reported lowest value of Simpson index. The rarefaction curve based on OTUs (Figure 4.4) showed that all soil sample reach saturation plateau producing sufficient sequencing data that covers the complexity of soil samples at range between 1,000 and 1,500 of bacterial species. Uncooled soils from the 1st harvest demonstrated the lowest bacterial species, while soil before growth reported the highest bacterial species as the sequencing depth increases. In contrast, cooled soils from the 1st harvest, as well as cooled and uncooled soils from the 3rd harvest represent the similar value of bacterial species from the sequencing reads.

Table 4.3: Number of sequencing reads and diversity of soil bacteria

Sample		Raw reads	Clean reads	Coverage (%)	OTU	Chao1	ACE	Simpson	Shannon
Before growth		687,932	686,149	99.74	1146	1213.988	1219.620	0.011	5.674
1st harvest	**Cooled**	835,577	833,656	99.77	1117	1251.864	1261.063	0.016	5.344
	Uncooled	775,305	773,407	99.76	980	1134.124	1120.520	0.020	5.009
3rd harvest	**Cooled**	775,834	774,097	99.78	1096	1226.712	1250.476	0.069	4.566
	Uncooled	785,763	784,091	99.79	1123	1260.764	1282.707	0.039	4.731

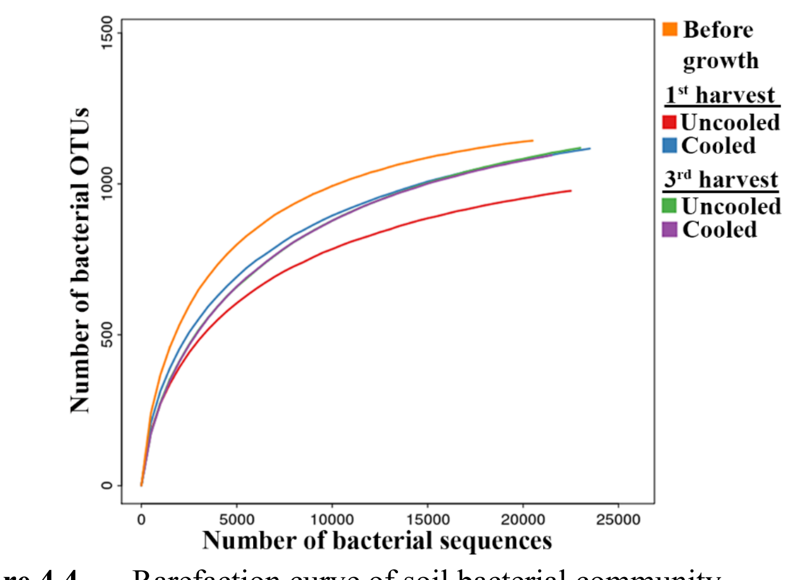


Figure 4.4 Rarefaction curve of soil bacterial community.

4.3.2 Soil fungal community

A total of 1,051,979 clean reads were generated from ITS sequencing for the following 4 samples: soil before growth, cooled and uncooled soils of the 1st harvest, and uncooled soil from the 3rd harvest. As for cooled soil from the 3rd harvest, sequencing was not performed due to failure in library preparation and purification. Nevertheless, reads from soil before growth and 1st harvest were able to provide the general overview amd shift in fungal community for lettuces grown under soil cooling. After trimming, screening and removing of chimeras, soil fungi generates 259,668 reads from soil before growth, 268,100 reads from cooled soil, and 255,924 reads from uncooled soil after the 1st harvest, and 268,287 reads from uncooled soil of the 3rd harvest (Table 4.4). As for the OTUs number, soil before growth, uncooled, and cooled soils from the 1st harvest were reported to be 128, 136, and 154, respectively, while uncooled soil from the 3rd harvest was reported to be 165.

Similar to soil bacteria, diversity of soil fungi was measured through indices including Chao1, ACE, Shannon and Simpson index. Soil before lettuce growth reported the lowest observed OTUs, chao1 and ACE indices that reflect species richness in fungal community, while uncooled and cooled soils from the 1st harvest showed the lowest value in Shannon and Simpson index, respectively (Table 4.4). The rarefaction curve showed steep increase in fungi species at the onset of sequencing process which then remain constant as the sequencing depth increases and reach plateau (Figure 4.5). Based on rarefaction curve, fungi species were the highest for uncooled soil from the 3rd harvest followed by cooled and uncooled soils of the 1st harvest with fungi ranging from 100 to 200 species. Meanwhile, the lowest fungi species were represented by soil before the lettuce growth.

Table 4.4: Number of sequencing reads and diversity of soil fungi.

Sample		Raw reads	Clean reads	Coverage (%)	OTU	Chao1	ACE	Simpson	Shannon
Before growth		284,132	259,668	91.39	128	128.750	130.068	0.151	2.449
1st harvest	**Cooled**	311,814	268,100	85.98	154	154.200	154.733	0.106	2.975
	Uncooled	265,118	255,924	96.53	136	137.500	138.430	0.274	2.161
3rd harvest	**Cooled**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Uncooled	278,494	268,287	96.33	165	166.500	166.218	0.221	2.504

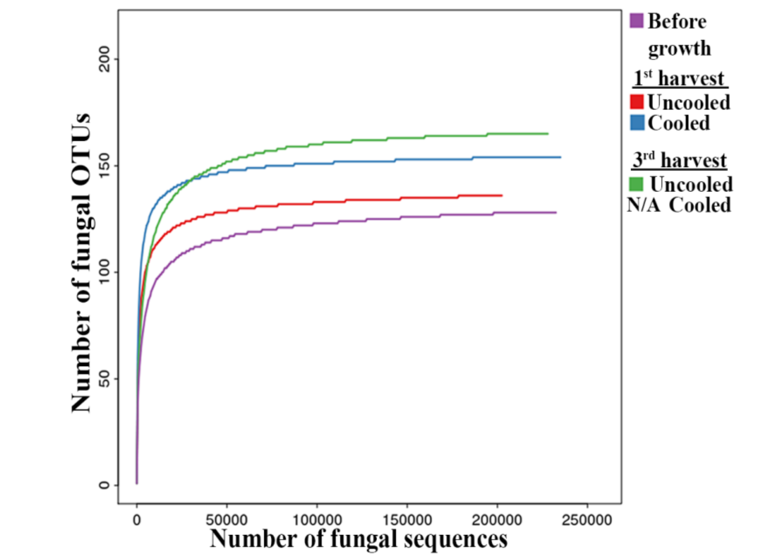


Figure 4.5 Rarefaction curve of soil fungal community.

4.4 Taxonomic composition

4.4.1 Soil bacterial community

The taxonomic abundance of bacterial community before the lettuce growth, cooled and uncooled soils of the 1st and 3rd harvest were compared. At phylum level, Proteobacteria was reported as the most abundant taxa in all soil (Fig 4.6a). Proteobacteria showed increase in abundance before the lettuce growth at 46.59% to 58.54% and 58.31% for cooled and uncooled soils of the 1st harvest, respectively. These phyla demonstrated continuous increase in the abundance for cooled (68.66%) and uncooled (68.38%) soils upon the 3rd harvest. Similar to Proteobacteria, Bacteroidetes demonstrated increase in the abundance from soil before growth to the 1st and 3rd harvest. Bacteroidetes in cooled soils for the 1st (13.44%) and 3rd (11.15%) harvest reported to be more abundant than uncooled soils in the 1st (10.61%) and 3rd harvest (6.86%). Acidobacteria and Actinobacteria were dominant phyla that abundant in soil before the lettuce growth but showed decline upon the 1st and 3rd harvest. Acidobacteria was higher in cooled soil during the 1st harvest (9.65%) than the 3rd harvest (7.61%), while Actinobacteria reported to be abundant in uncooled soil during the 1st (11.54%) and 3rd (5.34%) harvest as compared to cooled soils. In addition, Chloroflexi, Gemmatimonadetes, Nitrospirae, Planctomycetes and Verrucomicrobia were also reported to be abundant in soils particularly before the lettuce growth that decrease after the 1st and 3rd harvest.

At class level as shown in Fig 4.6b, Acidobacteria-6 (3.42%), Actinobacteria (8.24%), Saprospirae (7.31%), α-Proteobacteria (25.23%) and γ-Proteobacteria (16.80%) were abundant in uncooled soils after the 1st growth as compared to cooled soil (Acidobacteria-6: 2.86%; Actinobacteria: 3.69%; Saprospirae: 4.68%; α-Proteobacteria: 23.26% and γ-Proteobacteria: 10.58%). Similar to the 1st growth, these classes were also dominant in uncooled soil of the 3rd harvest. In contrast, β-Proteobacteria and Sphingobacteriia showed to be more abundant in cooled soils of

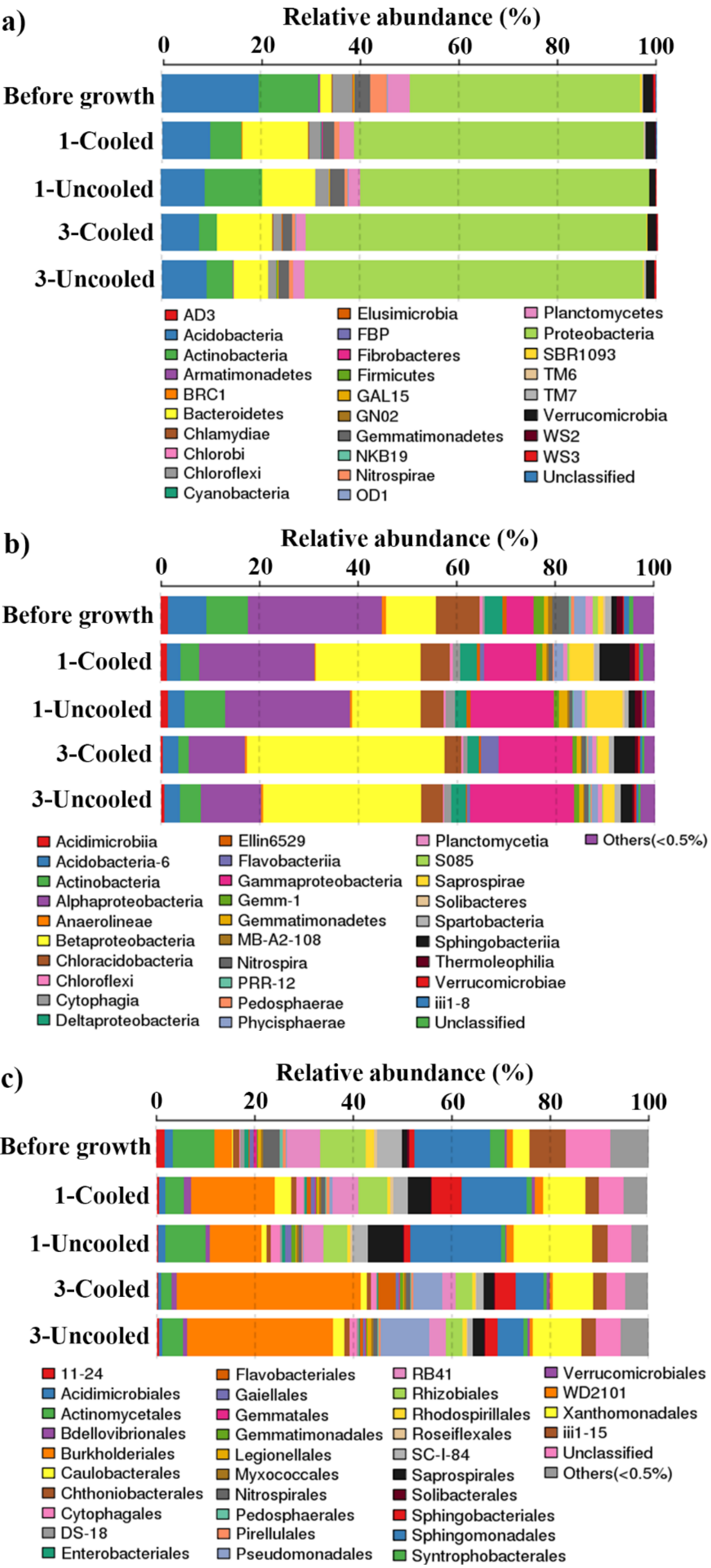


Figure 4.6 Taxonomical structure and relative abundance of soil bacteria at a) phylum; b) class; c) order; d) family; e) genus and f) species (Figure caption continued on next page).

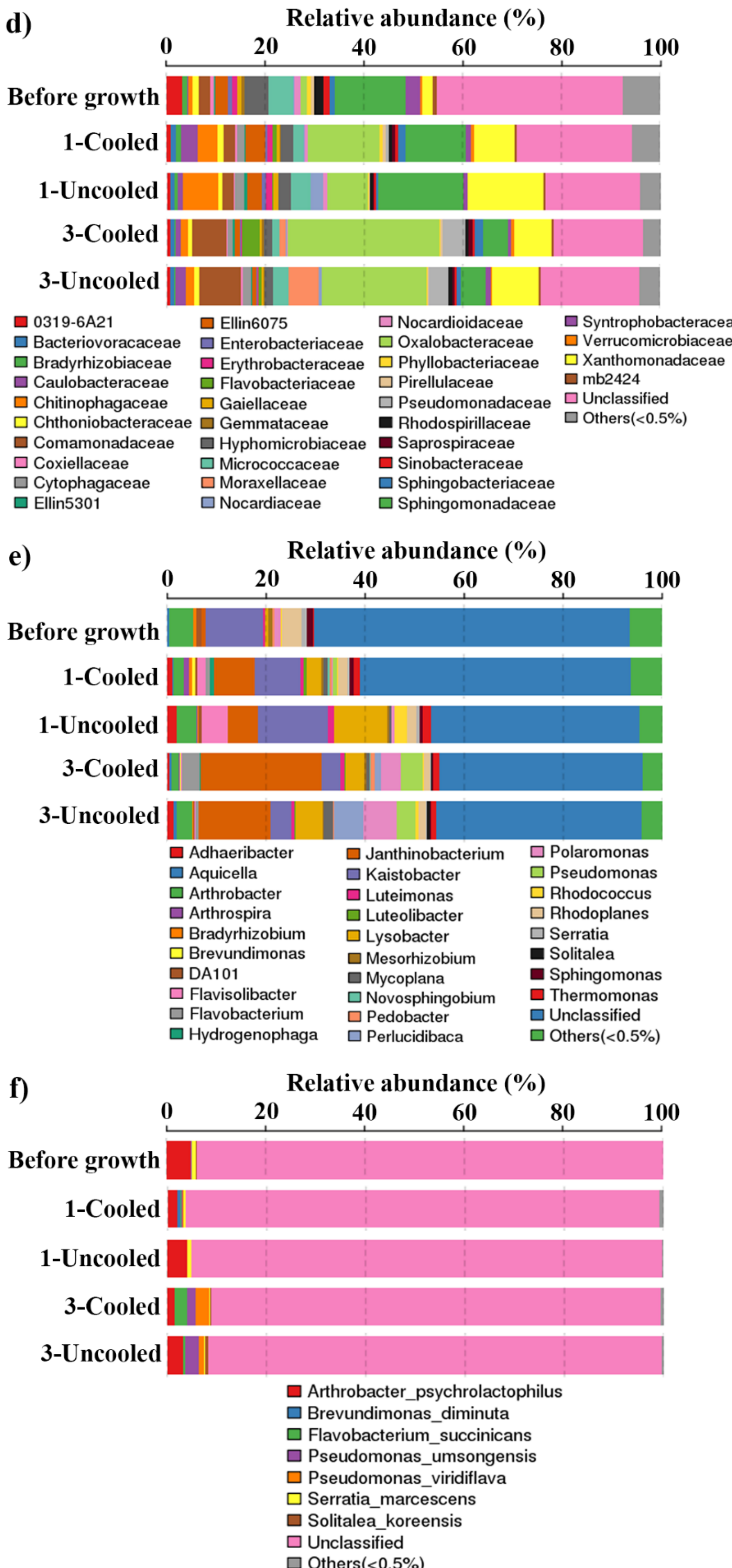


Figure 4.6 (Figure caption continued from previous page) Taxonomical structure and relative abundance of soil bacteria at a) phylum; b) class; c) order; d) family; e) genus and f) species.

the 1st and 3rd growth as compared to uncooled soil. As for δ-Proteobacteria, cooled soil reported to be dominant at the 1st growth (3.29%) and decease upon the 3rd growth (2.32%) as compared to uncooled soils (1st growth: 2.18% and 3rd growth: 2.89%).

Fig 4.6c reported Actinomycetales, Burkholderiales, RB41, Rhizobiales, Saprospirales, Sphingobacteriales, Sphingomonadales as the most abundant soil bacteria at order level. Actinomycetales showed dominant in soil before growth (8.39%) and uncooled soils of the 1st (8.20%) and 3rd (4.13%) harvest. Burkholderiales reported to be higher in cooled soils for the 1st (17.05%) and 3rd (37.59%) harvest as compared to uncooled soils (1st harvest: 10.44% and 3rd harvest: 29.66%). Likewise, Sphingobacteriales also reported to be dominant in cooled soils as compared to uncooled soils for both 1st and 3rd growth cycle. As for RB41 and Rhizobiales, these order showed higher abundance in cooled soil after the 1st growth but lower in the 3rd growth. Saprospirales was dominant in uncooled soils for the 1st (7.31%) and 3rd growth (2.21%), while Sphingomonadales was abundant in soil before the growth (15.38%), uncooled soil of the 1st harvest (18.55%) and cooled soil of the 3rd harvest (5.70%).

At family level, *Chitinophagaceaea* (7.14%), *Sphingomonadaceae* (17.12%) and *Xanthomonadaceae* (15.30%) reported to be the highest in uncooled soil of the 1st harvest (Fig 4.6d). Members of *Flavobacteriaceae* (1st growth: 0.87% and 3rd growth: 3.52%), *Oxalobacteraceae* (1st growth: 14.51% and 3rd growth: 30.75%), *Pseudomonadaceae* (1st growth: 0.73% and 3rd growth: 4.57%), and *Sphingobacteriaceae* (1st growth: 1.48% and 3rd growth: 1.80%) were reported to be dominant in cooled soils as compared to uncooled soils of the 1st and 3rd growth. As for uncooled soils, *Sphingomonadaceae* (17.12%) and *Xanthomonodaceae* (15.30%) dominated the 1st growth,while *Comamonadaceae* (8.25%) dominated the 3rd growth.

For the genus level (Fig 4.6e), *Arthrobacter* (4.10%), *Flavisobacter* (5.23%), *Kaistobacter* (14.23%) and *Lysobacter* (10.76%) reported as the most abundant in

uncooled soil of the 1st harvest than cooled soils (*Arthrobacter*: 2.00%; *Flavisobacter*: 1.75%; *Kaistobacter*: 9.24%; and *Lysobacter*: 3.06%). In contrast, *Flavobacterium* demonstrated increase in abundance for cooled soils from 0.86% to 3.51% during the 1st and 3rd growth as compared to uncooled soils (1st growth: 0.05% and 3rd harvest: 0.64%). Likewise, *Janthinobacterium* showed to be the highest in cooled soils after the 1st (8.14%) and 3rd harvest (24.32%). In addition to *Flavobacterium* and *Janthinobacterium*, *Pseudomonas* also reported increase in abundance from 1st to the 3rd growth at 0.69% to 4.51% for cooled soils and 0.02% to 3.83% for uncooled soils.

Upon identification of dominant genera, dominant species including *Arthrobacter psychrolactophilus* was reported to be abundant particularly in uncooled soils at the 1st (4.10%) and 3rd (3.20%) harvest as compared to cooled soils (Fig 4.6f). Likewise, *Serratia marcescens* and *Solitalea koreensis* also showed higher abundance in uncooled soils. *Serratia marcescens* reported 0.68% and 0.25% at 1st and 3rd growth as compared to cooled soils (1st growth: 0.30% and 3rd growth: 0.19%). *Solitalea koreensis* reported 0.03% and 0.61% for 1st and 3rd harvest while cooled soils showed 0.03% and 0.23% for 1st and 3rd growth. *Brevundimonas diminuta* was abundant in cooled soils at 1st (0.66%) and 3rd (0.05%) growth as compared to uncooled soils (1st growth: 0% and 3rd growth: 0.02%) as well as *Flavobacterium succinicans* that reported 0.09% and 2.50% at 1st and 3rd harvest of cooled soils. In addition, *Pseudomonas umsongensis* reported dominant in cooled soil at 1st growth (0.16%) but showed higher abundance in uncooled soil upon the 3rd growth (2.79%). The other members of Pseudomonas identified as *Pseudomonas viridiflava* also showed higher abundance in cooled soils at the 1st (0.39%) and 3rd harvest (2.88%) than uncooled soils (1st harvest: 0.02% and 3rd harvest: 1.02%). In all soils, most of the species were categorized as unclassified at ranges between 90.40% and 95.51%, as well as others at percentage of 0.05% to 0.87%.

4.4.2 Soil fungal community

The taxonomic composition of soil fungal community at phylum, class, order, family, genus and species level were compared between all soil samples in exception with cooled soil of the 3rd harvest due to failure in library construction (Fig. 4.7). At phylum level (Fig. 4.7a), Ascomycota reported as the most abundant in all soils with the highest in soil before growth (63.60%) followed by cooled soil of the 1st harvest (49.36%) and uncooled soils of the 1st (37.87%) and 3rd harvest (7.70%). Zygomycota also dominated the soils particularly uncooled soils of the 1st (54.92%) and 3rd growth (62.66%) as compared to cooled soil (37.08%). In addition, Basidiomycota demonstrated abundant in cooled soil (9.04%) and soil before growth (4.42%), while Chytridiomycota was more abundant in cooled soil (0.23%) and Glomeromycota was more dominant in uncooled soil of the 1st harvest.

The taxonomic abundance at class level (Fig. 4.7b) reported Incertae sedis as the highest fungal community in uncooled soils of the 1st (54.97%) and 3rd growth (62.66%) followed by cooled soil (37.12%) and soil before growth (30.60%). In cooled soil, Dothideomycetes (12.14%), Pezizomycetes (18.35%), Pucciniomycetes (3.53%), Sordariomycetes (15.86%) and Tremellomycetes (1.79%) were dominant. As for the order level (Fig. 4.7c), Mortierellales was the most abundant in all soils particulalrly uncooled soils of the 1st (54.92%) and 3rd harvest (62.66%) as compared to cooled soil (37.08%) and soil before growth (30.57%). In addition to Mortierellales, Pezizales (18.35%) and Pleosporales (11.70%) were dominant in cooled soil than uncooled soils of the 1st (Pezizales: 0.10% and Pleosporales: 3.15%) and 3rd growth (Pezizales: 0.08% and Pleosporales: 0.11%). Hypocreales reported highest abundance in soil before growth (10.01%) and cooled soil (7.70%) followed by uncooled soils (1st growth: 2.22% and 3rd growth: 1.24%). Likewise, Platygloeales was dominant in cooled soil (3.53%), while Sebacinales in soil before growth (4.01%) and cooled soil (2.68%). In contrast, Sordariales reported dominant in uncooled soil of the 3rd harvest (2.52%).

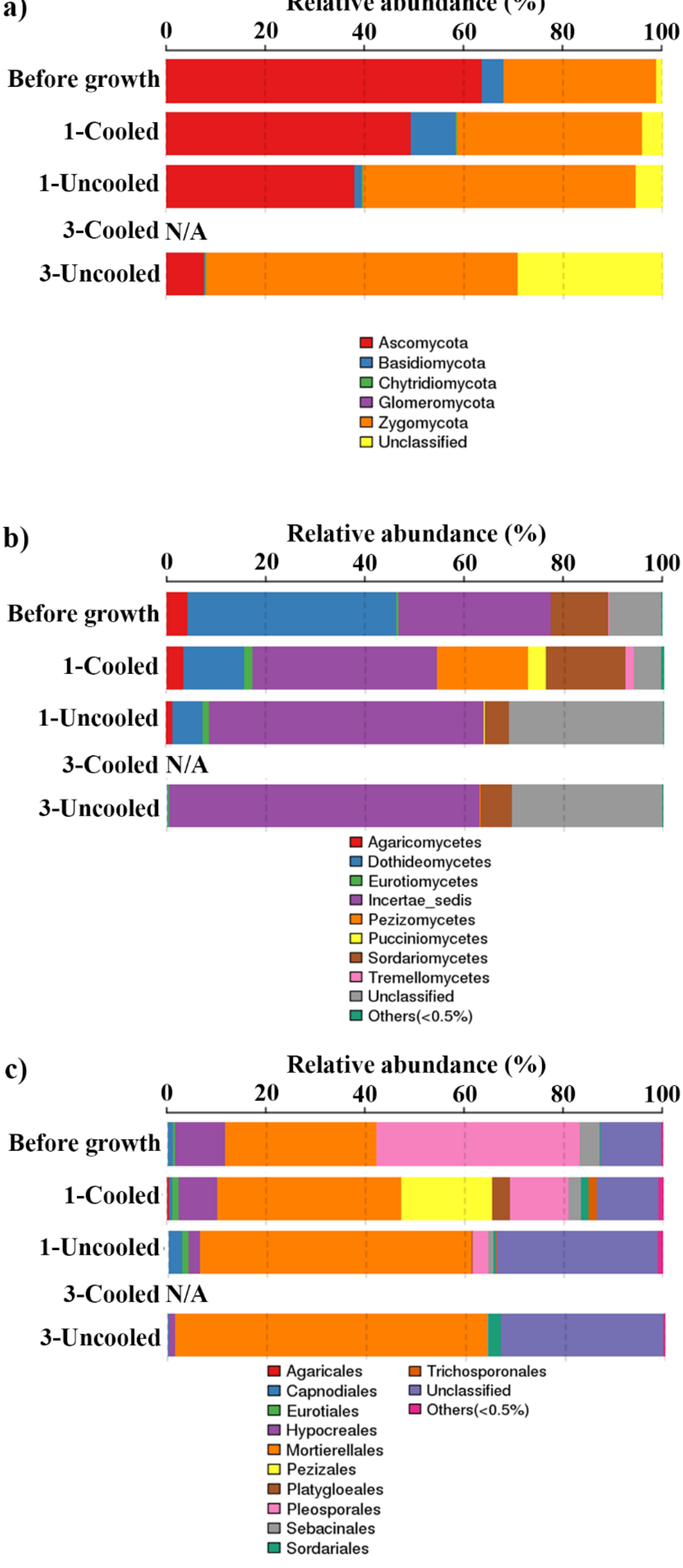


Figure 4.7 Taxonomical structure and relative abundance of soil fungi at a) phylum; b) class; c) order; d) family; e) genus and f) species (Figure caption continued on next page).

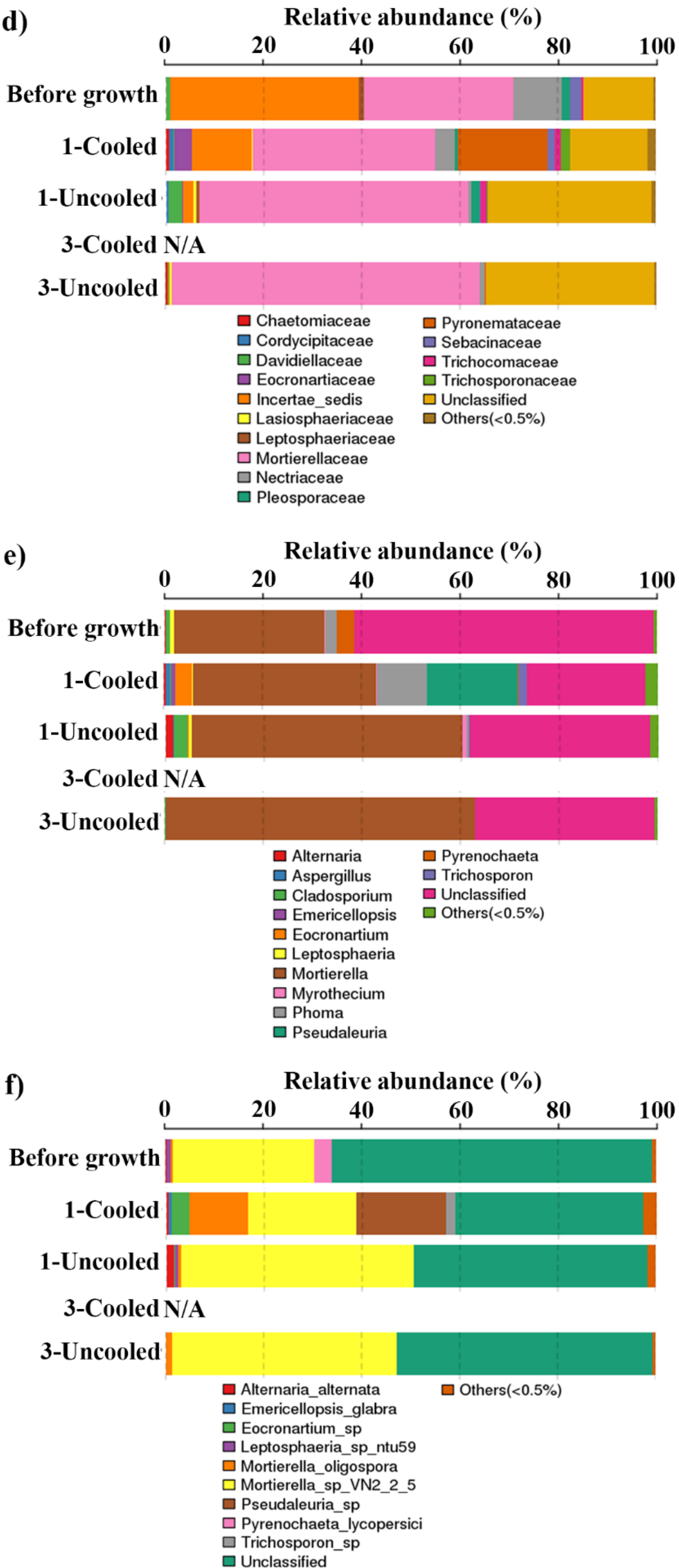


Figure 4.7 (Figure caption continued from previous page) Taxonomical structure and relative abundance of soil fungi at a) phylum; b) class; c) order; d) family; e) genus and f) species.

As for the family level (Fig 4.7d), *Mortierellaceae* and *Incertae sedis* were reported as the most dominant in uncooled soil of the 3rd harvest (62.66%) and soil before growth (38.38%), respectively. Members of *Pyronemataceae* reported abundant particularly in cooled soil at 18.31% followed by *Eocronartiaceae* at 3.53%. In addition, *Nectriaceae* showed dominancy in soil before growth (9.90%) and cooled soil (4.08%) as compared to uncooled soils of the 1st (0.68%) and 3rd growth (1.01%). Upon identification of dominant fungal family, genus including *Mortierella*, *Pseudaleuria*, *Pyrenochaeta* and *Eocronartium* were reported to be the most abundant in all soils (Fig 4.7e). *Mortierella* demonstrated the highest abundance in uncooled soil of the 3rd growth (62.66%) followed by the 1st growth (54.92%). *Pseudaleuria* (18.31%), *Phoma* (9.97%), *Eocronartium* (3.53%) and *Trichosporon* (1.79%) demonstrated abundant in cooled soil as compared to uncooled soils. As for *Pyrenochaeta*, these genus showed dominant in soil before growth (3.53%). At species level (Fig 4.7f), *Mortierella sp VN2-2-5* was the highest in uncooled soil of the 1st growth (47.53%) as compared to cooled soil (21.94%). In contrast, *Mortierella oligospora* showed dominant in cooled soil (12.01%) than uncooled soils of the 1st (0.59%) and 3rd growth (1.19%). Besides than the members of *Mortierella*, *Pseudalueria sp.* (18.31%), *Eocronartium sp.* (3.53%) and *Trichosporon sp.* (1.79%) were also reported to be dominant in cooled soil. In soil before growth, *Pyrenochaeta lycopersici* was shown as the highest at 3.53% as compared to other soils.

Soil is a complex ecosystem that serve as a source of nutrients to plants and home of bacteria, fungi, protists and animals. Plants directly and indirectly exhibit various interactions with abiotic (physical, chemical and biochemical) and biotic (soil biota) soil components. In this study, soil temperature was investigated for its effect on the growth of loose leaf lettuce via soil microbial diversity. Soil temperature was manipulated using soil cooling system based on the principle of OTEC that utilizes cold deep seawater for temperate agriculture as reported in Kume Island of Okinawa Prefecture and Hawaii (Yu, 2015; Martin, 2017). Loose leaf lettuce was grown under tropical climate of Malaysia with air temperature ranges between 26.0°C and 36.0°C. Study performed by Islam and collegues has reported a close correlation between air and soil temperature (Islam *et al.*, 2015). Soils receive, absorb and transmit heat mainly from solar radiation which varies on geographical location, particularly latitude. Tropical climate has the highest intensity for solar radiation between 11 am and 2 pm throughout the 11 hours of solar radiation, starting from 8 am to 7 pm which resulted to higher soil temperature than the temperate soils (Osman, 2012; Sanusi *et al.*, 2014). At high solar radiation intensity, the soil surface gets heated up and transmitted through underground soils. Study performed by Sanusi et al. has reported soil temperature between 26.9°C and 30.9°C, respectively for uncooled soils. However, to grow temperate crops, a lower soil temperature was required for optimum growth performance.

By using soil cooling method, soils were cooled to temperature ranges between 14.6°C and 20.1°C, which are conducive for the growth of temperate crop, particularly lettuce varieties including iceberg, butterhead, Romain and loose leaf lettuce. According to previous study, lettuces grow optimally under cool climatic condition at a temperature between 15°C and 20°C (Ogbodo *et al.*, 2010; Luo *et al.*, 2012; Lafta and Mou; 2013). This explained the higher average weight of lettuces grown on cooled soils during the 3-growth cycle. For uncooled soils, lettuces demonstrated a lower average weight after the 1st harvest and following cycle, attributed by high soil temperature. The adverse effect of high soil temperature to lettuce growth include leaf twisting, early bolting, stunted growth and reduced yields (Ogbodo *et al.*, 2010; Shaban *et al.*, 2016).

In addition, soil temperature has also demonstrated to be affecting the leaves, root and shoot phenology. At 3-growth cycle, lettuces grown under cooled and uncooled soils demonstrated decrease in height, length of roots, number and area of leaves. Soil temperature is closely correlated with the growth and physiology of plant root systems. Roots exert control over whole-plant growth and development by regulating the uptake of mineral nutrients. In root, cytokinins produced are translocated to shoots and involved in the control of leaf protein biosynthesis, as well as nitrogen assimilation in the root itself (Fageria and Moreira, 2011). In this study, lettuces grown under cooled soil showed higher shoot and longer roots than uncooled soil, although both soils demonstrated continues decrease towards 3rd growth cycle. Previous study has reported higher fibrous roots (involved in acquisition of water and nutrients): shoot ratio, thicker and smaller leaves, and higher taproot (stores carbohydrates and other compounds): shoot ratio of carrot cultivars at soil temperature of 12°C as compared to 25°C (González *et al.*, 2009). In addition to soil temperature, deficiency of mineral elements influence the plant growth and root-shoot relationship.

To understand the effect of soil temperature on plant growth via soil microbial diversity, no additional fertilizers was added throughout the 3-growth cycle. Temperate agricultural soils from Cameron Highland serve as a source of nutrients from previous agricultural activities of temperate vegetables including tomato, cabbage and green mustard for the growth of the lettuce under soil cooling. Soil chemical properties of cooled and uncooled soils showed rapid and unconsistent changes throughout the 3-growth cycle. N and P availability reported decreases during the 1st and 2nd growth for cooled and uncooled soils consistent with the growth of shoot, root and leaf phenology of loose leaf lettuce that decreases from the 1st to 2nd growth. N availability has been reported to affect the root production, biomass, elongation and mortality (Razaq *et al.*, 2017). Plant deficient in N lead to more photosynthate to be used by roots that develop in a greater length to help the plant in obtaining more N (Fageria and Moreira, 2011). Meanwhile, the uptake of P by lettuces grown on cooled and uncooled soils during the 1st and 2nd growth also showed positive effect on root elongation and plant height as reported by previous study (Hill *et al.*, 2006; Kim and Li, 2016). However, N and P level showed

unexpected increase during the 3rd growth. Nitrogen fixation reaction by free-living diazotrophs and microbial-mediated mineralization of organic forms to ammonium and subsequenct nitrification to nitrate may contribute to the N availability which influence the root behavious as well as rhizosphere dynamics in cooled soils (Richardson *et al.*, 2009; Jacoby *et al.*, 2017). Likewise, microbial biomass around the roots and bicarbonate-extractable organic P from microbial-mediated immobilization of orthophosphate may also influence the P availability in uncooled soils (Richardson *et al.*, 2005; Jacoby *et al.*, 2017). The result demonstrated that the availability of N and P can support the growth parameters to a certain extent but has a negative effect at the latter growth cycle.

In addition, K is also vital for plant growth and sustainable crop production including root system (Fageria and Moreira, 2011). K is available in soil in mineral form, as non exchangeable, readily available, in organic matter as well as within the soil microbial population (Prajapati and Kalavati, 2012). The total K in soils mostly existed in structural component of soil minerals and not available for the plant growth. In intensive agriculture, K is a limiting element particularly in coarse-textured or organic soils which require fertilizer to achieve optimum crop yield (Zörb *et al.*, 2014). However, in this study, K reported sudden increase after the 1st and 2nd growth on cooled and uncooled soils although no fertilizer was added during the lettuce growth. This result is speculated to be due to the long-term storage of soil sample prior to analysis that may changed the soil chemical properties of the cooled and uncooled soils after the harvest (Akpan and Mohammed, 2015). In addition, fixation of K reserve in soil minerals may also affect the K availability in cooled and uncooled soils. This involves the adsorption of K ions onto sites in the interlayers of weathered silicates or release of K from K-bearing minerals by excreting organic acids that either directly dissolve rock K or chelate the primary minerals's silicon ion into soil solution (Zörb *et al.*, 2014). Nevertheless, the availability of K in cooled and uncooled soils contributes to photosynthesis process, osmoregulation, transport of sugar and nutrients (Prajapati and Kalavati, 2012) for the lettuce growth. K uptake during the 1st growth promotes root elongation and leaf area (Wang *et al.*, 2013) for lettuces grown on cooled and uncooled soils. For soil low in exchangeable K, large

proportion of the K may be derived from non-exchangeable fraction for the uptake of plant growth (Marschner and Rengel, 2012).

K also form interaction with Ca and Mg (Malvi, 2011). Ca and Mg are adsorbed by plants, and their bioavailability mainly depends on mobility and extractability of the element from soil (Jodral-Segado *et al.*, 2006). Ca and Mg are present in mineral fraction of soils that depend on mineral weathering and rate of leaching that influence the availability of these elements in soil (Jakovljević *et al.*, 2003). Mg also present mainly in inorganic compounds and associated with organic material in humus. The exchangeable portion of magnesium is associated with clay particles and exhaustion of soluble magnesium cation in the soil by microorganisms and plant root which promote the uptake of non-soluble minerals thus affect the non-exchangeable fraction (Jodral-Segado *et al.*, 2006). These contribute to the availability of Ca and Mg in cooled and uncooled soils during the lettuce growth. As compared to other plant nutrients, Ca and Mg nutrition has not been studied extensively (Cakmak and Yazici, 2010). Some studies have reported weak correlation between Ca and lettuce yield indicated that lettuce only required low level of Ca for optimum growth (Hoque *et al.*, 2010). This is further supported by Hepler which reported small quantities of Ca uptake by plant despite high concentration of total Ca determined in the soil (Hepler, 2005). Previous studies have reported that the optimum levels of Ca and Mg for temperate crops, for example greenhouse tomatoes are strongly affected by climatic conditions including solar intensity, humidity and temperature (Papadopoulos, 2003; Rosales *et al.*, 2011). Plant growing under high light intensity has demonstrated higher requirement of Mg than the lower intensity (Cakmak and Yazici, 2010). Likewise, high concentration of Ca also shown to increase the tolerance of plants to environmental stress (Hong-Bo *et al.*, 2008).

As for micronutrients, the availability of these elements are essential for proper plant growth and development. Deficiency of micronutrients resulted to decrease in plant resistance to environmental stress that lead to reduce in yield and quality (Alloway, 2008). Concentration of micronutrients including Mn, Fe, Zn and

Cu in soil solution depends on soil organic matter and chelation by low molecular organic substances that affect the concentration of micronutrient cations in soil solution (Marschner and Rengel, 2012). Depending on soil organic matter, the proportion of complexed cations increase in the order of Mn < Zn < Cu, for 55%, 75% and 80% at low organic matter content, and 50%, 85% and >98%, for high organic matter (Marschner and Rengel, 2012) which may contribute to the availability of micronutrients in cooled and uncooled soils. Micronutrients are constituent of prosthetic group that catalyze redox processes by electron transfer (such as Cu, Fe, Mn, and Mo) and form enzyme-substrate complexes by coupling enzymes with the substrates (Fe and Zn), or influence molecular configurations between enzyme and substrate that affect enzyme reactions (Fageria and Moreira, 2011). Despite rapid and unconsistent changes of macro and micronutrients of cooled and uncooled soils, loose leaf lettuce showed consistent decrease on shoot, root and leaf phenology during the 3-growth cycle. Roots has been reported to exhibit high plasticity as a physiological response to localized patches of organic and inorganic nutrients in soil (Richardson *et al.*, 2009). Roots have 'foraging' capability to optimize resource allocation that varies within plant species in response to different environmental conditions as well as physical/chemical interactions in the soil and soil microorganisms (Richardson *et al.*, 2009).

Interaction between plant roots and soil microorganisms are ubiquitous and essential for ecosystem functioning. Root residue after the crop harvest improve SOM content and contribute to nitrogen cycle and microbial activity (Fageria and Moreira, 2011). Root turnover including shedding of root hairs by root elongation, plant death and other root-borne organic substances that is released into rhizosphere may contribute to an important addition of SOM to cooled and uncooled soils during the 3rd growth cycle (Kumar *et al.*, 2006). The abundance of soil bacterial community during the 1st and 3rd growth cycle also contribute to the addition of SOM in cooled and uncooled soils.

Plants are selective in the types of bacterial associations that they develop and promote, and recruit those that are beneficial for their growth. In this study, composition of bacterial and fungal community in cooled and uncooled soils of loose leaf lettuce was conducted using amplicon sequencing. Soil bacterial and fungal community in cooled and uncooled soils showed changes in abundance during the 1st and 3rd growth cycle in response to soil temperature and nutrient availability during the lettuce growth. To further understand the functional role of each microorganism that contribute to nutrient cycling and plant growth, the attributes of soil bacteria and fungi were discussed at species level. Based on sequencing data, soil bacteria that dominate the uncooled soil was reported to be *Arthrobacter psychrolactophilus*, *Serratia marcescens* and *Solitalea koreensis*. *Arthrobacter psychrolactophilus* is adapted to wide range of growth temperatures between 0°C and 30°C, and produced extracellular amylase (Smith and Zahnley, 2005). The capability of *Arthrobacter psychrolactophilus* to produce starch hydrolysing enzyme amylase allow breakdown of starch that converts substrates to glucose and/or oligosaccharides, while ß-amylase converts starch to maltose (Das and Varma, 2010). The roles and activities of amylase enzymes may be influenced by factors including vegetation, environment and soil types. Plants may influence the enzyme activity by directly supplying enzymes from their residues or excreted compounds, or indirectly provide substrate for the activities of microorganisms (Das and Varma, 2010). The abundance of *Arthrobacter psychrolactophilus* in uncooled soil aid in the breakdown of starch as energy source of soil microorganism that contribute to growth and nutrient uptake by loose leaf lettuce particularly in uncooled soils. As for *Serratia marcescens*, these species were also ubiquitous in soil as well as surface of plants. The presence of *Serratia marcescens* in rhizosphere is beneficial to plant growth and P supply in which these species display high mineral phosphate solubilizing activities (Chen *et al.*, 2006) that may contribute to high P availability in uncooled soils. In addition, these species also display important role in biotransformation of various compound including pesticides and chemicals (Salokhe and Govindwar, 1999). *Solitalea koreensis* grow optimally between 28°C and 30°C at pH 6 to 7. These species hydrolyse starch as well as tyrosine, and positive for *ß*-galactosidase (Weon *et al.*, 2009). The ability of *Solitalea koreensis* to produce *ß*-galactosidase aid in the breakdown of low-molecular-weight carbohydrates and produce glucose end product

that is vital in C cyling providing energy for soil microorganisms, as well as key indicators to evaluate the bioavailability of C in soil (Zhang *et al.*, 2015).

Brevundimonas diminuta and *Flavobacterium succinicans* were dominant in the cooled soil. *Brevundimonas diminuta* are frequently found in soils, glaciers, Antarctica, groundwater, rivers, sea, drinking water as well as endophyte bacteria, and has been characterized by limited predicted glycosyl hydrolase activity with only four genes encoding for ß-glucosidase activity (Talia *et al.*, 2012). These enzyme is vital for the hydrolysis and biodegradation of ß-glucosidase in plant debris that produce glucose, an important C energy source of soil microorganisms (Das and Varma, 2010). *Brevundimonas diminuta* also exhibited the attribute of plant growth promoting rhizobacteria (PGPR) including N-fixation and P solubilization (Rana *et al.*, 2011; Rana *et al.*, 2015) that contribute to the nutrient availability and lettuce growth in cooled soil. Likewise, *Flavobacterium* spp. is also found in soil, fresh and marine waters, sediments, microbial mats, glaciers as well as fish pathogens (Poehlein *et al.*, 2017). *Flavobacterium succinicans* has the capability to produce succinic acid that possess the ability to form complexes with positively charged ions such as Ca^{2+} and Fe^{3+} present in soil, and release P that is precipitated or occluded in the soil (Mardad *et al.*, 2013). The production of succinic acid contributes to plant growth, resistance to stress, and microbial growth in plant and soil that could participate in C cycling (Wu *et al.*, 2011). These species were also the only strain that reduces nitrate to nitrite (Bernardet, J. and Bowman, 2015). In addition, *Flavobacterium succinicans* has the ability to assimilate phosphite as a single P source that could be found in pesticide, supplemental fertilizer and biostimulant (Gómez-Merino *et al.*, 2015; Poehlein *et al.*, 2017) as a result of previous activities in MARDI, Cameron Highlands. The source of pesticide and fertilizer were contributed from the two projects conducted at sampling site which were the i) effects of pesticides on fungi population in Ringlet series soil (2012), and ii) organic project for green mustard, tomato and cabbage (2014/2015). The abundance of *Flavobacterium succinicans* in cooled soils may involved in the assimilation of pesticide and fertilizer from previous agricultural activities as a source of P for the lettuce growth.

Members of *Pseudomonas* was widely distributed in agricultural soils and possess various functional roles related to decomposition of organic matter, promotion of plant growth and also demonstrated pathogenic effects (Kwon *et al.*, 2003). In this study, *Pseudomonas umsongensis* was dominant in cooled soil at 1st growth but decreases during the 3rd growth. *Pseudomonas umsongensis* have been successfully isolated from strawberry root (Laili *et al.*, 2017). These species dominated the root and fix N_2, solubilize P and K, produce cellulase enzyme, siderophore as well as phytohormone. *Pseudomonas umsongensis* also influence the hydrolysis of arginine and nitrate reduction in soil that plays a key role in nitrogen distribution and recycling in plants (Kwon *et al.*, 2003). In contrast, *Pseudomonas viridiflava* showed higher abundance in cooled soils at the 1st and 3rd growth than uncooled soil. These pectinolytic species were reported to cause necrotic leaf, stem lesions as well as basal stem and root rots (Sarris *et al.*, 2012). *Pseudomonas viridiflava* was also reported to cause bacterial leaf spot disease in curly type lettuce (Aksoy *et al.*, 2018). The presence of these pathogenic species in cooled soil indicated the need of pesticides or antagonistic agent (Al-Karablieh *et al.*, 2017) in future agricultural activities under the soil cooling to supress pathogen development, although the pathogenic symptoms is less visible on lettuces grown on cooled soil.

Among microorganisms, some fungi have been found to play important role in soil ecosystem that contribute to the lettuce growth. In this study, identification of fungal species was limited to 1st growth due to failure in library preparation and purification of cooled soil of the 3rd growth. This is due to the presence of humic acid that interacts with template DNA and polymerase, which prevent enzymatic reaction even at low concentration (Schrader *et al.*, 2012). Nevertheless, as mentioned previously, ITS sequencing from soil before growth and the 1st growth were able to provide insight on the fungal community under the soil cooling, as well as the role of each species in soil functioning during the lettuce growth. ITS fungal sequencing reported *Mortierella sp VN2-2-*5, *Mortierella oligospora*, *Pseudalueria sp*, *Eocronartium sp* and *Trichosporon sp* from lettuces grown under soil cooling. *Mortierella sp VN2-2-5* was dominant in uncooled soils, while *Mortierella oligospora* was higher in cooled soil. Members of *Mortierella* are filamentous fungi that is commonly found in soil and often encountered as saprophytes (Kirk *et al.*,

2008). Members of *Mortierella* has been reported to increase soil K, Ca, Mg, P as well as Zn content through the production of oxalic acid from microbial decomposition of leaf litter in avocado plantations (Tamayo-Vélez and Osorio, 2018). The capability of *Mortierella* in producing organic acid may contribute to the release of P through phosphatase activity, as well as release of other nutrients through decomposition process in soil (Tamayo-Vélez and Osorio, 2018). *Mortierella sp. VN2-2-5* demonstrated the ability to degrade phenoxy acid compounds that have been widely used as a herbicide in agriculture as well as growth regulator on various crops ranging from potatoes to citrus fruit (Kennepohl *et al.*, 2010; Itoh *et al.*, 2013). In contrast, *Mortierella oligospora* showed dominant in cooled soils. These species was first discovered in soil of Dokdo in East Sea of Korea and was classified as zygomycetous fungi (Lee *et al.*, 2015). The functional role of *Mortierella oligospora* in soil was less discussed in the literature, but these Mortierellean species showed potential in biotransformations and endosulfan degrading capability in soil contaminated with organochlorine pesticide (Kataoka *et al.*, 2010; Lee *et al.*, 2015). The capability of *Mortierella sp.* to degrade herbicide and pesticide resulted to the higher abundance in the soil of lettuces grown under soil cooling. Besides *Mortierella*, agricultural projects conducted in MARDI also resulted to the abundance of *Pyrenochaeta lycopersici* in soil before growth as compared to other soils. *Pyrenochaeta lycopersici* is a filamentous ascomycete responsible for corky root rot disease of tomato as well as species of Eudicotyledones including melon, cucumber, safflower and spinach (Clergeot *et al.*, 2012). These species cause stunt growth and reduce yield by affecting the water and nutrient circulation of the infected plant (Golzar, 2009).

In this study, *Pseudalueria sp.*, *Trichosporon sp.* and *Eocronartium sp.* were also reported as dominant species in cooled soils. *Pseudaleuria sp.*, a member of phylum Ascomycota, was mostly abundant in healthy soils (Xu, 2011). These fungal species are nutritionally saprobic on soils and/or woods, or form ectomycorrhizal symbioses with host plants (Nedelin, 2014). Likewise, *Trichosporon sp.* is also saprophic and commonly found in temperate soil. These species have the ability to utilize polysaccharides that comprise of plant constituents (e.g. xylan, galactomannan) and products excreted by bacteria (e.g. dextran) as carbon and

energy sources (Middelhoven, 2005). *Eocronartium sp.* establishes a unique biotrophic relationship with temperate moss genera. This species was restricted to bryophytes which exploit the nutrient that pass through the transfer cells of moss (Frieders *et al.*, 2001). The abundance of *Eocronartium sp.* in cooled soils is due to the growth of mosses that coexist with lettuces grown in this study. However, the growth of lettuce was shown to be unaffected due to the abundance of *Eocronartium sp.* that subsequently parasitize the mosses and prevent nutrient competition with the lettuce.

Soil microorganisms play vital role in essential soil processes and functions including organic matter decomposition, nutrient cycling, soil structure formation, pest regulation and bioremediation of contaminants. Soil microorganisms serve as a biological indicator of soil health or soil quality in general besides than the physical and chemical indicators (Cardoso *et al.*, 2013). Soil health refer to ecological equilibrium and functionality of a soil and its capacity to maintain a well balanced ecosystem with high biodiversity above and below surface, and productivity (Cardoso *et al.*, 2013). Soil microorganisms indicate environmental changes through modification in quantity/biomass, structure and/or activity (Pulleman *et al.*, 2012). Each microorganism function in coordination with the overall soil microbiome to influence plant health and crop productivity. Plants are able to shape the soil microbiome through secretion of root exudates that involved in molecular communication during the plant development stage (Chaparro *et al.*, 2012). Therefore, identification of soil bacteria and fungi in this study allow understanding on the complex interrelationships between biological, physical and chemical components during the growth of loose leaf lettuce under the soil cooling.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This research marks the first insight of microbial diversity in soil of loose leaf lettuce grown under the soil cooling in a greenhouse in Malaysia. To the best knowledge, there are no published report on the study of soil temperature in relation to soil microbial diversity for the growth of temperate crops in Malaysia. In addition, this study also reported the possibility of the soil cooling as alternative of growing loose leaf lettuce or other temperate crops in lowland or greenhouse in Malaysia. Soil cooling system was able to prepare soil for the growth of the lettuce under tropical climate. A greenhouse with soil cooling system recorded air temperature between 26.0°C and 36.0°C during the lettuce growth. Under soil cooling, cooled soils showed temperature of 14.6 °C as the minimum and 20.1°C as the maximum, while uncooled soils recorded soil temperature between 26.4°C and 30.9°C. Model temperate crop, loose leaf lettuce grown on cooled soil showed higher average weight and positive development in the root, shoot and leaves for 3-growth cycle as compared to uncooled soil. Soil chemical analysis showed rapid changes in the availability and uptake of SOM, macro- (N, P, K, Ca and Mg) and micronutrients (Zn, Fe, Mn and Cu) during the 3-growth cycle. However, lettuces grown on cooled

and uncooled soil showed continues decrease in the root, shoot and leaves despite varying changes in the soil nutrients. To understand the effect of soil temperature on lettuce growth, soil microbial community was identified in cooled and uncooled soil upon harvesting. By using amplicon sequencing, soil bacteria reported *Arthrobacter psychrolactophilus*, *Serratia marcescens* and *Solitalea koreensis* as dominant species in uncooled soils, while *Brevundimonas diminuta* and *Flavobacterium succinicans* as dominat in the cooled soils. In addition, *Pseudomonas umsongensis* and *Pseudomonas viridiflava* were also identified in the soil of lettuces grown under soil cooling. As for soil fungi, result is limited to the 1st growth cycle due to failure in the preparation of sequencing library for cooled soil at the 3rd growth. Nevertheless, identification of soil fungi at 1st growth provide overview on the fungal diversity of the lettuce grown under the soil cooling which have never been reported in Malaysia. Sequencing result reported *Mortierella sp VN2-2-*5, *Mortierella oligospora*, *Pseudalueria sp*, *Eocronartium sp* and *Trichosporon sp* from lettuces grown under soil cooling, and *Pyrenochaeta lycopersici* from soil of MARDI, Cameron Highlands. Overall, soil bacteria identified in this study involved in decomposition, hydrolysing and breakdown of starch and low-molecular-weight carbohydrates, C and N cycling as well as phosphate solubilization, while soil fungi involved in the degradation and assimilation of herbicide and pesticide from previous agricultural activities in the MARDI, and several of the fungi were also identified as a plant pathogen. Identification of microbial diversity in lettuces grown under soil cooling provide general overview on the soil microbiome of the mountain agriculture of Cameron Highlands, Malaysia. Understanding the functional role of each microorganism allows effective monitoring of soil health to improve the yield of high quality temperate crops under soil cooling in Malaysia. This study also showed the potentiality of soil cooling as alternative of growing temperate crops on lowland or greenhouses in Malaysia, while preventing further illegal land clearing on mountain resort of Cameron Highlands as well as reducing the importation cost for temperate agricultural products.

5.2 Limitation and Recommendation

The present study has demonstrated the ability of soil cooling system to prepare soils to temperate soil temperature for the growth of temperate crops under tropical climate in a greenhouse without further addition of fertilizers throughout the growth cycle. Moreover, it also uncovers the overall community of soil microorganisms that involved in soil functioning during the growth of the lettuce which only possible at low soil temperature through sequencing approach, although almost 40% to 60% was unclassified possibly due to the lack of reference sequence in the database. However, gap in the current knowledge on soil bacterial and fungal diversity of temperate agricultural soils makes it difficult to understand the function of each microorganism during the crop growth. Hence, further studies are needed to identify the metabolic capabilities and disclose active functional genes of each soil microbial diversity during the growth of temperate crops at low soil temperature. Besides that, considering the importance of soil temperature for the production of temperate crops under tropical climate, these soil cooling systems could also be used for growing other temperate crop varieties including root crops (for example,carrots, radish, potato, beet, and others). The use of soil cooling for temperate agriculture under tropical climate on lowland or greenhouses allow production of temperate crops throughout the year. However, limitation of this study is that the current soil cooling system was only able to grow crops in a small-scale. In addition, fertilizers, pest management and other agricultural practices are also needed to produce large-scale marketable crops which are not the main concern of this study.

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APPENDIX

Appendix A: Soil was collected from 3 sampling points from agricultural sites in MARDI, Cameron Highlands at the depth of 20 cm using soil probe.

Appendix B: Soil physical properties of temperate agricultural soils in MARDI

Physical properties	
Sand (%)	89.1
Silt (%)	10.5
Clay (%)	0.4
Textural Class	Loamy Sand

Appendix C: Function of equipment in soil cooling system

Equipment (Model, Supplier)	Function
Chiller (DC-300, D-D The Aquarium Solutions)	- DC-300 chiller is a highly efficient compact refrigerant that have a low running noise, large refrigeration capacity that can cool water to any degree above 4°C in a short period of time. - The water chiller have a built in temperature memory system that ensures the selected temperature is maintained upon power failure. - The chiller has a cooling capacity of 300W and was able to refrigerate 50 to 300 litres of water at a flow rate of 200 to 1000 litres/hour.
 Custom-made chilled water storage tank with circulating pipes	- Custom-made chilled water storage tank store chilled tap water that was cooled by cold water pumped from DC-300 chiller. - Chilled water of constant temperature at 4±1°C was pumped through circulating pipes to adjacent pipes embedded within cooled soil plot.

<table>
<tr><td>
Cooled soil plot with embedded pipes</td><td>- Cooled soil plot was filled with eutrophic soils collected from MARDI, Cameron Highlands.
- The chilled water that flows through embedded pipes cooled the surrounding soils for the growth of temperate crops.</td></tr>
<tr><td>
Exhaust fan</td><td>- Exhaust fan provides a positive air movement through the greenhouse under all-weather condition.
- As the fan exhaust warm air, a slight vacuum was produced which draws in cooler air from the outside through the small openings of the greenhouse.</td></tr>
<tr><td>
Greenhouse</td><td>- Greenhouse protects soil cooling system and soil plots from all-weather conditions.</td></tr>
</table>

Appendix D: Materials and methods for the analysis of soil physical properties

Temperate eutrophic soils collected from Mardi, Cameron Highlands were analysed in terms of particle size distribution using a standard method of United States Department of Agriculture (USDA)/ Natural Resources Conservation Services Soils (NRCS) (Soil Survey Staff, 2009). Soil samples were sieved through 2.0 mm sieve, weighed and added with 50 ml of 30% hydrogen peroxide (R & M Chemical, Malaysia) before the mixture was left overnight to destroy organic matter. Water was added to the mixture and heated at 50°C to 60°C for 20 to 30 minutes. The mixture was then cooled prior to the addition of 20 ml of sodium hexametaphosphate (R & M Chemical, Malaysia) and shake for 2 hours. After 2 hours, mixture was transferred to 1000 ml measuring cylinder and mark up with distilled water. The percentage (%) of silt (<20 µm) and clay (<2 µm) were determined by mixing the sample vigorously for 30 seconds in a measuring cylinder using by plunger. The temperature of the sample was recorded and by referring to the settling charts (Table C.1), the time required to pipette out the clay and silt portion was determined. According to the settling chart, at 32°C, the time required to pipette clay and silt samples are 46 seconds after a vigorous mix at the depth of 20 cm. The pipetted sample was transferred into a pre-weigh beaker and left overnight in the oven at 105°C. Sample was then calculated to determine the weight of percentage silt and clay. The following day, temperature of sample in measuring cylinder was recorded and by using the other chart (Table C.2), depth of second pipetting was determined. By referring to the settling time and temperature of sample in the chart, the sample was pipetted at the depth of 25.2 cm, transferred to a pre-weigh beaker and left overnight in the oven prior to determination of clay. To determine the percentage of sand, the sample was poured in sink until it reached 200 ml and transferred to a beaker. The beaker was added with tap water, vigorously stir to ensure that the sample are suspended and poured into the sink. The water is added again and similar process was repeated until the water is clear. The sample was then placed in the oven for overnight and calculated for the percentage of sand. The final result of silt, clay and sand percentage was calculated and the textural class was determined using USDA Textural Class chart.

Table D.1: Pipetting sample (at the depth of 20 cm). For clay and silt (<0.060 mm)

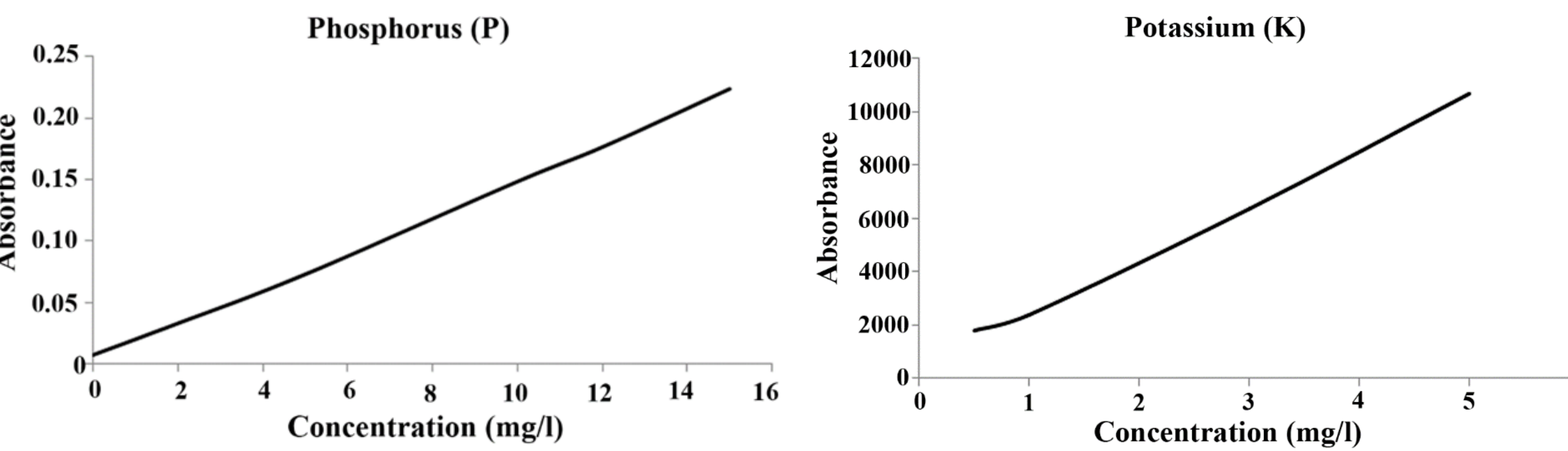
Temperature (°C)	23	24	25	26	27	28	29	30	31	**32**
Pipetting time (s)	56”	55”	54”	53”	52”	50”	49”	48”	47”	**46”**

Table D.2: Pipetting depth for clay (cm)

Time (h)	**Water temperature (°C)**											
	22	23	24	**25**	26	27	28	29	30	31	32	33
5	6.9	7.1	7.3	7.4	7.6	7.8	7.9	8.1	8.3	8.5	8.6	8.8
6	8.3	8.5	8.7	8.9	9.1	9.3	9.5	9.7	9.9	10.1	10.4	10.6
7	9.7	9.9	10.2	10.4	10.5	10.9	11.1	11.3	11.6	11.8	12.1	12.4
8	11.1	11.3	11.6	11.9	12.1	12.4	12.6	13	13.2	13.5	13.8	14.1
9	12.5	12.8	13.1	13.3	13.6	13.9	14.3	14.6	14.9	15.2	15.5	15.9
10	13.8	14.2	14.5	14.8	15.2	15.5	15.8	16.2	16.5	16.9	17.3	17.7
11	15.2	15.6	16.0	16.3	16.7	17	17.4	17.8	18.2	18.6	19	19.5
12	16.6	17.0	17.4	17.8	18.2	18.6	19.0	19.4	19.9	20.3	20.7	21.2
13	18.0	18.4	18.9	19.3	19.7	20.2	20.6	21.0	21.5	22.0	22.4	23.0
14	19.4	19.9	20.3	20.7	21.2	21.7	22.2	22.7	23.2	23.7	24.2	24.8
15	20.8	21.3	21.8	22.2	22.7	23.3	23.8	24.3	24.8	25.3	25.9	26.5
16	22.2	22.7	23.2	23.7	24.3	24.8	25.4	25.9	26.5	27.0	27.6	28.3
17	23.5	24.1	24.7	**25.2**	25.8	26.2	26.9	27.5	28.1	28.7	29.3	30.1

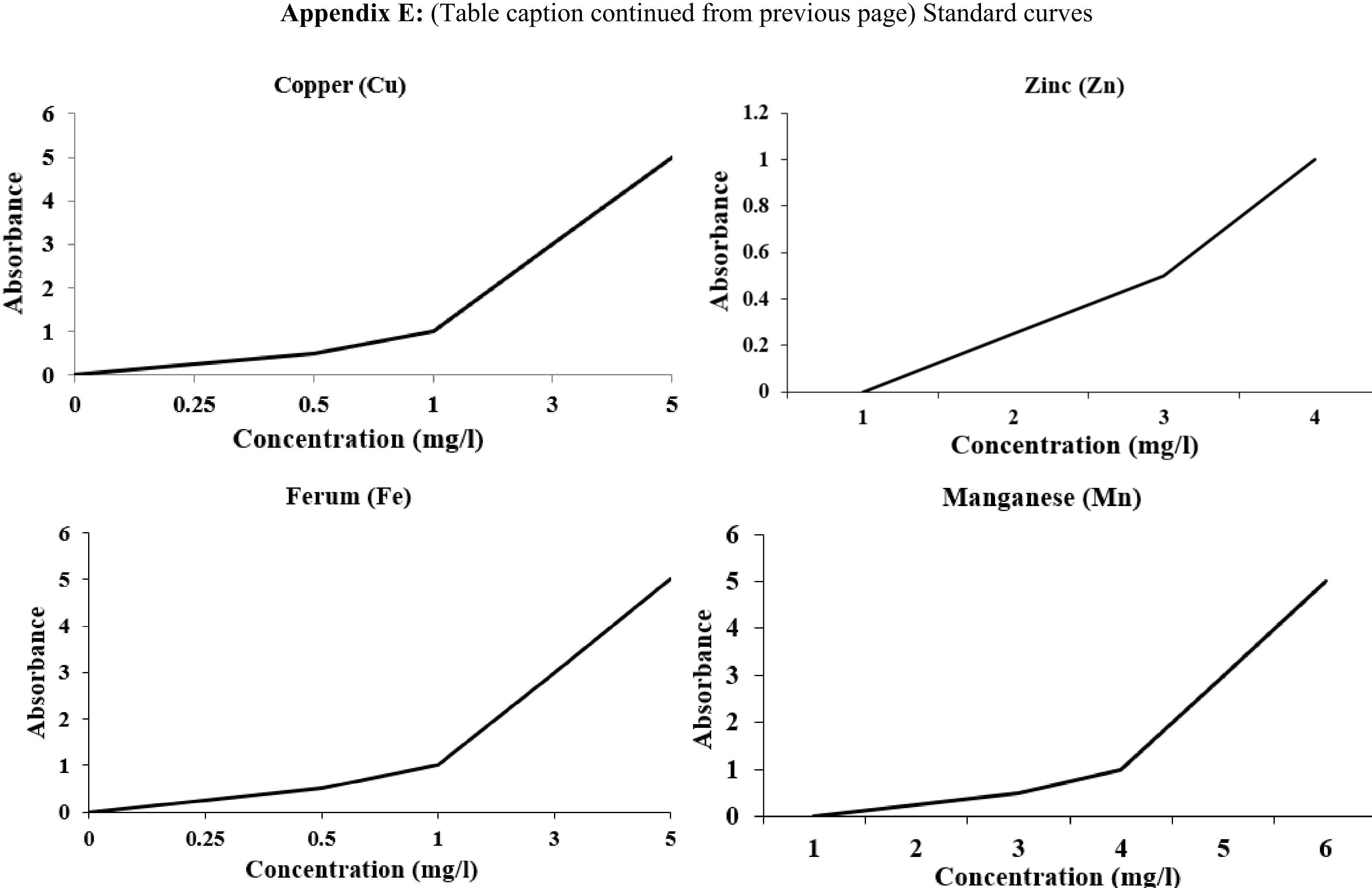
18	24.9	25.5	26.1	26.7	27.3	27.9	28.5	29.2	29.8	30.4	31.1	31.8
19	26.3	26.9	27.6	28.2	29.8	29.5	30.1	30.8	31.4	32.1	32.8	33.6
20	27.7	28.4	29.0	29.6	30.3	31.0	31.7	32.4	33.0	33.8	34.6	35.4
21	29.1	29.8	30.5	31.0	31.8	32.6	33.3	34.0	34.9	35.5	36.2	37.1
22	30.5	31.2	31.9	32.6	33.4	34.1	34.9	35.6	36.4	37.2	38.0	38.9
23	31.8	32.6	33.4	34.0	34.8	35.7	36.5	37.3	38.0	38.9	39.7	40.7

Appendix E: Standard curves (Table caption continued on next page)

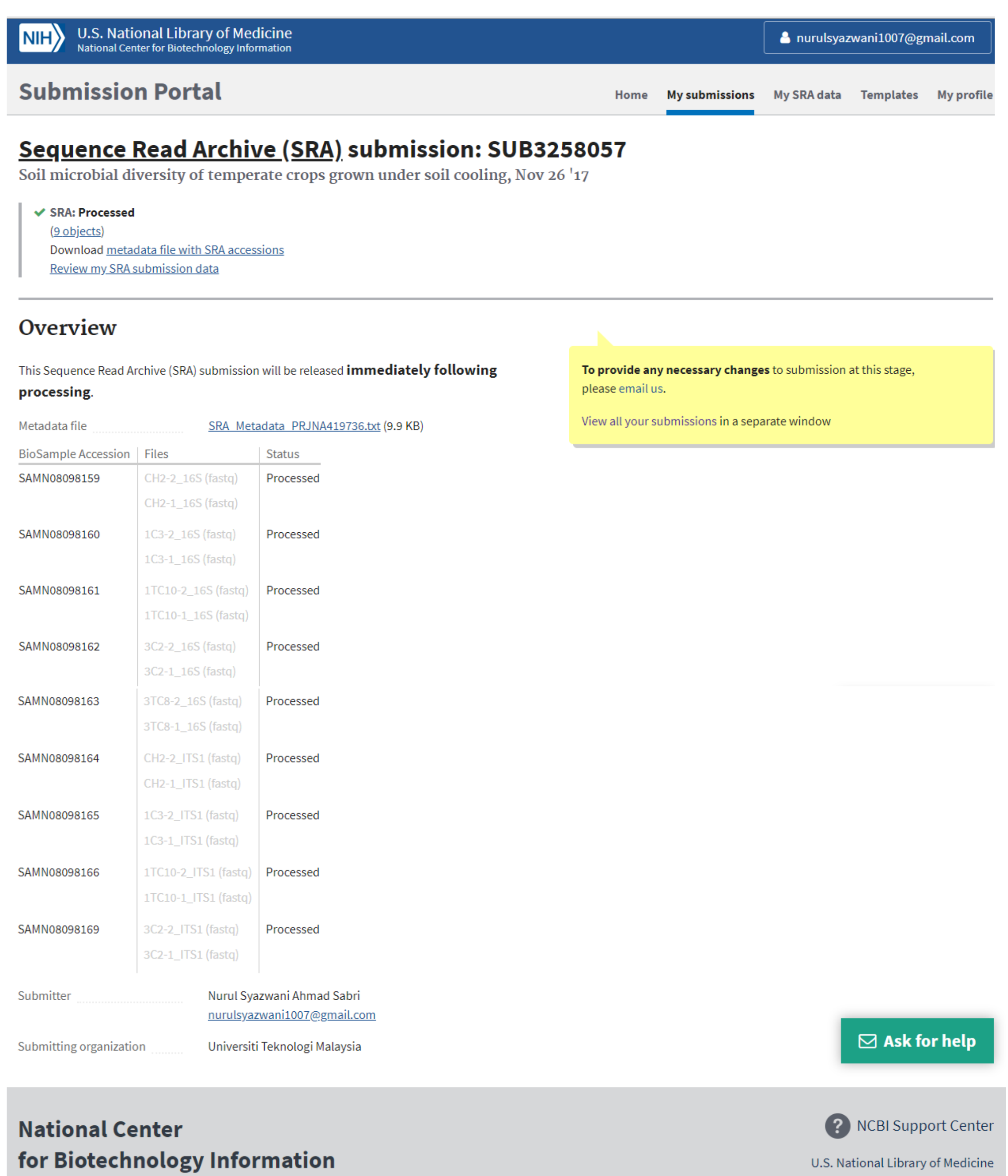


Note: Absorbance of K was measured using copper lamp that was increased in the energy level to wavelength for K, 766.5 nm. Standard curve was plotted upon stability in the energy level.

Appendix E: (Table caption continued from previous page) Standard curves



Appendix F: Submission of sequencing reads to NCBI



Appendix G: Data statistics

16S sequencing

#Sample Name	Reads Length (bp)	Raw Data (Mbp)	Adapter (%)	Nbase (%)	Ploy Base	Low Quality (%)	Clean Data (Mbp)	Data Utilization Ratio (%)	Raw Reads	Clean Reads	Read Utilization Ratio (%)
Before	250:250	343.97	0	0.088	0.055	0	343.07	99.74	687932*2	686149*2	99.74
I-Uncooled	250:250	387.65	0	0.088	0.053	0	386.7	99.76	775305*2	773407*2	99.76
1-Cooled	250:250	417.79	0	0.088	0.041	0	416.83	99.77	835577*2	833656*2	99.77
3-Uncooled	250:250	392.88	0	0.086	0.033	0	392.05	99.79	785763*2	784091*2	99.79
3-Cooled	250:250	387.92	0	0.094	0.031	0	387.05	99.78	775834*2	774097*2	99.78

ITS sequencing

#Sample Name	Reads Length (bp)	Raw Data (Mbp)	Adapter (%)	Nbase (%)	Ploy Base (%)	Low Quality (%)	Clean Data (Mbp)	Data Utilization Ratio (%)	Raw Reads	Clean Reads	Read Utilization Ratio (%)
Before	250:249	141.78	0	0.051	0.353	3.759	114.71	80.91	284132*2	259668*2	91.39
I-Uncooled	249:249	132.03	0	0.02	0.466	1.301	113.01	85.6	265118*2	255924*2	96.53
1-Cooled	244:243	151.85	0	0.014	0.762	10.68	114.5	75.4	311814*2	268100*2	85.98
3-Uncooled	246:243	136.18	0	0.015	0.015	2.299	114.6	84.15	278494*2	268287*2	96.33

Appendix H: Merge paired-end reads to tags

The following transcript merge paired end reads to single reads for downstream analysis.

```
C:\Users\user\Desktop\Analysis>flash CH2_1.fq CH2_2.fq --min-overlap=15 --max-overlap=250 --max-mismatch-density=0.1
[FLASH] Starting FLASH v1.2.11
[FLASH] Fast Length Adjustment of SHort reads
[FLASH]
[FLASH] Input files:
[FLASH]     CH2_1.fq
[FLASH]     CH2_2.fq
[FLASH]
[FLASH] Output files:
[FLASH]     ./out.extendedFrags.fastq
[FLASH]     ./out.notCombined_1.fastq
[FLASH]     ./out.notCombined_2.fastq
[FLASH]     ./out.hist
[FLASH]     ./out.histogram
[FLASH]
[FLASH] Parameters:
[FLASH]     Min overlap:           15
[FLASH]     Max overlap:           250
[FLASH]     Max mismatch density:  0.100000
[FLASH]     Allow "outie" pairs:   false
[FLASH]     Cap mismatch quals:    false
[FLASH]     Combiner threads:      4
[FLASH]     Input format:          FASTQ, phred_offset=33
[FLASH]     Output format:         FASTQ, phred_offset=33
[FLASH]
[FLASH] Starting reader and writer threads
[FLASH] Starting 4 combiner threads
[FLASH] Processed 25000 read pairs
[FLASH] Processed 50000 read pairs
[FLASH] Processed 75000 read pairs
[FLASH] Processed 100000 read pairs
[FLASH] Processed 125000 read pairs
[FLASH] Processed 150000 read pairs
[FLASH] Processed 175000 read pairs
[FLASH] Processed 200000 read pairs
[FLASH] Processed 225000 read pairs
[FLASH] Processed 250000 read pairs
[FLASH] Processed 275000 read pairs
[FLASH] Processed 300000 read pairs
[FLASH] Processed 325000 read pairs
[FLASH] Processed 350000 read pairs
[FLASH] Processed 375000 read pairs
[FLASH] Processed 400000 read pairs
[FLASH] Processed 425000 read pairs
[FLASH] Processed 450000 read pairs
[FLASH] Processed 475000 read pairs
[FLASH] Processed 500000 read pairs
[FLASH] Processed 525000 read pairs
[FLASH] Processed 550000 read pairs
[FLASH] Processed 575000 read pairs
[FLASH] Processed 600000 read pairs
[FLASH] Processed 625000 read pairs
[FLASH] Processed 650000 read pairs
[FLASH] Processed 675000 read pairs
[FLASH] Processed 686149 read pairs
[FLASH]
[FLASH] Read combination statistics:
[FLASH]     Total pairs:      686149
[FLASH]     Combined pairs:   674035
[FLASH]     Uncombined pairs: 12114
```

```
[FLASH] Percent combined: 98.23%
[FLASH]
[FLASH] Writing histogram files.
[FLASH]
[FLASH] FLASH v1.2.11 complete!
```

Appendix I: Tags statistics

16S sequencing

#Sample Name	Total Pairs Read Number	Connect Tag Number	Connect Ratio (%)	Average Length And SD	Tags Without Primer	Tag Utilization Ratio (%)	Average Length And SD
Before	686149	674035	98.23	253/0	-	-	-/-
1-Uncooled	773407	759326	98.18	253/0	-	-	-/-
1-Cooled	833656	818427	98.17	253/0	-	-	-/-
3-Uncooled	784091	770555	98.27	253/0	-	-	-/-
3-Cooled	774097	760395	98.23	253/0	-	-	-/-

Summary of Sample's Reads and Tags

5 Samples Clean Reads

Total pairs read number: 3851400

Average read number and SD: 770280/53173

5 Samples Tags

Total connect tag number: 3782738

Average connect tag number and SD: 756547/52096

Average tag length and SD: 253/0

ITS sequencing

#Sample Name	Total Pairs Read Number	Connect Tag Number	Connect Ratio (%)	Average Length And SD	Tags Without Primer	Tag Utilization Ratio (%)	Average Length And SD
Before	259668	253223	97.52	277/19	247872	95.46	235/18
1-Uncooled	255924	225531	88.12	282/17	219515	85.77	240/16
1-Cooled	268100	251770	93.91	291/28	237061	88.42	249/28
3-Uncooled	268287	267574	99.73	267/27	232494	86.66	229/25

Summary of Sample's Reads and Tags

4 Samples Clean Reads

Total pairs read number: 1051979

Average read number and SD: 262994/6195

4 Samples Tags

Total connect tag number: 998098

Average connect tag number and SD: 249524/17513

Average tag length and SD: 279/25

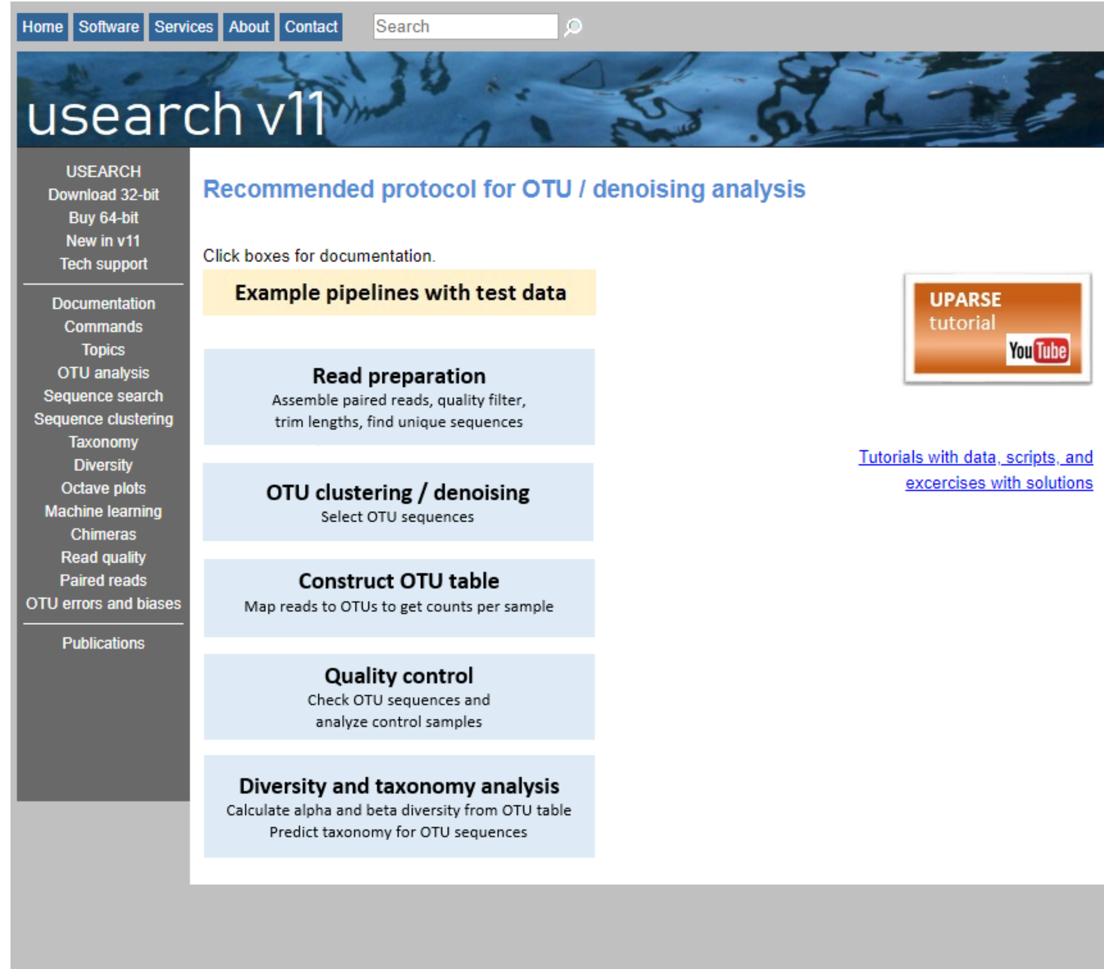
4 Samples Tags without primer:

Total noprimer tag number: 936942

Average noprimer tag number and SD: 234235/11742

Average noprimer tag length and SD: 238/24

Appendix J: Clustering of tags into OTU using UPARSE in USEARCH



The following transcript cluster tag into OTU using pipeline of USEARCH.

C:\Users\user\Desktop\Analysis\USEARCH>usearch.exe -fastx_info CH.Tags.fasta
usearch v11.0.667_win32, 2.0Gb RAM (6.3Gb total), 4 cores
(C) Copyright 2013-18 Robert C. Edgar, all rights reserved.
https://drive5.com/usearch

License: achiksonata@yahoo.com

00:06 42Mb 100.0% Processing
File size 181M, 674.0k seqs, 170.6M letters
Lengths min 250, lo_quartile 253, median 253, hi_quartile 253, max 334
Letter freqs G 36.6%, A 24.6%, C 19.9%, T 18.8%
0% masked (lower-case)

```
C:\Users\user\Desktop\Analysis\USEARCH>usearch.exe -fastx_uniques CH.Tags.fasta -sizeout -
relabel Uniq -fastaout uniques.fasta
usearch v11.0.667_win32, 2.0Gb RAM (6.3Gb total), 4 cores
(C) Copyright 2013-18 Robert C. Edgar, all rights reserved.
https://drive5.com/usearch

License: achiksonata@yahoo.com

00:03 241Mb   100.0% Reading CH.Tags.fasta
00:05 239Mb   100.0% DF
00:05 247Mb  674035 seqs, 197788 uniques, 168802 singletons (85.3%)
00:05 247Mb  Min size 1, median 1, max 18621, avg 3.41
00:09 238Mb   100.0% Writing uniques.fasta

C:\Users\user\Desktop\Analysis\USEARCH>usearch.exe -cluster_otus uniques.fasta -otus otus.fasta -
uparseout uparse.txt -relabel  Otu
usearch v11.0.667_win32, 2.0Gb RAM (6.3Gb total), 4 cores
(C) Copyright 2013-18 Robert C. Edgar, all rights reserved.
https://drive5.com/usearch

License: achiksonata@yahoo.com

00:28 57Mb    100.0% 4556 OTUs, 3204 chimeras

C:\Users\user\Desktop\Analysis\USEARCH>usearch.exe -  -uchime_ref otus.fasta -db gold.fa -
uchimeout results.uchime -strand plus

C:\Users\user\Desktop\Analysis\USEARCH>usearch.exe -otutab otus.fasta -otus otus.fasta -otutabout
otutab.txt -mapout map.txt
usearch v11.0.667_win32, 2.0Gb RAM (6.3Gb total), 4 cores
(C) Copyright 2013-18 Robert C. Edgar, all rights reserved.
https://drive5.com/usearch

License: achiksonata@yahoo.com

00:01 7.4Mb   100.0% Reading otus.fasta
00:01 6.2Mb   100.0% Masking (fastnucleo)
00:01 7.0Mb   100.0% Word stats
00:01 7.0Mb   100.0% Alloc rows
00:01 11Mb    100.0% Build index
00:08 150Mb   100.0% Searching, 100.0% matched
4556 / 4556 mapped to OTUs (100.0%)
00:08 150Mb  Writing otutab.txt
00:08 150Mb  Writing otutab.txt ...done.

C:\Users\user\Desktop\Analysis\USEARCH>usearch.exe -usearch_global CH.Tags.fasta -db
otus.fasta -strand plus -id 0.97 -uc readmap.uc -maxaccepts 8 -maxrejects 64 -top_hit_only
usearch v11.0.667_win32, 2.0Gb RAM (6.3Gb total), 4 cores
(C) Copyright 2013-18 Robert C. Edgar, all rights reserved.
https://drive5.com/usearch

License: achiksonata@yahoo.com

00:00 7.4Mb   100.0% Reading otus.fasta
00:00 6.2Mb   100.0% Masking (fastnucleo)
00:00 7.0Mb   100.0% Word stats
00:00 7.0Mb   100.0% Alloc rows
00:00 11Mb    100.0% Build index
02:19 51Mb    100.0% Searching, 84.3% matched
```

Appendix K: Analysis of alpha (α)-diversity by Mothur

Windows version

mothur v.1.41.1
Last updated: 11/26/18
by
Patrick D. Schloss

mothur > count.seqs(name=CH2.Tags.fasta)

It took 5 secs to create a table for 674035 sequences.

Total number of sequences: 674035

Output File Names:
CH2.Tags.count_table

mothur > unique.seqs(fasta=CH2.Tags.fasta)

Output File Names:
CH2.Tags.names
CH2.Tags.unique.fasta
----
mothur > summary.seqs(fasta=CH2.Tags.unique.fasta)

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	250	250	0	3	1
2.5%-tile:	1	253	253	0	4	4945
25%-tile:	1	253	253	0	4	49448
Median:	1	253	253	0	5	98895
75%-tile:	1	253	253	0	5	148342
97.5%-tile:	1	254	254	0	8	192844
Maximum:	1	334	334	0	9	197788

Mean: 1 253 253 0 4
# of Seqs: 197788

It took 6 secs to summarize 197788 sequences.

Output File Names:
CH2.Tags.unique.summary
----
mothur > align.seqs(fasta=CH2.Tags.unique.fasta, reference=core_set_aligned.imputed.fasta, processors=4)

Reading in the core_set_aligned.imputed.fasta template sequences... DONE.
It took 2 to read 4938 sequences.
Aligning sequences from CH2.Tags.unique.fasta ...

It took 497 secs to align 197788 sequences.

[WARNING]: 8 of your sequences generated alignments that eliminated too many bases, a list is provided in CH2.Tags.unique.flip.accnos.
[NOTE]: 2 of your sequences were reversed to produce a better alignment.

Output File Names:

```
CH2.Tags.unique.align
CH2.Tags.unique.align.report
CH2.Tags.unique.flip.accnos
----------------------------------------------------------------------------------------------------------
mothur > summary.seqs(fasta=CH2.Tags.unique.align)

                 Start   End    NBases  Ambigs  Polymer  NumSeqs
Minimum:         0       0      0       0       1        1
2.5%-tile:       2264    4050   253     0       4        4945
25%-tile:        2264    4051   253     0       4        49448
Median:          2264    4051   253     0       5        98895
75%-tile:        2264    4051   253     0       5        148342
97.5%-tile:      2266    4051   254     0       8        192844
Maximum:         6843    6849   305     0       9        197788
Mean:   2264     4051    253    0       4
# of Seqs:       197788

It took 110 secs to summarize 197788 sequences.

Output File Names:
 CH2.Tags.unique.summary

mothur > screen.seqs(fasta=CH2.Tags.unique.align, count=CH2.Tags.unique.count_table,
summary=CH2.Tags.unique.summary, start=2264, end=4051, maxhomop=8)


It took 99 secs to screen 197788 sequences, removed 13059.
----------------------------------------------------------------------------------------------------------
Running command: remove.seqs(accnos=CH2.Tags.unique.bad.accnos,
count=CH2.Tags.unique.count_table)
Removed 0 sequences from your count file.

Output File Names:
CH2.Tags.unique.pick.count_table

Output File Names:
CH2.Tags.unique.good.summary
CH2.Tags.unique.good.align
CH2.Tags.unique.bad.accnos
CH2.Tags.unique.good.count_table


mothur > filter.seqs(fasta=CH2.Tags.unique.good.align, vertical=T, trump=.)

It took 95 secs to create filter for 184729 sequences.
It took 38 secs to filter 184729 sequences.

Length of filtered alignment: 476
Number of columns removed: 7206
Length of the original alignment: 7682
Number of sequences used to construct filter: 184729


Output File Names:
CH2.filter
CH2.Tags.unique.good.filter.fasta

mothur > unique.seqs(fasta=CH2.Tags.unique.good.filter.fasta)

Output File Names:
CH2.Tags.unique.good.filter.names
```

CH2.Tags.unique.good.filter.unique.fasta
----

mothur > summary.seqs(fasta=CH2.Tags.unique.good.filter.unique.fasta)

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	476	249	0	3	1
2.5%-tile:	1	476	253	0	4	4619
25%-tile:	1	476	253	0	4	46181
Median:	1	476	253	0	5	92362
75%-tile:	1	476	253	0	5	138543
97.5%-tile:	1	476	254	0	8	180105
Maximum:	2	476	267	0	8	184723
Mean:	1	476	253	0	4	
# of Seqs:	184723					

It took 8 secs to summarize 184723 sequences.

Output File Names:
CH2.Tags.unique.good.filter.unique.summary
----

mothur > pre.cluster(fasta=CH2.Tags.unique.good.filter.unique.fasta,
name=CH2.Tags.unique.good.filter.names, diffs=1)

Total number of sequences before precluster was 184723.
pre.cluster removed 473 sequences.

It took 369 secs to cluster 184723 sequences.

Output File Names:
CH2.Tags.unique.good.filter.unique.precluster.fasta
CH2.Tags.unique.good.filter.unique.precluster.names
CH2.Tags.unique.good.filter.unique.precluster.map
----

mothur > chimera.vsearch(fasta=CH2.Tags.unique.good.filter.unique.precluster.fasta,
name=CH2.Tags.unique.good.filter.unique.precluster.names)

Reading file CH2.Tags.unique.good.filter.unique.precluster.temp 100%
46634569 nt in 184250 seqs, min 249, max 267, avg 253
Masking 100%
Sorting by abundance 100%
Counting unique k-mers 100%
Detecting chimeras 100%
Found 17 (0.0%) chimeras, 184232 (100.0%) non-chimeras,
and 1 (0.0%) borderline sequences in 184250 unique sequences.
Taking abundance information into account, this corresponds to
17 (0.0%) chimeras, 184711 (100.0%) non-chimeras,
and 1 (0.0%) borderline sequences in 184729 total sequences.

It took 1134 secs to check your sequences. 17 chimeras were found.

Output File Names:
CH2.Tags.unique.good.filter.unique.precluster.denovo.vsearch.chimeras
CH2.Tags.unique.good.filter.unique.precluster.denovo.vsearch.accnos

mothur > classify.seqs(fasta=CH2.Tags.unique.good.filter.unique.precluster.pick.fasta,
template=trainset6_032010.rdp.fasta, taxonomy=trainset6_032010.rdp.tax, cutoff=60)

Reading in the trainset6_032010.rdp.tax taxonomy... DONE.
Calculating template taxonomy tree... DONE.
Calculating template probabilities... DONE.
Classifying sequences from CH2.Tags.unique.good.filter.unique.precluster.pick.fasta ...

It took 1333 secs to classify 184233 sequences.

It took 18 secs to create the summary file for 184233 sequences.

Output File Names:
CH2.Tags.unique.good.filter.unique.precluster.pick.rdp.wang.taxonomy
CH2.Tags.unique.good.filter.unique.precluster.pick.rdp.wang.tax.summary
----
mothur > remove.lineage(fasta=CH2.Tags.unique.good.filter.unique.precluster.pick.fasta, name=CH2.Tags.unique.good.filter.unique.precluster.names, taxonomy=CH2.Tags.unique.good.filter.unique.precluster.pick.rdp.wang.taxonomy, taxon=unknown)

Output File Names:
CH2.Tags.unique.good.filter.unique.precluster.pick.rdp.wang.pick.taxonomy
CH2.Tags.unique.good.filter.unique.precluster.pick.names
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta
----
mothur > cluster.split(fasta=CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta, name=CH2.Tags.unique.good.filter.unique.precluster.pick.names, taxonomy=CH2.Tags.unique.good.filter.unique.precluster.pick.rdp.wang.pick.taxonomy, splitmethod=classify, taxlevel=4, cutoff=0.03, processors=8)

It took 841 seconds to split the distance file.
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.5.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.54.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.10.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.2.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.30.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.59.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.19.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.73.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.77.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.21.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.82.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.66.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.88.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.100.dist

Clustering CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.109.dist

Clustering CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.103.dist

Clustering CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.100.dist

Clustering CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.85.dist

Clustering CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.104.dist

Clustering CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.86.dist

Clustering CH2.Tags.unique.good.filter.unique.precluster.pick.pick.fasta.94.dist
It took 95 seconds to cluster
Merging the clustered files...
It took 2 seconds to merge.

Running command: sens.spec(cutoff=0.03,
list=CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.list,
column=CH2.Tags.unique.good.filter.unique.precluster.pick.pick.dist,
name=CH2.Tags.unique.good.filter.unique.precluster.pick.names)

NOTE: sens.spec assumes that only unique sequences were used to generate the distance matrix.

label cutoff numotus tp tn fp fn sensitivity specificity ppv npv fdr accuracy
mcc f1score
0.03 0.03 60557 9227048 16947355899 3134101 3537288 0.722877 0.999815
0.7464560.999791 0.746456 0.999607 0.734375 0.734477

It took 140 to run sens.spec.

Output File Names:
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.sensspec

-Done.

label cutoff numotus tp tn fp fn sensitivity specificity ppv npv fdr accuracy
mcc f1score
0.03 0.03 60557 9227048 16947355899 3134101 3537288 0.7229 0.9998 0.7465 0.9998
0.7465 0.9996 0.7344 0.7345

Output File Names:
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.dist
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.sabund
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.rabund
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.list
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.sensspec
----

mothur >
rarefaction.single(list=CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.list)

Output File Names:
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.rarefaction
----

mothur >
rarefaction.single(list=CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.list,
calc=sobs-chao-ace)

Output File Names:
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.rarefaction
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.r_chao
CH2.Tags.unique.good.filter.unique.precluster.pick.pick.opti_mcc.r_ace