# EVALUATION OF DEVELOPED FUNGAL-BACTERIAL BIOFILMS AS MICROBIAL AMELIORATORS FOR POTATO (*SOLANUM TUBEROSUM* L.) CULTIVATION

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# DECLARATION

I do hereby declare that the work reported in this project report/thesis was exclusively carried out by me under the supervision of Prof. G. Seneviratne and Dr. C.L Abayasekara. It describes the results of my own independent research except where due reference has been made in the text. No part of this project report/thesis has been submitted earlier or concurrently for the same or any other degree.

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# EVALUATION OF DEVELOPED FUNGAL-BACTERIAL BIOFILMS AS MICROBIAL AMELIORATORS FOR POTATO (*SOLANUM TUBEROSUM* L.) CULTIVATION

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Though chemical inputs have created devastating impacts on soil biological properties due to excessive and indiscriminate use, they seem necessary for crop production. Long term application of chemicals causes collapse of beneficial soil microbial communities, increase environmental pollution due to accumulation of soil toxicities. As potato tuber is entirely an underground plant part, there is a possibility to contaminate tubers with numerous soil toxins. Rhizosphere associated beneficial microbial inoculants such as biofilmed biofertilizers (BFBFs) have emerged as an environmental friendly biofertilizing method. Therefore, current study aimed to develop and assess BFBFs as an ameliorator for crop productivity of potato and to improve soil quality. In developing BFBFs, fungal-bacterial biofilms (FBBs) were first constructed using beneficial microorganisms isolated from the rhizosphere of potato crop cultivated at Regional Agriculture Research and Development Center, Bandarawela, Sri Lanka. Then, different combinations of the beneficial biofilms were applied to potato plants as biofertilizers (BFBF1 and BFBF2) in combination with chemical fertilizers (CF). Growth and biochemical responses of potato plants, soil quality amelioration and rhizoremediation of nitrosamine and heavy metals by different BFBF and CF treatments were evaluated under greenhouse and different agroclimatic conditions for tuberization (Bandarawela, Bibile and Padukka). Results showed that treatment BFBF1 + 50% CF increased tuber weight at Bandarawela and Padukka field sites compared to recommended CF (100%). BFBF1 + 50% CF showed the lowest soil and plant tissue pH, the highest soil organic carbon (SOC) and microbial biomass carbon (MBC), and the highest leaf chlorophyll and tissue calcium ($Ca^{2+}$) contents compared to the 100% CF at all three field sites. Further, soil $Ca^{2+}$ content was significantly enhanced by the same treatment compared to the 100% CF at Bandarawela and Bibile field sites. In addition, internal sugar and starch contents were altered by BFBF1 + 50% CF, inducing tuberization. The same treatment enhanced rhizoremediation of soil nitrosamines and heavy metals. Thus, it can be concluded that the exogenous, favorable biochemical and physiological conditions created by the BFBF1 induced internal biochemical mechanisms, leading to enhanced tuberization, while improving rhizo-remediation. Further, the climatic requirements for potato tuberization have been compensated by the biofilm microbial actions. The findings also confirmed that the CF can be reduced by 50% along with the developed BFBF, and this is an enormous environmental and economic gain that improves soil health.

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# LIST OF ABBREVIATIONS

AAS – Atomic Absorption Spectrophotometer

ABA- Abssisic Acid

ANOVA- Analysis of Variance

ARA- Acetylene Reduction Assay

BFBFs- Biofilmed Biofertilizers

BLAST- Basic Local Alignment Search Tool

BNF- Biological Nitrogen Fixation

C- Carbon

$Ca^{2+}$ - Calcium

CCM –Combine Carbon Medium

CF – Chemical Fertilizer

CFU – Colony Forming Unit

CRD – Complete Randomized Design

CTAB - Cetyl Trimethyl Ammonium Bromide

CZ- Czapek Dox

DNA – Deoxy Ribose Nucleic Acid

DOA – Department of Agriculture

EDTA- Ethylenediaminetetraacetic Acid

EPS – Extra Polymeric Substances

EtBr – Ethidium Bromide

FBBs – Fungal Bacterial Biofilms

FTIR- Fourier Transform Infrared Spectroscopy

GA – Gibberellic Acid

HBF – Higher Order Biofilms

IAA – Indole Acetic Acid

ITS – Internal Transcribed Spacer

K - Potassium

MBC- Microbial Biomass Carbon

MOP- Muriate of Potash

MR-VP- Methyl Red and Vogas Proskauer

$N/N_2$- Nitrogen

NA- Nutrient Agar

NB – Nutrient Broth

NTDMA- *N*-nitro dimethyl amine

NTMA- *N*-nitro methyl amine

OUT- Operational Taxonomic Unit

P- Phosphorus

PCR – Polymerase Chain Reaction

PD – Potato Dextrose

PDA – Potato Dextrose Agar

PGPRs – Plant Growth Promoting Rhizobacteria

RCBD – Randomized Complete Block Design

RDP- Ribosomal Database Project

RFLP- Restriction Fragment Length Polymorphism

RNA- Ribonucleic Acid

SDS- Sodium Dodecyl Sulfate

SOC- Soil Organic Carbon

SSU- Small Subunit

TAE- Tris-Acetate EDTA

TE-Tris- EDTA

TR- Treatment

TRFLP- Terminal Restriction Fragment Length Polymorphism

TSI- Triple Sugar Iron

TSP- Triple Super Phosphate

# CHAPTER 1

# OVERVIEW AND OBJECTIVES OF THE STUDY

## 1.1 Overview of the study

Application of Chemical Fertilizer (CF) in crop production has become a common practice and many governments even in developing countries resorted to provide heavy subsidies on fertilizers to meet the escalating food demand. Fertilizers are natural or manufactured chemicals containing nutrients known to improve the fertility of soils. When crops are grown under modern high-input systems, substantial amounts of nutrients are removed from the soil (Taiz and Zeiger, 2002). Then, to prevent deficiencies, nutrients can be added back to soil in the form of fertilizers. In the last few decades the rate of CF application has tremendously increased globally in crop production (Adesmoye and Kloepper, 2009). Since, yields of most crop plants increased linearly with the amount of fertilizer that they absorb (Loomis and Conner, 1992), agricultural sector is strongly depending on fertilization with mineral nutrients. This is very common practice in cultivation of annual crops including cash crops, vegetables and potato (*Solanum tuberosum* L.).

The importance of potato as one of the world's major staple crops is increasingly being recognized, because it produces more dry matter and protein per hectare than the major cereal crops. The nutritional value of potato tubers is a key factor for its progressive production, along with the economic benefits that potato cultivation can bring to developing countries (Van Gijessel, 2005; McGregor, 2007). Potato popularly known as 'The king of vegetables', is considered as one of the most important and leading cash crops in Sri Lanka after rice and few other crops like chilies, onion, maize etc. Potato cultivation is an integral part of the up country vegetable farming system because of its high income generating potential. Currently, in Sri Lanka around 6,000 hectares are cultivated with potato including Badulla (68%) and Nuwara-Eliya (38%) (Weerarathna, 2011). However, the annual potato production (approximately 70,000 MT) in Sri Lanka is not adequate to cater to the growing demand (140,000 MT) as a staple food (Department of Agriculture, 2013). Further, a sharp decline in potato production has been observed recently in Sri Lanka due to the increased cost of production and high prices of farm inputs like CF.

Generally the potato crop is considered as a heavy remover of soil nutrients and removes 1.5 times the amount of nitrogen (N) and 4 to 5 times the amount of phosphate compared to other vegetable crops ( Bansal and Trehan, 2011). Therefore as in most agricultural crops, large amounts CF inputs are usually used in potato cropping systems in order to enhance the yield. CF requirement for potato cultivation is around 1000 kg $ha^{-1}$ and from that N requirement is as high as 330 kg $ha^{-1}$ for an expected yield of 5000 kg $ha^{-1}$ (Davies *et al.*, 2005b; Lang *et al.*, 1999). Further, intensive potato cultivation on the steep slopes of up-country hills in Sri Lanka requires extremely high levels of agrochemicals (fungicides and pesticides) other than the CF to maintain high yields and profitability. According to DOA (Sri Lanka), 70 to 80% of farmlands in Welimada and Nuwara-Eliya have soil phosphate levels exceeding the agronomic critical level (30-40 ppm) which means that any further addition of phosphate fertilizer to these lands is an absolute waste, in economic terms, and crops do not respond to phosphate beyond this level (Bandara and Thiruchelvam, 2008). However, farmers do not follow the doses and frequencies recommended in the instructions, but apply higher doses more frequently, as they believe that this will increase yields. For instance, farmers who grow potato use as much as ten times the amount of CF and other agrochemicals recommended by the Department of Agriculture (DOA), Sri Lanka and the manufacturers in terms of enhancing crop yield. This can lead to reduced crop quality, increased environmental pollution and ultimately deterioration of soil quality.

Fertilizer application is required to replace crop land nutrients that have been consumed by previous plant growth with the ultimate goal of maximizing productivity and economic returns. Balanced application of CF may also contribute indirectly to an increase in soil microbial populations by increasing the quantity of crop residues returned to the soils (Rai *et al.*, 2014). For sustainable crop production, integrated use of chemical and organic fertilizer has proved to be highly beneficial (Seran, Srikrishnah and Ahamed, 2010). However, excessive use of CF and agrochemicals in conventional agriculture poses a major environmental threat globally to the functions of entire agroecosystems including reduced crop productivity by collapsing the soil microbial diversity (Seneviratne, 2009). The relationship between the soil microorganisms and crop productivity is very critical because the soil microbial diversity increases the soil fertility. Soil bacteria are more sensitive to chemical N fertilizer application during the plant growth cycle. Long term and irregular CF and agrochemical application in conventional agriculture also affect negatively to control aboveground disease and pests, possibly due to reduced microbial diversity (Seneviratne,

2009). Further, this irresponsible and harmful agricultural habits contribute to the accumulation of different compounds in soil (e.g. heavy metals, nitrosamines, mycotoxins) which are harmful for soil microorganisms and cultivated plants, and then for animals and humans (Seran *et al.,* 2010). The over application of N also represents an unnecessary economic expenditure for farmers leading to increased production cost. Therefore, there is an urgent need to reduce the usage of synthetic agrochemicals and CF and in turn increase the usage of non-CF while maintaining soil fertility through sustainable agricultural practices.

Sustainability in agriculture without compromising environmental quality, agro-ecosystem function and diversity conservation is among the major concerns of present day agriculture worldwide. To overcome the environmental problems resulting from extensive applications of CF, environmental friendly inputs such as natural and biological fertilizers provide sustainable solutions to improve soil fertility and plant growth. Biofertilizers are products containing living cells of different types of microorganisms, which have the ability to convert nutritionally important elements from unavailable to available form through biological process, such as $N_2$ fixation and solubilization of phosphate containing minerals (Narula *et al.,* 2000). Biological fertilizers are cost effective, ecofriendly and renewable sources of plant nutrients to supplement chemical fertilizers in sustainable agricultural systems.

Application of rhizosphere associated beneficial microbial inoculants like plant growth promoting rhizobacteria (PGPRs) as bio-fertilizers has been realized as an alternative option due to their promising role in sustainability. Evidence shows that maintenance of sustainable soil fertility depends greatly on the ability to harness the benefits of PGPRs (Wu *et al.,* 2013). Beneficial activities exerted by the microbial inoculants may be considered in three basic modes, i) as biofertilizers and soil ameliorators to improve the availability of mineral nutrients for plants; ii) as phytostimulators due to the production of phytohormones, and iii) as biocontrol agents to protect plants against phytopathogenic organisms. Studies suggested that PGPR-based biofertilizers can be used as effective supplements to CF and can reduce its use by many fold. PGPRs have been shown to increase plant yields 10 to 30% in many non-legume crops such as potato, radish, and sugar beet (Kloepper *et al*., 1980a, b). In recent years, studies have focused on the effects of microbial inoculation as biofertilizers on potato, including yield and quality enhancement (Duffy and Cassells, 2000; Yao *et al*., 2002). For

instance, application of microbial based biofertilizers has been reported to increase the production efficiency of potato seed tubers from minitubers and reduce the production costs (Davod *et al.,* 2011).

Direct application of microbial communities like biofilms has been introduced recently, and observed to be multi-functional and more effective than conventional microbial biofertilizers (Seneviratne *et al.*, 2011). In soil, beneficial bacteria like PGPRs, are frequently found to form micro-colonies or biofilm-like structures on plant roots (Bandara *et al.*, 2006). Biofilms are naturally existing complex communities of multiple microbial species (Lynch *et al.*, 2003), expressing enormous metabolic activity with great survival ability under adverse environmental conditions (Stewart, 2002). Different types of biofilms can be found naturally in the soil; bacterial (including *Actinomycetes*), fungal and fungal- bacterial biofilms (FBBs). The FBBs are formed on abiotic surfaces in the soil. In the case of non-filamentous fungi, both bacteria and fungi can act as the biotic surface (Seneviratne *et al.*, 2006). It is also reported that the self-produced extra cellular polymeric substances (EPS) play an important role in the colonization of bacterial biofilms by non-filamentous fungi. These biofilmed communities could be harmful/pathogenic or beneficial (Morikawa, 2006). However, beneficial biofilms that is being developed *in vitro* using rhizosphere associated beneficial microorganisms, can be used effectively as biofertilizers, known as biofilmed biofertilizers (BFBFs), to enhance plant growth including non-legumes and to ameliorate soil nutrients (Seneviratne *et al.*, 2009). Development of such biofertilizers provides a new mean in biofertilizer research to address current issues arising in the agriculture (Seneviratne, 2009).

However, the potential negative environmental impacts related to biofertilizer inoculation were always neglected. Since inoculation consists in supplying high densities of viable and efficient microbes for a rapid colonization of the host rhizosphere, it would induce at least a transient perturbation of the equilibrium of soil microbial communities. Changes in microbial composition may be undesirable if important native species are lost, thus affecting subsequent crops. Further, the potential of inoculating pathogenic microbial species in the form of biofertilizer can become a devastating effect for the entire soil ecosystem (Trabelsi and Mhamdi, 2013). Therefore, almost all the microbial inoculants should be undergone several pre- screening steps to select the correct beneficial inoculants to enhance the crop productivity and the soil amelioration.

Beneficial biofilms developed *in vitro* have already shown numerous favorable effects on agricultural and biotechnological applications (Seneviratne *et al.*, 2008). Formation of beneficial FBBs gives the biofilms enhanced metabolic activities compared to monocultures, through a range of mechanisms. As such, diverse forms of the FBBs inocula have been shown to improve nodulation and $N_2$ fixation in *Rhizobium*–legume symbiosis (Jayasinghearachchi and Seneviratne, 2004a), improve growth of non-legume plants by colonizing on plant roots (Seneviratne *et al.*, 2009), ameliorate soil nutrients (Jayasinghearachchi and Seneviratne, 2004b), produce higher acidity and plant growth-promoting hormones (Bandara *et al.*, 2006), generate bioactive compounds (Zavahir and Seneviratne, 2007), and increase the biodegradability of synthetic polymers (Seneviratne *et al.*, 2006). Interestingly, the BFBFs have significant biological functions over mono or mixed cultures of biofertilizers, on reducing CF dependency by 50% while ameliorating soil nutrients. For instance, a liquid formulation of BFBFs together with 50% of recommended CF for tea increased soil organic carbon (C) by 30%, compared to the application of 100% of the recommended fertilizers alone (Jayasekara *et al.*, 2008). Further, it has been reported that the application of BFBFs to agricultural soil induces dormant microbial cells, to be transferred them to active cells, thereby reinstate depleted soil microbiome and consequences the increased microbial diversity (Seneviratne and Kulasooriya, 2013). Diversity of soil microbes in soil-plant system assists in many roles since microbial diversity is the major indicator in soil quality and health to build up a robust soil and thus to lead onto sustainable agroecosystems (Tilman *et al.*, 1996). These views are similar with the previous report of Seneviratne *et al* (2011) to restore the degraded agroecosystems in the application of BFBFs in tea cultivation. Moreover, detoxification of toxins accumulated in the rhizosphere by the microbial communities has been reported in several studies (Mallik, 2006; Yan *et al*, 2007). Therefore, the effect of BFBFs on plant growth promotion, soil amelioration and rhizo-remediation open up avenues for the sustainability of agriculture sector in Sri Lanka with increased biological functioning which cannot be observed in the CF alone application.

## 1.2 Objectives of the study

Primary objective of the study

- To develop a BFBFs from rhizosphere associated beneficial fungal and bacterial species for crop improvement of potato.

Specific objectives

- To identify the microbial isolates obtained from potato rhizosphere.
- To screen the microbial isolates and produce beneficial BFBFs for potato crop.
- To evaluate the crop responses of potato for the developed BFBFs under greenhouse and different field conditions
- To evaluate the responsive BFBFs as a soil ameliorator for the enhancement of soil nutrients.
- To evaluate the influence of most effective biofilm on tuberization through biochemical, physiological and physicochemical processes of soil-plant systems.
- To evaluate the rhizo-remediation abilities of the responsive BFBFs on heavy metals and nitrosamine contaminations.

# CHAPTER 2

# ISOLATION AND IDENTIFICATION OF SOIL MICROORGANISMS FROM POTATO RHIZOSPHERE

## 2.1 Introduction

Soil microbial biomass represents the living portion of the soil inhabitant microorganisms which is the main driving force for most of the soil activities (Hirsch, Mauchline and Clark, 2010). The enormous phenotypic and genetic diversity found in soil microbial communities makes it one of the most difficult communities to study. Soil contains many microhabitats that are suitable for microbial growth. As a result, microorganisms are highly aggregated in soil existing in clumps or ''hot spots''. Microbes are aggregated in hot spots like nutrient rich top soil layer and the region around the plant root, which is known as rhizosphere (Gothwal *et al.*, 2007). Plants also influence the spatial distribution of soil bacteria (Wall and Virginia, 1999) and fungi. Roots secrete nutrients that stimulate microbial life and allow fast spreading of microbes through soils (Walker *et al.*, 2003). Further, the heterogeneity of the soil composition is also a factor for the determination of microbial diversity in a particular soil (Hirsch *et al.*, 2010).

An important consequence of the high diversity near the rhizosphere is an intense microbial activity with feedback effects on root development and plant growth in general. Most of these soil microbial communities naturally exist in the rhizosphere zone play an important role in crop production and have been used for sustainable agricultural development as growth promoters (Buddhika *et al.*, 2014). Their functions involve in different direct and indirect mechanisms by synthesizing growth promoting hormones, fixing atmospheric $N_2$, facilitating the uptake of mineral nutrients from the crop rhizosphere and preventing crops from pathogens and diseases. These beneficial soil microorganisms are classified as PGPRs (Hayat *et al.*, 2010).

The use of PGPRs has been found a potential role in developing sustainable systems in crop production (Sturz *et al.*, 2000; Hayat *et al.*, 2010). In this regard, soil naturally inhabitant PGPRs are highly focused as they are completely adapted to the environment and can be more competitive than the foreign microbial inoculants (Hayat *et al.*, 2010). Therefore,

naturally existing beneficial microbial strains adapted to the environment are now being used as microbial inoculants or as biofertilizers to reduce the use of CF in current agriculture (Karagoz *et al.*, 2012; Buddhika *et al.*, 2014). The advantage of using locally collected soil isolates is the easier adaptation and succession while showing beneficial activities when inoculated into the plant rhizosphere (Seneviratne, 2009). Microbial inoculants of PGPRs have been used for plant growth and development in different leguminous and non-leguminous crops for several decades (Bashan, 1998). It has been reported that 1-4% of microorganisms isolated from naturally existing potato rhizosphere showed significant plant growth promotion effects on potato i.e. enhancement of the stolon length, early tuber setting and enhancement of tuber yield (Suslow *et al.*, 1979). Moreover, another study has reported the importance of characterization for soil inhabitant microbial isolations for their beneficial effects on growth enhancement of sweet potato (Yasmin *et al.*, 2007). Rhizo-bacterial strains isolated from sweet potato have shown beneficial characters like higher indole acetic acid (IAA) production and phosphate solubilization (Yasmin *et al.,* 2007). Therefore, isolation of soil inhabitant microorganisms from the rhizosphere region is very important when evaluating the beneficial activities. On the other hand, characterization and identification of these bacteria are necessary for wide ecological studies of the plant rhizosphere.

Over the first half of the $20^{th}$ century, numerous approaches for the identification and classification of bacteria have been entertained. Traditionally, microbial diversity was assessed using selective plating and direct viable counts. These methods are fast, inexpensive and can provide information on the active, heterotrophic component of the population. Limitations include the difficulty in dislodging bacteria or spores from soil particles, growth medium selections (Tabacchioni *et al.*, 2000; Krick *et al.*, 2004), growth conditions (temperature, pH, light), the inability to culture a large number of bacterial and fungal species with current techniques and the potential for colony–colony inhibition or of colony spreading (Trevors, 1998; Krick *et al.*, 2004). Phenotypic grouping of closely related species based on colony morphology, fatty acid composition and physiological characteristics is often misleading (Coorevits *et al.*, 2008). However, phenotypic approach is time consuming, particularly for analysis of diversity in complex environments, such as soil, and when quantification of measures of diversity is required. Those methods can allow identification of bacteria to the genus level, or minimize the probability that they belong to other groups.

When microscopy and culturing methods alone are not adequate enough to identify a species, specific biochemical tests are carried out. In order to have a definitive identification of bacteria, microbiologists have developed a series of biochemical tests that can be used to differentiate even closely related organisms. Biochemical identification uses chemical and biochemical markers to differentiate the products obtained by the metabolism from bacteria. Unambiguous identification of microorganism can be obtained by most of the biochemical tests in conjunction with a dichotomous trees (Willey, Sherwood and Woolverton, 2008). These may include testing for products the bacterium may produce (due to a presence of specific enzyme/s) or even their ability to grow on either selective or differential media or a combination of the two. The shape of the bacteria can be determined by microscopy, and culturing of the bacteria on various media – selective, differential and certain characteristic (metabolic) media. Selective media only allow certain bacteria to grow whereas differential media are used to distinguish bacteria from others in the presence of some form of dye or indicator (Madigan *et al.,* 2009). With biochemical identification, bacteria can be classified to species level, however the level of classification can vary between groups (Baron, 1996; Fox, Wisotzkey and Jurtshuk, 1992).

### 2.2 Objectives

1. To isolate fungi and bacteriafrom potato rhizosphere.
2. To isolate diazotrophic bacteria.
3. To identify the isolated microorganisms up to their genus level.

## 2.3 Methodology

### 2.3.1 Isolation of bacteria and fungi from potato rhizosphere

#### 2.3.1.1 Selection of a suitable location for sample collection and field activities

The ultimate aim of this research was to develop a microbial biofetilizer from soil inhabitant microorganisms to enhance the crop productivity of potato. Therefore, the isolation of beneficial microorganisms from soil was considered as one of the key factors in this research. Since potato cultivation is restricted to certain areas with low atmospheric temperature at night, the selection of a suitable location to isolate soil microorganisms which are adapted to the relevant soil climatic conditions was another key consideration. Therefore, a field site at Regional Agriculture Research and Development Center, Bandarawela, was selected as a suitable site to collect soil samples and to conduct field experiments of this research.

#### 2.3.1.2 Collection of soil samples

Soil samples and plant specimens (*Solanum tuberosum* L.) were collected randomly from five locations in an abandoned potato crop land (the location had been used for organic potato cultivation before two years and the vegetation type at the time of the harvest was entirely wild grasses) located at the Regional Agriculture Research and Development Center, Bandarawela, (6° 48' 0" N, 80° 58' 0" E) [up country intermediate zone ($IU_3$) 1506 m amsl, rainfall 1100 mm-1400 mm], Sri Lanka. Soil samples were collected from 0-15 cm top soil layer using a metal corer of 5 cm diameter.

#### 2.3.1.3 Isolation of fungi and bacteria

Disease free 'Granola' potato seed tubers were obtained from the Regional Agriculture Research and Development Center, Bandarawela and were grown in black plastic pots (diameter- 6 inch) with the soil samples. All pots were kept under greenhouse conditions (average day temperature 30 °C and average night temperature 19 °C) until the establishment of root system. Watering was done two times a day till harvesting. After eight weeks from seed sowing, plants were uprooted carefully and soil samples and root parts were collected and placed in black polythene bags separately.

Thereafter, samples were brought to the laboratory of the Microbial Biotechnology Unit (MBU), Institute of Fundamental Studies (IFS), Kandy, Sri Lanka for the isolation of rhizosphere associated microorganisms.

Isolation of microorganisms, attached to the root surface, was performed by dipping the root portions in potato dextrose (PD) and nutrient broth (NB) media separately for four days. Further isolation was carried out using streak plate technique on NA and PDA media. Microorganisms in soil samples were isolated using dilution plate technique on same nutrient media as follows. Ten grams of soil from each random sample was aseptically weighed and transferred to a flask with 100 ml sterile water, and was shaken for 30 min in an orbital shaker. Immediately after shaking, the soil samples were serially diluted (10 fold-pipetting 1 ml aliquots into 9 ml sterile water. The final dilution was 100- fold; 0.1 ml of each dilution of the series was placed onto a Petri dish.) and inoculated on sterile nutrient agar [NA, (Himedia™, India,) 20 g per liter of medium] and PDA plates for bacteria and fungi respectively. Three replicate dishes were made for each dilution (Karagoz *et al.*, 2012; Afzal, Shazad and Un Nisa, 2013). Then, all inoculated plates were incubated for 24 hours at 33 °C. That specific temperature was the optimum temperature for the isolated bacterial (measophylic) samples (Pietikainen, Pettersson and Baath, 2005: Tourna *et al.*, 2008). Bacterial colonies were differentiated on the basis of colony morphology and pigmentation. Colonies were sub-cultured on agar-based subculture medium plates by streaking technique and re-incubated at 33 °C for 48- 72 hours. This isolation process was carried out by further sub-culturing on agar medium until monocultures were obtained. Monocultures were inoculated on the agar-based culture medium slant in the test-tube (12 ml) and incubated at 33 °C for 48-72 hours followed by storing at 4 °C in a refrigerator (Herath *et al.*, 2013; Nhu and Diep, 2014). Single spore isolation technique was used to prepare fungal pure cultures. Spores of the isolated fungi were picked up directly from the culture using a fine sterile needle and then placed on a sterilized PDA medium. Subsequently, all inoculated plates were incubated for 3-4 days at 30 °C. Purified samples were preserved on agar slants and were stored at 4 °C.

#### 2.3.1.4 Isolation of diazotrophic bacteria

Bacterial isolates on NA media were transferred to combine carbon medium (CCM, Koomnok *et al.*, 2007), a selective modified N free medium for $N_2$ fixing microorganisms (pH= 6.8). The inoculated plates were incubated for five days at 33 °C. Sub culturing was performed with the same medium using streak plate technique to maintain the cultures.

### 2.3.2 Identification of isolated soil microorganisms

#### 2.3.2.1 Morphological and biochemical identification of bacterial isolates

Colony characters were recorded for the pure bacterial isolates inoculated on NA medium. Microbiological and biochemical analysis were carried out for the isolated microorganisms in order to identify them. Biochemical identification was performed using different biochemical experiments such as Gram's staining, catalase test, citrate utilization test, triple sugar iron (TSI) test, urease test, oxidase test, indole test, methyl red and vogas proskauer (MR-VP) test and motility test (Buchanan and Gibbons, 1974). All tests were performed for 24 hours old bacterial pure cultures grown on NA.

##### 2.3.2.1.a Gram's Staining

Smears were prepared from each isolated pure cultures and were heat fixed. Then the cultures were stained with primary stain (crystal violet) followed by addition of mordant (Gram's iodine) which binds to crystal violet and traps it in the cell. Then stain was decolorized with 90% alcohol for a few seconds. Finally the smear was counterstained for three minutes with safranin (2.5%) followed by washing with water. The prepared smear was observed through the oil immersion lens of the light microscope.

##### 2.3.2.1.b Catalase test

Cells from the center of a colony were transferred to a glass slide with a sterilized loop followed by the addition of two drops of 3% $H_2O_2$. Visual observation were recorded for the rapid emergence of bubbles with in one to two seconds (Abdulkadir and Waliyu, 2012). Bubble formation would indicate a positive result of this reaction taking place.

#### 2.3.2.1.c Citrate test

Isolated colonies were inoculated on Simmons citrate agar using a sterilized loop and incubated for 24- 72 hours at 35 °C. Colour change of the medium was recorded after the incubation period (Abdulkadir and Waliyu, 2012).

#### 2.3.2.1.d TSI test

Isolated colonies were inoculated on TSI slants by first stabbing the butt down to the bottom and then streaked the surface of the slant and incubated at 35 °C. Colour change of the medium and gas accumulation at the bottom of the medium were recorded after the incubation period (Abdulkadir and Waliyu, 2012).

#### 2.3.2.1.e Urease test

Isolated colonies were inoculated on urea agar slants (20 g/l of urea, 9.5 g/l of $Na_2HPO_4$, 9.1/l g of $KH_2PO_4$, 0.1 g/l of yeast extract and 0.01/l g of phenol red. The pH was made to 6.8 ± 0.2 at 25 °C) and incubated at 35 °C. Colour change was observed after 48 hours (Abdulkadir and Waliyu, 2012).

#### 2.3.2.1.f Oxidase test

A drop of oxidase regent was applied on the isolated cultures streaked on a filter paper. Purple colour appearance at the culture streaked regions was recorded within 30 seconds after adding oxidase regent (Abdulkadir and Waliyu, 2012). The oxidized reagent forms the colored compound indophenol blue which is the positive response (Behera *et al.,* 2014).

#### 2.3.2.1.g Indole test

A loop of each pure bacterial culture was inoculated into sterile peptone broth and incubated at 35 °C for 48 hours. Following incubation, five drops of Kovac's reagent (isoamyl alcohol, *p*-Dimethylaminobenzaldehyde, and concentrated hydrochloric acid) were added to the culture broth and the formation of a purple ring on the surface was observed (Abdulkadir and Waliyu, 2012).

### 2.3.2.1.h MR-VP test

Five milliliters of fresh MR-VP medium was pipetted in to a test tubes and the tubes were inoculated with fresh (18 - 24 hours) pure bacterial culture followed by incubation for 48 hours at 35 °C.

MR test- 2.5 ml of the incubated culture was transferred into a new sterile culture tube followed by adding five drops of the methyl red reagent. Colour change of the medium was observed and compared with the control.

VP test- 0.6 ml of Barritt's reagent A and 0.2 ml of Barritt's reagent B were added to the remaining 2.5 ml of the MR-VP culture medium and mixed the tube for one miniute to expose the medium to atmospheric oxygen. Colour change was observed after keeping the tube to stand for at least 30 minutes (Buchanan and Gibbons, 1974).

### 2.3.2.1.i Motility test

Wet mount was prepared by placing 0.5 ml of each bacterial culture on a clean glass slide. The prepared slide was observed through the light microscope to identify the motile bacteria (Buchanan and Gibbons, 1974).

### 2.3.2.2 Morphological and microscopic observations of fungal isolates

Isolated fungal colonies were transferred to czapek dox (CZ) medium, a specific medium for fungi (Afzal *et al.*, 2013) in triplicates and incubated for 48-72 hours at 30 °C. After the incubation, plates were observed for macroscopic characteristics such as colony diameter, colour changes of the media due to exudates and microscopic characteristics for the presence of conidiophore, vesicle, phialides and conidia. For microscopic characteristics slides were stained with cotton blue and mounted in Lactophenol (Afzal *et al.*, 2013). Fungal identification guides were used to identify the fungal isolates (Dugan, 2008).

## 2.4 Results and Discussion

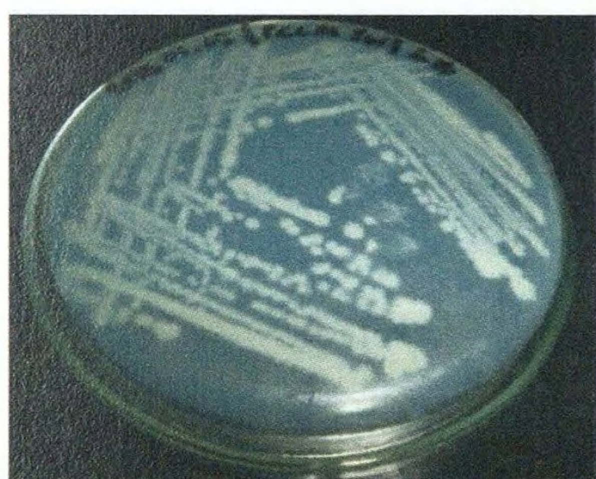
### 2.4.1 Isolation of microorganisms

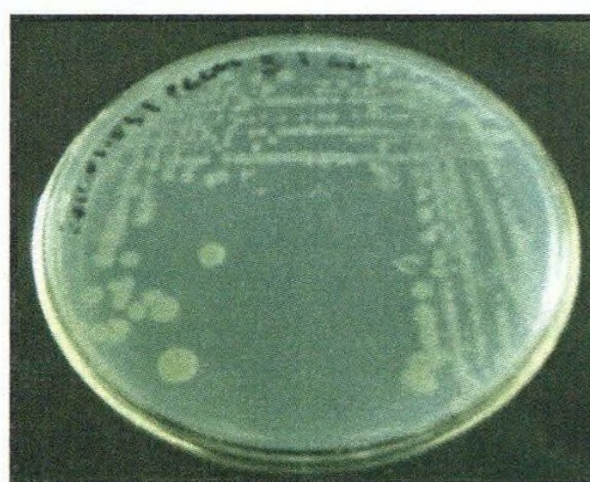
Bacterial colonies were observed ($10^{-6}$ dilution plate) on NA plates after 24 hours of incubation. Fourteen different bacterial isolates and three fungal isolates (F1, F2 and F3) were isolated from potato rhizosphere region using NA and PDA media. Out of them, eight bacterial strains (B1, B2, B3, B4, B5, B6, B7 and B8) were selected as diazotrophic bacteria through screening with CCM.

### 2.4.2 Identification of isolated microorganisms

#### 2.4.2.1 Morphological identification of bacterial isolates

Clear isolated bacterial colonies were observed on CCM plates after five days from incubation (plate 2.1). The isolates were categorized according to the colour change of the medium from green (pH 6-7.6) to blue (pH >7.6) or yellow (pH< 6) due to the acidic or basic nature of different bacterial isolates (Colour change is due to Bromothymol blue indicator in CCM).





**(a)** **(b)**

Plate 2.1- Bacterial growth pattern on CCM plates. (a)- Bacterial isolates with basic secretions. (b)- Acidic bacteria grown on CCM plates.

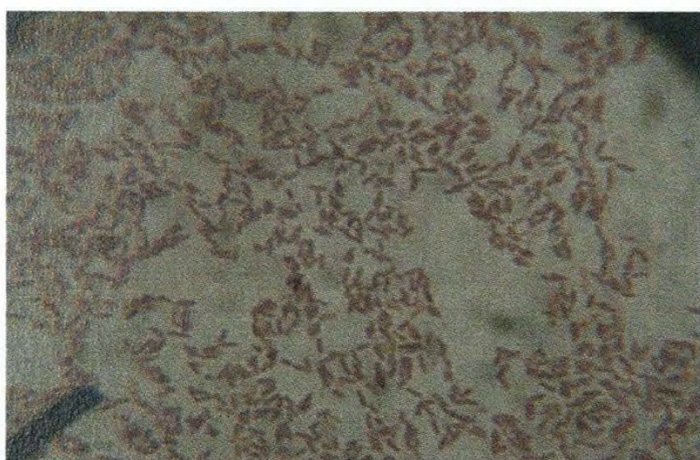
CCM is a modified N free medium which can be used intensively to screen diazotrophic microorganisms (Koomnok *et al.*, 2007). It has been reported that the bacteria especially PGPRs and some fungi can produce plant growth promoting hormones such as IAA and other complex organic acids like gluconic and ketogluconic acids which can create acidic environment around the rhizosphere (Blume *et al.*, 2002). In the current study showed that all bacterial isolates except the isolates B2, B4 and B6, changed the colour of the medium

from green to yellow. This could be the reason of producing different organic or inorganic acids and acidic derivatives to the medium. Further, following observations were made with reference to colony morphology and the Gram's staining of the bacterial isolates on NA plates.

Six bacterial isolates (B2, B3, B4, B5, B6 and B7) were identified as Gram's negative bacteria whereas two bacterial isolates (B1 and B8) were identified as Gram's positive rod shape bacteria (table 2.1). Except for B5 (plate 2.2b), all other Gram negative isolates were rod shape bacteria.

Table 2.1- Colony characteristics and the Gram's staining of different bacterial isolates

| Bacterial strain | Colony characters | Gram's staining and the shape |
|---|---|---|
| B1 | White creamy colonies | Positive, short rod |
| B2 | opaque creamy colonies | Negative, rod |
| B3 | opaque creamy colonies | Negative, rod |
| B4 | Off white colonies | Negative, rod |
| B5 | White creamy colonies with brown pigments | Negative, Cocci |
| B6 | Grayish white gummy colonies | Negative, rod |
| B7 | White creamy colonies | Negative, rod |
| B8 | Grayish white rough colonies | Positive, Rod |



(a)

(b)

Plate 2.2- Microscopic view of the bacterial monocultures after Gram's staining (Magnification 1000X). (a)- Gram's negative rod shape bacteria (B7). (b)- Gram's negative cocci shape bacteria (B5).

It has been reported that microbial communities generally shift from greater Gram's negative dominance at the soil surface to greater Gram's positive dominance at deeper soil depths (Fierer, Schimel and Holden, 2003). Most of the rhizobacteria are rod shape Gram's

negative bacteria which are concentrated in the rhizosphere, (Blume *et al.*, 2002). Availability of Gram's positive rods or cocci bacteria are lower near the rhizosphere (Bhattacharyya and Jha, 2012). It has been recorded that the characterization of the isolates on the basis of their fatty acid methyl esters profiles revealed the greater abundance of Gram's negative bacteria within rhizosphere soils (Karagoz *et al.*, 2012).

### 2.4.2.2 Biochemical identification of bacterial isolates

All bacterial isolates were considered as aerobic or facultative anaerobic bacteria since they positively responded for catalase test. Catalase test has been reported to be used to detect the presence of catalase enzymes by the decomposition of $H_2O_2$ to release oxygen and water. The ability of bacteria to produce catalase is an important biochemical characteristic and this characteristic can be used for the identification (Madigan *et al.*, 2009). Bi-functional enzymes combining both catalatic and peroxidatic activities known as catalase-peroxidase are found in aerobic and facultative bacteria and marginally in some fungi and some protists (Za mocky, Furtmuller and Obinger, 2008).

Out of all bacterial isolates, B1, B3, B4, B6, B7 and B8 isolates were categorized as aerobic isolates due to the positive responses given for Oxidase test (table 2.2). Oxidase positive microorganisms contain cytochrome C oxidase enzyme and therefore can utilize oxygen for energy production with an electron transfer chain. The cytochrome system is only present in aerobic organisms which are capable of utilizing oxygen as the final hydrogen receptor (Forchetti *et al.*, 2007). The oxidase reagent contains N,N,N,N'-tetra-methyl-p-phenylenediamine dihydrochloride which acts as an artificial electron donor for the enzyme oxidase.

Bacterial isolates B2, B3 and B7 showed organic acid production since giving positive responses for TSI test (plate 2.3). TSI test reflects the ability of the production of organic acids by microorganisms through the fermentation which change the color of the medium red pH-sensitive dye (phenol red) to a yellow color. Further, all bacterial isolates were considered as motile isolatesexcept B2 strain since they positively responded for the motility test. According to all biochemical experiments, bacterial isolates B1, B2, B3, B5 and B7 were identified as *Bacillus* sp., *Acinetobacter* sp., *Acidomonas* sp., *Serratia* sp. and *Rhizobium* sp. respectively. Further, bacterial isolates B4 and B6 were identified

*Pseudomonas* sp. Therefore, all most all isolates can be considered as common soil bacteria associated with the rhizosphere. Detailed identities of bacterial isolates B1 and B7 were mentioned in chapter five.

Table 2.2- Responses shown by different bacterial isolates for biochemical experiments

| Test | Bacterial Isolates | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 |
| Catalase test | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Oxidase test | ✓ | x | ✓ | ✓ | x | ✓ | ✓ | ✓ |
| Citrate test | ✓ | x | x | ✓ | ✓ | ✓ | x | ✓ |
| TSI test | x | ✓ | ✓ | x | x | x | ✓ | x |
| Urease test | x | x | ✓ | ✓ | x | ✓ | ✓ | x |
| Indole test | x | x | x | x | x | x | ✓ | x |
| MR test | ✓ | x | x | x | x | x | x | ✓ |
| VP test | ✓ | x | x | x | ✓ | x | x | ✓ |
| Motility test | ✓ | x | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |

Positive Negative

(a)

Negative Positive

(b)

Plate 2.3- Responses of different bacterial isolates on TSI test and urease test. (a)- Biochemical responses for TSI test. (b)- Biochemical responses for urease test.

### 2.4.2.3 Morphological and microscopic identification of fungal isolates

Fungal colonies of F1 and F2 were observed on CZ plates after six days from inoculation. Fungal colonies of isolate F3 were observed after nine days from inoculation. According to the colony characteristics and microscopic observations (table 2.3), fungal strain F1, F2 and F3 were identified as *Aspergillus* sp., *Trichoderma* sp. and *Phytopthora* sp. respectively (Dugan, 2008). Morphological observations and the microscopic observations for the fungal isolates are illustrated in table 2.4, plate 2.4 and plate 2.5.

Table 2.3- Colony characteristics of the fungal isolates

| Fungal isolate | Observations |
|---|---|
| F1 | Light brown colour powdery colonies were observed on CZ medium. |
| F2 | White colour colonies were observed on CZ medium. Yellow pigments were secreted into the medium. |
| F3 | White/ gray colour colonies were observed on CZ medium. |

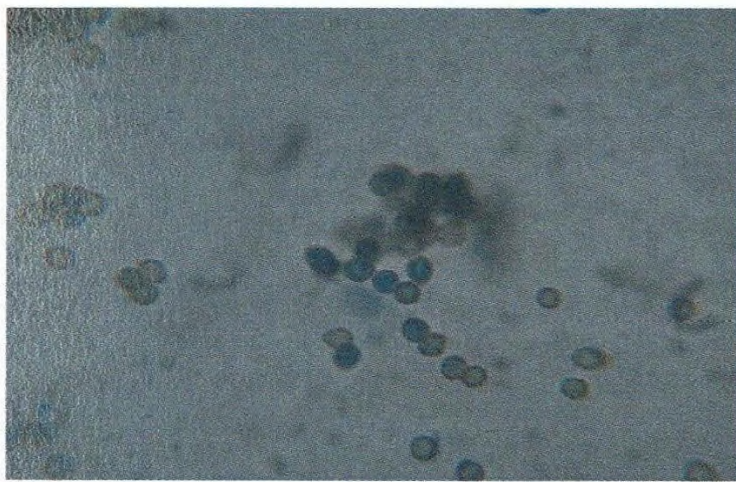
(a)

(b)

Plate 2.4- External colony appearances of the isolated fungi on CZ medium. (a) – Colony appearance of fungal strain F1. (b) – Colony appearance of fungal strain F2.

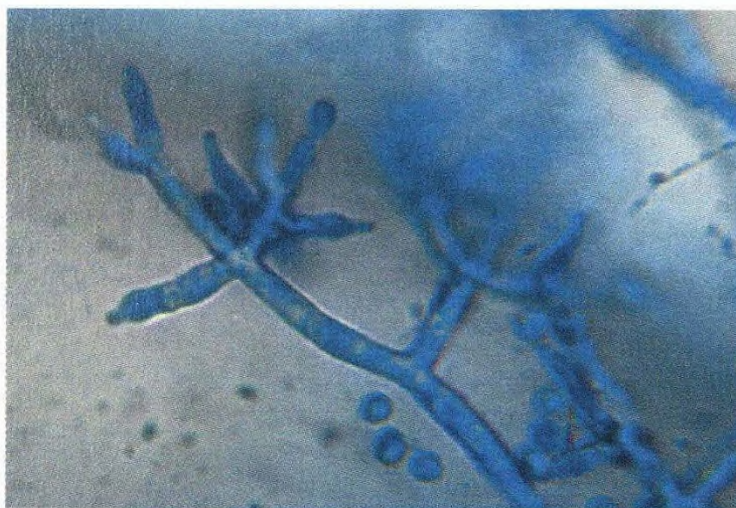
Table 2.4- Microscopic observations of the fungal isolates

| Fungal isolate | Observations |
|---|---|
| F1 | Hyphae were branched and septate. Brown colour spherical spores were observed. Conidial heads were typically radiate, Conidiophore was hyaline and coarsely rough, Conidia were |
| F2 | Hyphae was branched and aseptate. Conidiophores were highly branched. Main branches of the conidiophores produced lateral side branches. Typically the conidiophore terminated in one or a few phialides. |
| F3 | The mycelium was hyaline and septate. The sporangiophore was ended up with large ovoid shape sporangia. |

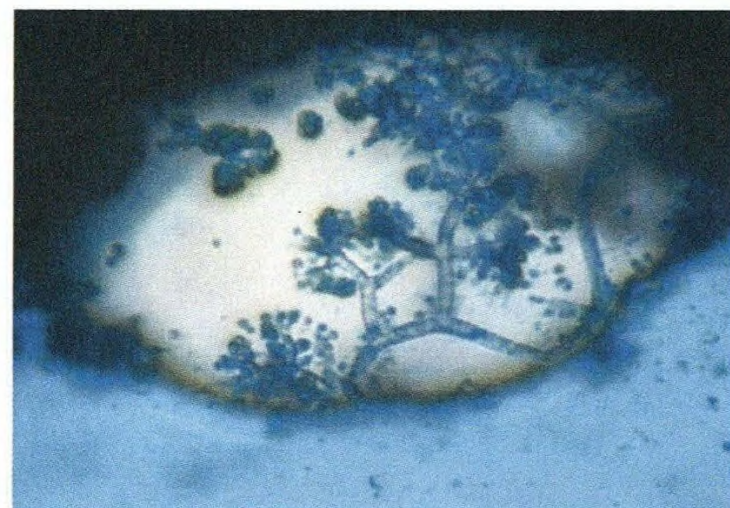


(a)

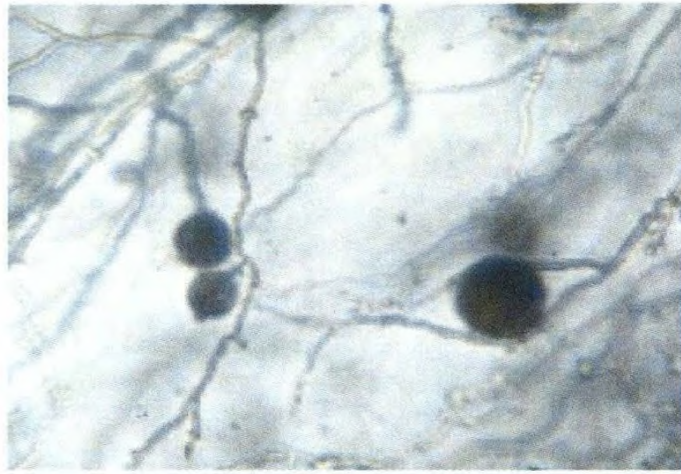
(b)



(c)



(d)



(e)

Plate 2.5- Microscopic view of the isolated soil fungi staining with Lacto phenol Cotton Blue. (a) and (b)- Microscopic view of the spores and conidia of fungi type F1 (Magnification 1000x). (c)- Microscopic view of the conidia of fungal type F2 (Magnification 1000x). (d)- Microscopic view of the conidia and the sporangium of the fungal type F2 (Magnification 400x). (e)- Microscopic view of the sporangia of the fungal type F3 (Magnification 1000x).

It has been reported that the genus *Trichoderma* comprises a great number of fungal isolates that act as biological control agents. It is more efficient in acidic than alkaline soils (Benitez *et al.*, 2004). *Trichoderma* can even exert positive effects on plants with an increase in plant growth (biofertilization) and the stimulation of plant-defense mechanisms. Further, it has a superior capacity to mobilize and take up soil nutrients compared to other organisms. Genus *Aspergillus* is also considered as beneficial fungal group since some of the species in *Aspergillus* are also used in the bioindustry, especially for biotransformations (Frisvad *et al.,* 2004). Fungal identifications confirmed the identity of the fungal strain F3 as *Phytopthora* sp. According to the literature, *Phytophthora* sp. attack a wide range of plants, and are responsible for some of the world's most destructive plant diseases such as potato late blight, root rots and stem cankers (Drenth and Sendall, 2001).

## 2.5 Conclusions

Fourteen bacteria and two fungi were isolated from potato rhizosphere. Out of the fourteen bacterial isolates, eight isolates were potential diazotrophic bacteria. *Acinetobacter* sp., *Acidomonas* sp., *Serratia* sp. and *Rhizobium* sp. (B2, B3, B5 and B7 respectively) were among the isolates made from potato rhizosphere. Further, bacterial isolates B1 and B8 were identified as *Bacillus* sp. and bacterial isolates B4 and B6 were identified as *Pseudomonas* sp. Microscopic observations revealed that the fungal isolates F1, F2 and F3 were *Aspergillus* sp., *Trichoderma* sp. and *Phytopthora* sp. respectively.

# PRELIMINARY SCREENING OF MICROORGANISMS ISOLATED FROM SOIL AND CHARACTERIZATION OF ESTABLISHED BENEFICIAL FUNGAL-BACTERIAL BIOFILMS

## 3.1 Introduction

Soil microbial communities like PGPRs in the rhizosphere zone play an important role in crop production and have been used for sustainable agricultural development as growth promoters (Hayat *et al.*, 2010). Beneficial PGPRs strains use one or more direct or indirect mechanisms to enhance the growth and health of plants. PGPRs have been reported to directly enhance plant growth by a variety of mechanisms *viz.* fixation of atmospheric $N_2$, solublization of phosphorus (P) containing minerals to enhance P level, production of siderophores and synthesis of plant growth hormones i.e. IAA, gibberellic acid (GA), cytokinins, and enhance seedling emergence, vigor, and yield (Kloepper, 1997; Kumar, Khare and Dubey, 2012). Therefore, PGPRs are now being used as microbial inoculants or as biofertilizers to enhance crop productivity and to reduce the use of CF in current agriculture (Karagoz *et al.*, 2012; Buddhika, Seneviratne and Abayasekara, 2014). For instance, a bio fertilizer, developed with *Azospirillum* sp. has been applied to an agricultural soil for which fertilizer inputs are impractical or undesirable (Pacovsky, 1990). Further, diazotrophic microbial inoculation on crops has been reported to enhance plant growth () and improve nutrient assimilation (Saharan and Nehra, 2011).

Most of rhizosphere microbes including PGPRs prefer to exist in soil as multicellular complex communities or as biofilms with other microorganisms by attaching to biotic or abiotic surfaces. A biofilm is an aggregation of microbial cells (algal, fungal, bacterial and/or other microbial) in an extracellular biopolymer (Seneviratne, 2003). Many bacteria produce a layer of polysaccharides or glycoproteins that coats the surface of the cell. Some form a slime layer while others form a thick gelatinous capsule which is known as an extracellular polymeric substance (EPS) and is used by the bacteria to form biofilms so that they can attach to structures (Ravneet and Shruti, 2013). Bacterial, fungal, and fungal-bacterial biofilms are the major types of biofilms that can occur in natural soil. Both bacterial and fungal biofilms are formed on abiotic surfaces, while fungi act as the biotic

surface in formation of fungal-bacterial biofilms. Such microbial communities frequently found to form micro-colonies or biofilm-like structures on plant roots (Bandara *et al.*, 2006).

It has been reported that the community interactions like biofilms can fluctuate between parasitism and mutualism through time (Toft and Andersson, 2010). Changes in microbial composition by the inoculation of microbial community or individual microbial strains may be undesirable if the inoculating microorganisms are pathogenic. The possibility of inoculating pathogenic microbial species in the form of biofertilizer can become a devastating effect for the entire soil ecosystem especially for agricultural soil (Trabelsi and Mhamdi, 2013). Further, it causes to lose the important native species from soil, thus affecting subsequent crops. However, beneficial microbial interactions are a source of evolutionary innovation through genetic rearrangements that give rise to metabolic capabilities and emergence of syntrophy to exploit resources, stabilizing in a mutualistic relationship (Garcia *et al.*, 2014). It has been well documented that the increment of the complexity of the microbial communities in rhizosphere results syntrophy which help to exchange the essential metabolites known as metabolic cross feeding between different communities to the benefit of the community members (Mee *et al.*, 2014).

Studies have suggested that biofilm community formation from such beneficial microorganisms play diverse activities in terms of improving the productivity of crops (Morris and Monier, 2003). For example, *Paenibacillus polymyxa*, a beneficial PGPRs bacterium, has been reported to protect the plants against pathogenic infections by forming biofilm-like structures and colonizing on plant root tips (Timmusk, Grantcharova and Wagner, 2005). When such beneficial bacteria attached and colonized on fungal mycelia to form the biofilms, known as beneficial FBBs. In FBBs fungi act as the biotic surface to which the bacteria adhere. Enhanced metabolic activities have been reported due to the formation of FBBs by bacterial colonization on biotic fungal surfaces, compared to monocultures (Seneviratne *et al.*, 2007). Further, it has been well documented that the microorganisms showed higher beneficial activities when they form biofilms with other microorganism rather than when they exist as mono or mixed cultures (Bandara *et al.*, 2006: Seneviratne *et al.,* 2007). However, because the density of biofilms in natural soil is not adequate to give maximum beneficial effects on plants, biofilms with beneficial microorganisms developed *in vitro* can be applied as biofertilizers to improve the beneficial activities like $N_2$ fixation and plant growth promotion (Bandara *et al.*, 2006).

Different biofertilizing methods have been developed to enhance biological $N_2$ fixation (BNF) for cereals and other non- legumes by establishing $N_2$ fixing bacteria in plant roots (Cocking, 2000; Mia *et al.*, 2010). It was clearly observed that PGPRs which consist of $N_2$ fixing bacteria, fix $N_2$ biologically through the nitrogenase activity and enhance the N availability to plant (Jayasinghearachchi and Seneviratne 2004a; Seneviratne *et al.*, 2007). Previous reports have suggested positive impacts of microbes on N uptake in non-legume by BNF (Adesemoye, Torbert, and Kloepper, 2009). For instance, application of PGPR strains, especially *Azospirillum* sp. and *Bacillus subtilis* have been reported to improve N availability in soil while enhancing crop productivity of non- legumes such as oil palm (*Elaeis guineensis*), sweet potato (*Ipomoea batatas*) and potato (*Solanum tuberosm*) (Amir *et al.*, 2001; Yasmin *et al.*, 2009). It has been reported that PGPRs inoculation along with 33% N fertilizer enhanced crop productivity and produced a similar plant biomass and yield like the fully fertilized plants while reducing chemical inputs by 67% in sweet potato (Saad *et al.*, 1999; Mia *et al.*, 2010). In addition, PGPRs inoculation has been reported to contribute 89% of the total N requirement of oil palm plantlets grown under *in vitro* conditions. Numerous studies have shown greater $N_2$ fixation activities in inoculated plants than in un-inoculated controls (Okon, 1985). This might be due to the increment of number of nitrogenous compound and nitrogenase activity in inoculated plants (Bashan and Holguin, 1997).

However, beneficial PGPRs living in a biofilm show significantly different $N_2$ fixing abilities compared with the same species that are free-living (Seneviratne *et al.*, 2008). For instance, incorporation of $N_2$ fixing strains to FBBs has been shown to improve N content significantly in N deficient conditions. Approximately 30% enhancement of $N_2$ fixation in soybean has been reported by the inoculation of FBBs including $N_2$ fixing bacteria compared to a conventional inoculant of *Rhizobium* alone (monoculture inocula) (Jayasinghearachchi and Seneviratne, 2004b). Thus, it is clear that the beneficial activities such as $N_2$ fixation shown by microbial communities and free living stages are highly variable. Therefore, a proper quantification method for $N_2$ fixation by such beneficial microorganisms, is required to obtain a better understanding of their involvements. One of the most reliable and easiest method of quantifying $N_2$ fixation of such soil bacteria or microbial communities is through measuring the ethylene production by acetylene reduction assay. The enzyme nitrogenase, universally responsible for biological $N_2$ fixation, is also capable of reducing acetylene to ethylene. Both gases can be readily detected and

quantified using gas chromatography (GC) (Schollhorn and Burris 1967; Hardy *et al.*, 1968). Thus, the acetylene reduction assay (ARA) is a sensitive measure of nitrogenase activity at a point in time and can be very useful for detecting $N_2$ fixation activity (Mcnabb and Geist, 1979; Herridge, Peoples and Boddey, 2008). Further, associative $N_2$ fixing bacteria have been found to be capable of producing growth regulators like IAA, GA and cytokinins, which were thought to contribute to the stimulated plant growth (Umali-Garcia *et al.*, 1980).

IAA production and seedling vigor are another two important criteria to evaluate beneficial activities of different soil microorganisms. It has also been well documented that the biosynthesis of auxins with their excretions into soil makes a major contribution to the bacterial plant growth-promoting effect (Lambrecht *et al.*, 2000). Evidences can be found for the beneficial bacterial effects on the enhancement of seedling emergence, seedling vigor, seed germination and plant growth due to the excretion of plant growth hormones i.e. IAA, GA, cytokinins, and ethylene (Nelson, 2004; Kumar *et al.*, 2012). IAA is one of the most important phytohormones and function as an important signal molecule in the regulation of plant development. It has been reported that IAA production by PGPRs can vary among different species and strains, and also influenced by culture conditions, growth stage and substrate availability (Mirza *et al.*, 2001). Further, some beneficial bacterial strains such as *Pseudomonas putida* and *Pseudomonas fluorescens* have been reported to increase root and shoot elongation in canola, lettuce, and tomato as well as crop yields in potato, tomato, lettuce, beans, and wheat due to the effect of plant growth promoting hormones (Rodriguez and Fraga, 1999). Further, it has been reported that soil beneficial microorganisms can produce significant amount of IAA and the production ranging from 11.6 to 60.0 μg/ml (Malleswari *et al.*, 2013). However, many studies have recorded higher acidity and higher IAAs production from biofilm communities than their mono or mixed cultures (Jayasinghearachchi and Seneviratne 2005; Seneviratne and Indrasena 2006). For example, FBBs of beneficial endophytes were observed to produce higher acidity and plant growth promoting hormones like IAA than their mono- or mixed cultures with no biofilm formation (Bandara *et al.*, 2006). Further, it has been reported that FBBs inocula which excrete such growth promoting substances like IAA led to 25% increment in plant dry weight in early growth of rice compared to conventional mono or mixed cultures with no biofilm formation (Bandara *et al.*, 2006).

Moreover, a research on seed germination and seedling vigor of tomato has shown that the seed germination has increased significantly due to the influence of seed bacterization with different rhizobacterial isolates (Agrawal and Agrawal, 2013). Increase of seed germination due to the PGPRs has been reported in crops such as rice (Ng *et al.*, 2012), maize (Nezarat and Gholami 2009) and soybean (Sreenivasa *et al.*, 2009). Further, enhanced seedling vigor (Vessey 2003; Ashrafuzzaman *et al.*, 2009) and early seedling establishment (Noel *et al.*, 1996; Khalid, Arshad and Zahir, 2004) have also been noted due to the PGPRs applications. For example, rapid seed germination of *Dianthus caryophyllus* has been observed to be caused by the production of plant growth regulators like IAA, which overcomes seed dormancy (Buddhika *et al.*, 2014). However, it has been well recorded that biofilms especially FBBs significantly enhanced the seedling vigor and seed dormancy compared with their monocultures. Maize seeds inoculated with FBBs has been shown higher root lengths (which is a factor effecting seedling vigor) than the seeds inoculated with bacterial monocultures, whereas no such a difference has been found in shoot length (Buddhika *et al*, 2014). This might be due to the production of different metabolic compounds by the microbial communities over their monoculture inoculants (Hoflich, Wiehe and Kuhn, 1994).

Since soil microbial communities play divers behavioral activities including parasitism and mutualism, pre- screening and characterization of microbial inoculants are essential to minimize the adverse effect on entire soil microbial community and crop productivity. In that sense, molecular characterization of biofilm inoculants and screening for beneficial activities to make a solid confirmation on the behavioral activity and the identity of the microbial inoculants are greatly required. Therefore, strong molecular identification tools such as 16S r-RNA gene sequencing and RFLP techniques and basic screening experiments such as IAA production, nitrogenase activity and seedling vigor index can be used to estimate microbial community composition and beneficial activities in complex communities. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker. 16S rRNA gene sequencing provides unambiguous data even for rare isolates, which are highly reproducible. These small subunit (SSU) rDNA molecules are particularly suited for such studies for a number of reasons. First, they are found universally in all three forms of life: the domains Bacteria, Archaea, and Eucarya (Woese, Kandler and Wheelis, 1990). Second, these molecules are composed both of highly conserved regions and also of regions with

considerable sequence variation. Third, the phylogenetic information held in the SSU rDNA molecule is further enhanced by its relatively large size (e.g. 1.5 kb for the 16S rDNA molecule) and the presence of many secondary structural domains. Consequently, evolutionary changes in one domain do not affect the rate of change in other domains. Finally, SSU rDNA can be easily amplified using polymerase chain reaction (PCR) and rapidly sequenced (Hill *et al.*, 2000). However, there are some pitfalls in the use of 16S rRNA for studies of biodiversity. One is that in terms of size the genes for the 16S molecule are extremely conserved and therefore different genes cannot be easily separated by size (Martinez *et al.*, 1999).

DNA fingerprinting using restriction fragment length polymorphism (RFLP), is another tool used to study microbial diversity which the PCR amplified rDNA is digested with cutting restriction enzymes (Liu *et al.*, 1997). Different fragment lengths are detected using agarose gel electrophoresis in the case of community analysis (Liu *et al.*, 1997; Tiedje *et al.*, 1999). Though this method is useful for detecting structural changes in microbial communities, diversity analysis or detection of specific phylogenetic groups cannot be achieved (Liu *et al.*, 1997). Further, RFLP provides complex banding patters for diverse microbial communities thereby the analysis becomes confusing (Krick *et al.*, 2004; Fakuruddin *et al.*, 2013). Therefore, more advanced techniques like terminal restriction fragment length polymorphism (TRFLP) can be used to address some of the limitations of RFLP (Tiedje *et al.*, 1999).

TRFLP analysis is an automated and sensitive fingerprinting method which follows the same principle as RFLP except that one PCR primer is labelled with a fluorescent dye and allows detection of only the labelled terminal restriction fragment using a DNA sequencer (Liu *et al.*, 1997). The sequencer recognizes only the fluorescently labeled terminal fragments, and therefore, in principle each fragment represents a unique operational taxonomic unit (OTU) in the sample. The relative quantitative distribution within a profile can be determined, since the fluorescence intensity of each peak is proportional to the amount of genomic DNA present for each OTU in the sample. Further, TRFLP extended to analysis of multiple species by the use of primers with a high degree of species cross-reactivity. Since fragments amplified from different species typically differ in nucleotide sequence, they produce fragments of different sizes on digestion with a restriction enzyme. This simplifies the banding pattern, thus allowing the analysis of complex communities as

well as providing information on diversity as each visible band represents a single operational taxonomic unit or ribotype (Tiedje *et al.*, 1999). The banding pattern can be used to measure species richness and evenness as well as similarities between samples (Liu *et al.*, 1997). Further, TRLFP has been used to study complex bacterial communities and it has proved to be more consistent and reproducible than other fingerprinting methods because of its automated analysis mode and because an internal size standard is included in every sample (Acinas *et al.*, 2005).

## 3.2 Objectives

1. To screen isolated microorganisms based on specific biochemical experiments.
2. To evaluate the microbial isolates for their pathogenicity.
3. To evaluate the microbial isolates for their growth responses on potato seed tubers.
4. To establish biofilms and select the beneficial biofilms through screening experiments.
5. To characterize the biofilm forming microbial isolates using molecular methods.

### 3.3 Methodology

### 3.3.1 Preliminary screening of bacterial isolates

Preliminary screening experiments were carried out for the isolated diazotrophic bacteria to select beneficial microorganisms. Screening experiments were based on inoculum pH, nitrogenase activity through ARA (Husen, 2003), seedling vigor (Mia, Shamsuddin and Mahmood, 2012: Pawar *et al.*, 2014), and production of IAA (Husen, 2003; Buddhika *et al.*, 2014).

#### 3.3.1.1 Broth culture pH of microbial isolates

Isolated eight different diazotrophic bacterial isolates (2.3.1.4) were inoculated on CCM broths and incubated at 33 °C for five days. Fungal isolates were inoculated on CZ broth separately and incubated at 30 °C for five days. After incubation, pH of each culture media was measured separately. Three replicates were maintained per treatment.

#### 3.3.1.2 Nitrogenase activity of microbial isolates

Analysis of nitrogenase activity of different bacterial isolates was accomplished by GC using Porapak N column (200 x 0.2 cm) equipped with a flame ionization detector (FID) in isocratic condition. The operating conditions were as follows: temperature of oven at 80 °C; the injector temperature was kept at 200 °C; and the detector temperature was maintained at 200 °C. The flow of $N_2$ gas was maintained 42 ml/min, $H_2$ at a flow rate of 64 ml/min and air at a flow rate of 42 ml/min respectively. Bacterial isolates were inoculated into CCM broths and incubated at 33 °C for five days. Subsequently, 100 μl of each broth culture (approximate CFU-2 x $10^7$/ml) was mixed with one milliliter of Glucose Malate (ML) medium in an ARA vial separately and incubated at 33 °C for 24 hours. After the incubation, 0.5 ml of gas was withdrawn from each ARA vial and injected 0.5 ml of acetylene gas in to the same vial. After the incubation of each vial for one hour at 33 °C, 0.5 ml of gas was withdrawn again and injected in to GC (Model No. SHIMADZU, GC-9AM) to obtain the readings. The accuracy of the data was confirmed by triplicating the experiment along with a control (without inoculation). According to the readings (peak areas), concentration of the ethylene accumulated in the ARA vial due to nitrogenase activity was calculated for each

bacterial monocultures using the following equation. The mean values of each experiment were calculated and reported.

Concentration of ethylene = (0.0008 x area of the peak) + 2.5712

### 3.3.1.3 Seedling vigor through lettuce seed germination assay

The experiment was designed in an aseptic conditions to prevent cross-contaminations of culture bottle and seeds. Isolated bacteria were inoculated (approximate CFU-2 x $10^7$/ml) in ML medium and incubated at 33 °C for 24-48 hours. Selection of good quality seed was considered as one of the major influencing factor for this experiment since germination is highly depended on the viability of seeds (Harris and De Mason, 1989). Therefore, lettuce seeds were obtained from certified seed company (CIC Agri Business (Pvt) Ltd) to confirm the germination and to minimize the pathogenic effects. Lettuce seeds were surface sterilized by dipping the seeds with 1% Clorox for 3 min followed by repeated washings (3 times) with sterilized distilled water. Then surface sterilized lettuce seeds were soaked separately with 10 times diluted each bacterial cultures in ML medium for 2 hours. The seeds which were soaked in un-inoculated ML medium was kept as the control. Sterilized 250 ml culture bottles were used for the experiment to provide enough aeration since the seed germination is highly effected by the availability of Oxygen in the atmosphere (Van der Valk *et al.,* 1999). About 50 soaked seeds from each broth culture were placed equidistance on the sterilized sand medium inside the sterilized culture bottles under aseptic conditions. The sand medium was moistened with 15 ml of each diluted bacteria broth cultures separately. Favorable and equal environmental conditions were provided for each culture bottle with inoculated seeds throughout the experiment. Then the seeds were allowed to germinate in the dark for four days. The effect of different bacterial inoculations on seed germination was evaluated using germination percentage, root and shoot lengths of the seedling. Seedling vigor for each bacterial treatment (vigor index) was calculated based on the data follows (Agrawal and Agrawal, 2013). The accuracy of the data was confirmed by triplicating the experiment and the mean values of each experiment were calculated and reported.

Germination (%) = $\frac{\text{Number of emerged seedlings}}{\text{Number of seeds sown}} \times 100$

Total length of seedling = root length + shoot length

Vigor Index (VI) = germination (%) x mean total length of seedling

**3.3.1.4 Quantification of IAA production**

Bacterial isolates were inoculated (approximate CFU- 2 x $10^7$/ml) on Tris-YMRT medium [mannitol, 10 g; $CaCl_2.2H_2O$, 0.15 g; $MgSO_4.7H_2O$, 0.25 g; TRIS (hydroxymethyl) amino methane,1.21 g; casamino acids, 1.0 g; yeast extract, 0.2 g; water,1000 ml; pH 6.8] (Biswas *et al.,* 2000) and incubated at 33 °C for seven days. Incubated cultures were then centrifuged at 4025×*g* for 20 min at 4 °C. 1ml aliquot of the supernatant from each culture was vigorously mixed with 4 ml of Salkowski's reagent (150 ml of concentrated $H_2SO_4$, 250 ml of distilled $H_2O$, 7.5 ml of 0.5 M $FeCl_3.6H_2O$) and allowed to stand at room temperature for 20 min. Subsequently, the absorbance was measured using colorimetric method at 535 nm in UV/Visible Spectrophotometer (Model No. Genetics 6). IAA production was calculated from the regression equation of standard curve and the result was expressed as $\mu g\ ml^{-1}$ (control- Un- inoculated YMRT medium); 100 mg/l of an IAA solution was prepared by dissolving 0.005 g of IAA in 50 ml of ethyl acetate solution. From the prepared solution, 0.25, 0.5, 0.75, 1.0 and 1.25 ml of aliquots were mixed with 25 ml of ethyl acetate to make 1, 2, 3, 4 and 5 mg IAA/ml, respectively to prepare standard series.

**3.3.2 Preliminary screening of fungal isolates**

It was decided not to use *Phytopthora* sp. for further evaluations with potato crop as it was identified as plant pathogenic fungi. *Aspergillus* sp. and *Trichoderma* sp. were screened based on the pH of the broth medium. Therefore, both isolates were inoculated in CZ medium and incubated for four days at 30 °C followed by measuring the pH of the broth medium.

### 3.3.3 Responses of fungal and bacterial isolates on potato plant

Efficient fungal and bacterial isolates selected from screening experiments were evaluated for their growth responses on potato using a pot experiment under greenhouse conditions at the Regional Agriculture Research and Development Center, Bandarawela, Sri Lanka. Further, pathogenicity of the selected microbial isolates were evaluated as a separate experiment simultaneously with the evaluation of growth responses. Experiments were conducted from October 2011 to February 2012. Growth was evaluated using tuber fresh weight, tuber number, stolon number, shoot dry weight and root dry weight of potato plant. Pathogenicity was evaluated using number of wilted plants due to bacterial wilt disease and number of leaves with brown patches due to the late blight disease.

#### 3.3.3.1 Media preparation and planting of seed potato

Top loamy soil was used (particle size $\leq 2$ mm) as the medium for the pot experiment. The medium was heat sterilized using a dry oven at 160 °C for 2 hours. Six inch diameter black plastic pots were used for the pot experiment and were filled (3/4 of the pot) with sterilized soil medium after washing the pots with clean water.

A separate experiment was conducted to evaluate the pathogenicity of the inoculated microbial isolates using sterilized river sand as the medium under the same greenhouse conditions. River sand was sieved (particle size $\leq$ 1mm) and washed three times with tap water before sterilization. Subsequently, the sand medium was heat sterilized using a dry oven at 160 °C for two hours after adjusting the pH to 5-7. Six inch diameter black plastic pots were used for the experiment and were filled with sterilized sand medium.

Since potato is highly susceptible to fungal and bacterial diseases, selection of good quality disease free planting material (seed tubers) is one of the key considerations in this research. Therefore, disease free seed tubers ('Granola' variety) were obtained from government certified Regional Agriculture Research and Development Center, Bandarawela. Two seeds were grown in each pot with soil and sand separately. Subsequently, all the pots were arranged according to the CRD inside the greenhouse till harvesting. Five replicates were maintained for each treatment.

### 3.3.3.2 Preparation and application of microbial cultures

Selected bacterial and fungal isolates were inoculated in CCM and CZ broth separately and incubated at suitable temperatures (bacteria-33 °C and fungi- 30 °C). Bacterial colony forming units (CFU) were counted for each bacterial isolates after 48 hours of incubation using a heamocytometer. Suitable dilutions (bacteria- with CCM and fungi- with CZ) were performed for bacterial isolates in order to equalize the CFU for all the bacteria. . Total dry mass of fungal mycelium in one milliliter of broth culture was determined for each fungal isolates after 48 hours of incubation. Required dilutions were prepared for fungal cultures in order to equalize the concentration.

After 10 days from seed sowing (approximately two weeks required for root establishment), 20 ml of the diluted eight different diazoptrophic bacterial cultures (CFU = $10^7$/ml) and two fungal (Dry mass = 0.12 mg/ml) broth cultures were applied separately around the root zone of the potato plants grown in pots. Potato plant with un-inoculated CCM and CZ media were considered as the control experiments.

### 3.3.3.3 Fertilizer application

A mixture of urea (2.0 g), Triple Super Phosphate (TSP) (3.33 g) and Muriate of Potash (MOP) (1.33 g) was mixed with 1 kg of soil as a basal fertilizer mixture before filling soil in to pots. Fertilizer application (2.0 g of urea and 1.33 g of MOP) was repeated after five weeks from seed sowing simultaneously with biofertilizer application.

Albert solution (1%) was used as the basal CF until the establishment of the root system for the plants which were grown in sand pots for the evaluation of pathogenecity. After 8- 10 days, the concentration of Albert solution medium was increased to 10% and applied simultaneously with the microbial inoculations. Rates of CF application was calculated per plant basis according to the DOA recommendations. Water level of the medium was maintained constantly by applying 250 ml of water for each pot every day. Plants were grown with a daily minimum-maximum temperature range of 20 °C – 30 °C.

### 3.3.3.4 Evaluation of pathogenicity

Visual observations and field evaluations were made for the plants which were grown in sand pots once a week to check the emergence of any harmful pest attacks or disease infections. Bacterial wilt and potato late blight diseases are considered as severe diseases which can reduce crop yield significantly in potato. Therefore, number of wilted plants were counted after four weeks and eight weeks from seed sowing to determine the emergence of bacterial wilt disease due to inoculation. Leaves with brown/ black lesions were collected once in two weeks to evaluate the emergence of late blight disease due to inoculation. Microscopic observations of leaf section cuttings were carried out to confirm the infections. Results were compared with non-treated controls.

### 3.3.3.5 Harvesting

After 90 days from seed sowing, plants were uprooted carefully without damaging the root system and were washed carefully with water to remove adhered soil materials attached with the root system. Then the individual plants were transferred in to black polythene bags accordingly with the different treatments and were brought in to the laboratory to measure different parameters of the plants. Fresh weights of the tubers, number of stolons and tubers, dry weights of roots and shoots were measured to evaluate the growth responses for the treatments.

### 3.3.3.6 Statistical analysis

Normality of the data and constancy of residuals were confirmed. Statistical data analyses were performed on all data collected using the one way Analysis of Variance (ANOVA) Model in MINITAB 16 Statistical Software. The mean values of fresh weights of the tubers, dry weights of roots and shoots, number of tubers and number of stolons were compared on treatment basis using the tukey's simultaneous test at 5% significance level. Correlations between different growth parameters and other specific characteristics (pH and IAA etc...) were constructed using non-linear regression analysis.

Microbial isolates selected from each screening experiments (pH, ARA, seedling vigor, IAA production and growth responses on potato) were statistically ranked using MINITAB

16 Statistical Software to evaluate the beneficial fungi and bacterial isolates. Most efficient organisms (bacteria and fungi), which were able to produce high concentrations of ethylene, high concentration of IAA, high seedling vigor and low pH were selected for biofilm establishment.

### 3.3.4 Establishment of simple FBBs from selected bacterial and fungal isolates

Each isolate of efficient responsive bacteria (selected after screening) was inoculated in 25 ml of CCM broth and incubated at 33 °C for 2 days. Isolates of fungi were grown separately in 25 ml of CZ medium and incubated at 30 °C for 5 days. The bacteria and fungi were mixed with each other into desired combinations (table 3.1) to form simple biofilms in a special biofilm forming medium (patented). Those mixed cultures were incubated at 30 °C for 5 days with continuous mixing in an orbital shaker to produce simple biofilms (Gunathilake *et al.*, 2013). The progress of biofilm development was recorded at 2 day intervals by visual and microscopic observations using a light microscope by staining with lactophenol cotton blue. Bacterial attachments along the fungal mycelium were taken as fungal-bacterial biofilms according to the definition by Seneviratne *et al.* (2008).

Table 3.1: Possible biofilm combinations of fungi and bacteria

| | Bacterial isolates | | | | |
|---|---|---|---|---|---|
| Fungal isolate | *Bacillus* sp. | *Acidomonas* sp. | *Serratia* sp. | *Rhizobium* sp. | *Bacillus* sp. |
| *Aspergillus* sp. | B1F1 | B3F1 | B5F1 | B7F1 | B8F1 |
| *Trichoderma* sp. | B1F2 | B3F2 | B5F2 | B7F2 | B8F2 |

### 3.3.5 Screening experiments for simple biofilms

Screening experiments were carried out for fungal-bacterial simple biofilms to select the beneficial cultures based on pH of the biofilm broth medium, nitrogenase activity through ARA, seedling vigor through lettuce seed germination assay (Pawar *et al.*, 2014) and production of IAA (Husen, 2003) as performed in bacterial isolates (3.3.1). Biofilm combinations selected from each screening experiment (pH, nitogenase activity, seedling

vigor and IAA concentration) were statistically ranked using MINITAB 16 Statistical Software to evaluate the beneficial biofilms. Most efficient biofilms, which were able to produce high concentrations of ethylene, high concentration of IAA, higher seedling vigor and lower pH were selected as best performing biofilms.

### 3.3.6 Establishment of higher order biofilms from selected bacteria and fungi

Fungal and bacterial mono cultures were combined in all possible combinations to produce higher order biofilms (more than one bacteria with a fungus). The bacteria and fungi from the screened best performed biofilms were mixed with each other into desired combinations (table 3.2) to form higher order biofilms in a special biofilm forming medium (patented). Those mixed cultures were incubated for 5 days with continuous mixing in an orbital shaker at 30 °C to produce higher order biofilms (Gunathilake *et al.*, 2013). The progress of biofilm development was recorded at two day intervals by visual and microscopic observations using a light microscope by staining with lactophenol cotton blue.

Table 3.2: Possible higher order biofilm combinations of fungi and bacteria

| Bacterial isolates | Fungal isolates | Biofilm combinations |
|---|---|---|
| *Bacillus* sp. and *Rhizobium* sp. | *Trichoderma* sp. | HBF1 |
| *Serratia* sp. and *Bacillus* sp. | *Apergillus* sp. | HBF2 |

Selection of the bacterial and fungal combinations for the production of higher order biofilms was based on the ranking scores of simple biofilms in the screening experiments. Since four simple biofilm combinations performed well in the screening experiments, those combinations were selected to prepare higher order biofilms with respective fungal isolates. All the screening experiments were performed for the higher order biofilms as done in simple biofilms.

### 3.3.6.1 GA production by higher order biofilms

GA production by the higher order biofilms was determined as the method described by Umamaheshwari *et al.* (2013). The cultures were grown in nutrient broth for 7 days at room temperature and centrifuged for 10 min at 10,000 rpm. The supernatants were collected and acidified to pH 2.0 with 5 N HCl and extracted with equal volumes of ethyl acetate twice. The ethyl acetate phase was evaporated at 32 °C and the residue was re-dissolved in 10 ml of distilled water containing 0.05% of Tween 80. Subsequently, 2 ml of zinc acetate and 2 ml of potassium ferrocyanide were added and centrifuged at 1000 rpm for 15 min. The supernatant was acidified with 30% HCl and incubated at 20 °C for 75 min. The blank sample was treated with HCl and the absorbance of the sample as well as blank was measured at 254 nm in a spectrophotometer (Model No. Genetics 6).

Statistical data analyses were performed on all data collected (monoculture treatment/ biofilm treatments) using the one way ANOVA Model in MINITAB 16 Statistical Software. Normality of the data and constancy of residuals were confirmed. The mean values of pH, ethylene concentrations, seedling vigor (Pawar *et al.*, 2014) and production of IAA (Husen, 2003) were compared on treatment basis using the tukey's simultaneous test at 5% significance level. Correlations between different parameters for all bacterial monocultures and biofilm combinations were constructed by using correlation analysis.

### 3.3.7 Molecular identification of microorganisms in the most effective higher order biofilm

### 3.3.7.1 Microbial culture preparation

Species identification of *Bacillus* sp. (B1), *Rhizobium* sp. (B7) and *Trichoderma* sp. (F2) were performed using molecular techniques at the Biomedical building C8, Faculty of Agriculture and Environment, University of Sydney, Australia. The experiment was conducted from June 2014 to September 2014.

All bacterial isolates were sub cultured on the agar-based subculture medium (NA medium) plates and incubated at 33 °C for 48-72 hours. Fungal isolate was sub cultured on PDA medium and incubated at 30°C for 48-72 hours. Broth cultures were prepared from all bacterial isolates using NA medium and fungal isolates using CZ medium.

### 3.3.7.2 Extraction of genomic DNA from bacteria and fungi using CTAB (Cetyl trimethyl ammonium bromide) method

Genomic DNA was extracted from 1.5 ml of each bacterial and fungi samples sepetately using CTAB method (Appendix 1). After the extraction, DNA samples (high molecular weight) with 50μl of TE buffer were stored in low temperature until further processing.

### 3.3.7.3 Assessing the purity, quantity and RNA contamination of the extracted DNA

Quantity, quality and RNA contamination of the extracted DNA from CTAB method were assessed using agarose gel electrophoresis. Agarose gel (1%) was prepared by adding 1 g of agarose to 100 ml of 1X TAE buffer and were completely dissolved by heating using a microwave oven. Approximately 1-2μl of ethidium bromide (EtBr) was added to the melted agarose and the mixture was poured in to the gel tray of the BIORAD mini gel electrophoresis unit. 10μl of DNA extracted from each microorganism was mixed with 5μl of gel loading dye separately and were loaded into the wells of the agarose gel. DNA bands were visualized using gel documentation system (BIORAD ChemiDoc$^{TM}$ MP imaging System at Faculty of Agriculture and Environment, University of Sydney) after running the samples through the agarose gel with 110-150 V maximum voltage. A 1Kb (Hyper ladder 1Kb, BioLine) DNA size marker was included in the gel for size analysis.

### 3.3.7.4 Amplification of the extracted DNA using PCR method

Extracted DNA samples were subjected to PCR amplification using 16S rRNA –specific primers. 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-CGG TTA CCT TGT TAC GAC TT-3') were used as forward primer and reverse primers respectively for the amplification of bacterial 16S rRNA and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used as forward and reverse primers for the amplification of fungal DNA. PCR was carried out in a 25 μl reaction mixture, containing 5X My *Taq* polymerase buffer, 10 μM forward primer, 10 μM reverse primer, 5U/ μl My *Taq* polymerase and genomic DNA samples (appendix 2). PCR reaction mixture (25 μl reaction mixture) was prepared by mixing 24 μl of master mix with 1 μl of each DNA samples in PCR strip tubes. Sterilized dd $H_2O$ (1μl) was used as the negative control and previously tested DNA sample was used as the positive control.

PCR amplification was performed in a thermal cycler (BIO-RAD C1000 Touch ™ ) at the Biomedical building C81, Faculty of Agriculture and environment University of Sydney. DNA amplification was performed under the following conditions: initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), and 72 °C for 1.0 min (extension), with a final extension at 72 °C for 5 min. The PCR products were fractionated on a 1.5% agarose gel using 1X TAE buffer (0.04M Tris-acetate, 0.001M EDTA). Gels were stained with EtBr and photographed under UV light using BIORAD Gel Doc system. A 100bp Plus DNA size marker (Hyper ladder 100bp Plus, BioLine) was included on the gel for size analysis. Reproducibility of PCR reactions was evaluated by performing duplicate reactions for each template DNA isolated. DNA templates used in the study were able to give reproducible PCR amplification results in duplicated experiment.

**3.3.7.5 Purification of the PCR products for restriction digestion**

PCR products from each microbial isolates were transferred to clean sterile eppendorf tubes separately and the volume of each sample was recorded. Subsequently, 0.1 volumes of 3 M Sodium Acetate (pH 5.2) and 2 volumes of absolute ethanol were added simultaneously to each tube followed by vigorous mixing. Tubes were incubated for 30 min on ice followed by centrifugation at 10,000 g for 20 min in a bench top centrifuge. Then, 500 µl of 70% ethanol was added to each pellet after removing the supernatant. Tubes were mixed minimally using vortex mixture and were centrifuged again at 10,000 g for 10 min. Bulk of the supernatant was removed carefully and centrifuged again at 10,000 g for 5 min to adhere the purified DNA to bottom of the tubes. After the centrifugation, ethanol remaining in the tubes was removed as much as possible carefully using a micropipette without disturbing the pellet. Tubes were kept open for 10 min to evaporate the trace amount of ethanol remaining in the tube followed by dissolving the DNA pellet in 25 µl of sterile water. The concentration of each purified DNA was measured using UV spectrophotometer (Nano-drop 2000 C) at 260 nm wavelength and the samples were stored in low temperature until further processing. The purity of the extracted DNA was quantified by calculating the ratio of the absorbance at 260 nm and 280 nm ($A_{260}/A_{280}$).

### 3.3.7.6 Restriction digestion of the purified PCR products and RFLP analysis

RFLP analysis was performed for the purified PCR products of each microbial strains to assess the quality (pure or contamination) of the DNA samples. Restriction digestion was carried out in a 12 µl reaction mixture, containing 10X NEB cut smart buffer and restriction enzymes with the purified PCR products (appendix 2). Three different restriction enzymes (Taq α I, HhaI and MSPI) were used for the digestion.

After mixing, the reaction mixture was incubated at 65 °C for one hour followed by incubation at 80 °C for 20 min to inactivate the enzyme activity. The digested DNA samples were fractionated on a 2.5% agarose gel using 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). Gels were stained with EtBr and photographed under UV light using BIORAD Gel Doc system. A 100 bp plus DNA size marker was included on the gel for size analysis.

### 3.3.7.7 Extraction of genomic DNA using a crude method

Analysis of the RFLP bands confirmed that two bacterial DNA samples were contaminated. Therefore, bacterial genomic DNA extraction was repeated again using a rapid DNA extraction method as follows. Small amount of contaminated mother culture was serially diluted (10 fold) and inoculated on sterile diluted (5 times) R-2A medium followed by incubation at 33 °C for 24 hrs. Isolated colonies were sub-cultured on same diluted R-2A medium plates again by streaking technique and incubated at 33 °C for 48-72 hours to prepare pure cultures (B1, B7 and contaminated sample B9). Small amount of pure bacterial sample from each colonies was obtained using a sterile inoculation needle and mixed separately with 50 µl of sterile double distilled water in a clean 1.5 ml eppendorf tube. The samples were mixed vigorously using a vortex mixture. Tubes were first heated at 95 °C for 5 min and subsequently incubated on ice to remove the crude DNA by damaging the cell membranes. PCR was performed for the crude DNA samples as explained in 3.3.7.4 above. RFLP analysis was proceeded after the purification of PCR products as explained in 3.3.7.5.

### 3.3.7.8 DNA sequence analysis

#### 3.3.7.8.a Preparation of DNA samples for sequence analysis

Purified PCR products were prepared for sequence analysis by mixing 100-200 ng of each PCR products obtained from different microbial strains, with 1 µl of each forward and revers primers separately in a 1.5 ml eppendorf tube. Subsequently, the final volume was adjusted to 12 µl with sterile distilled water and samples were submitted to MACROGEN Company, South Korea for sequence analysis.

#### 3.3.7.8.b Assessing DNA sequence and performing sequence analysis using online databases

DNA sequence traces frequently contain errors. There are numerous possible causes for sequence errors and it is essential that all sequence traces are thoroughly checked for any potential errors and are corrected where possible. Therefore, sequence chromatograms were visualized and edited in FASTA format using a soft wear known as 'Finch TV'(http://www.geospiza.com/Products/finchtv.shtm) and analyzed using software known as BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) which were freely available. The sequence alignment of the edited sequences from bacterial samples (FASTA format) was performed using available online databases with Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu/) and the sequence alignment of the edited sequences from fungi samples was performed using GenBank database using the Basic Local Alignment Search Tool (BLAST) search algorithm (Altschul *et al.*, 1990).Partial forward sequences were used as query sequences to find similar sequences in data bases for all of the microbial isolates. The most similar reference sequences with the query sequences were obtained and used to identify the genus or species of the fungal and bacterial strains. The isolates were assigned to a species if the sequence was $\geq$ 99% similar to a valid species sequence deposited with respective data bases, and to a genus if the species identity was not conclusive, but the similarity was $\geq$ 97% (Thomas *et al.*, 2008).

### 3.3.8 Community analysis of fungal-bacterial biofilm through TRFLP

#### 3.3.8.1 Extraction of DNA from a fungal-bacterial biofilm community

*Bacillus* sp. (B1), *Rhizobium* sp. (B7) and bacterial isolate B9 were combined with *Trichoderma* sp. in a biofilm forming broth culture and incubated for 4-5 days at 30 °C to produce biofilms. The progress of biofilm development was recorded at two days intervals through visual and microscopic observations using a light microscope (Olympus BX51). Three replicates were maintained for the biofilm (BF1, BF2 and BF3) one biofilm sample ( BF4) was prepared using contaminated mother cultures and maintained under similar incubation conditions.

All biofilm cultures including all replicates were transferred to clean sterile 50 ml centrifuge tubes followed by centrifugation at the minimum speed (1000 g) for 30 s to separate the attached biofilm mass out from free floating un-attached microorganisms. The entire supernatant portion was carefully removed using a micropipette without disturbing the pellet. Subsequently, the pellet (attached biofilm mass) was washed several times with sterile double distilled water until obtaining a clear supernatant. Entire removal of free floating un- attached microorganisms was confirmed by obtaining a clear transparent supernatant. At the final washing, the clear transparent supernatant was removed carefully and centrifuged at 3,000 g for 20 min to stick the purified biofilm mass to the bottom of the tube.

DNA extraction from the purified biofilm community was performed using MO BIO Power Soil DNA isolation kit (Cat No- 12888-100) with the bead beating method, as explained in appendix 3. The extracted DNA samples were stored in low temperature (-20 °C).

#### 3.3.8.2 Amplification of the extracted DNA using labelled TRFLP primers

Amplification of the extracted DNA samples was performed using labelled TRFLP primers. Purified DNA samples obtained from bacteria (*Bacillus* sp., contaminated bacterial sample B9 and *Rhizobium* sp.) and one set of DNA samples extracted from biofilm community (BF1, BF2, BF3 and BF4) were amplified using labelled 27F primer and un-labelled 1492R primer whereas purified DNA samples obtained from *Trichoderma* sp. and another set of DNA samples extracted from biofilm community (FF1, FF2, FF3 and FF4) were amplified

using labelled ITS1 and un- labelled ITS4 primers. PCR was performed according to the same protocol explained in 3.3.7.4 above. The PCR products were fractionated on a 1.5% agarose gel using 1X TAE buffer (0.04 M Tris-acetate, 0.001M EDTA). Gels were stained with EtBr and photographed under UV light using BIORAD Gel Doc system. A 100bp DNA size marker was included on the gel for size analysis.

### 3.3.8.3 Purification of the PCR products obtained using labelled primers

Purification of the PCR products was performed by the ethanol precipitation method using the same protocol as explained in 3.3.7.5 above. The concentration of each purified DNA was measured using UV spectrophotometer (Nano-drop 2000 C) at 340 nm wavelength and stored at low temperature until further processing.

### 3.3.8.4 Restriction digestion of the purified PCR products and RFLP analysis

Selection of a suitable restriction enzyme for restriction digestion of the PCR products was performed using online tool 'cutter' (http://rna.lundberg.gu.se/cutter2/).Restriction digestion was performed for the purified PCR products obtained from each individual microbial samples and biofilm communities. Restriction digestion was carried out in a 30 µl reaction mixture, containing 10X NEB cut smart buffer and suitable restriction enzymes with the purified PCR products (appendix 4). DNA samples amplified with 16S rRNA primers were digested using HhaI enzyme and the DNA samples amplified with ITS primers were digested using Taqα I enzyme. Subsequently, the reaction mixture was incubated at 65 °C for one hour followed by incubation at 80 °C for 20 min to inactivate the enzyme activity.

### 3.3.8.5 Preparation of the samples for TRFLP analysis

#### 3.3.8.5.a Preparation of bacterial and fungal labelled DNA samples

Approximately 40 ng of the bacterial PCR products (after restriction digestion) amplified using 16S rRNA labelled primers and 20 ng of the fungi PCR products amplified using labelled ITS primers were added in 1.5 ml eppendorf tubes separately. Subsequently, the final volume of each tube was adjusted to 20 μl with nano pure water.

#### 3.3.8.5.b Preparation of labelled DNA samples from biofilm communities

Approximately 40 ng of the biofilm PCR products (depending on the concentrations) amplified using 16S rRNA labelled primers and 20 ng of biofilm PCR products amplified using labelled ITS primers were mixed together in 1.5 ml eppendorf tubes. Subsequently, the final volume of each tube was adjusted to 20μl with nano pure water. All samples were sent to a sequencing company (MACROGEN, South Korea) for T-RFLP analysis.

### 3.3.8.6 Data Analysis

Electropherograms obtained from the TRFLP profiles (peaks) of each biofilm combinations and the individual microorganisms were analyzed using PEAK SCANNER software version 1.

### 3.4 Results and Discussion

#### 3.4.1 Screening experiments for microbial isolates

##### 3.4.1.1 Broth culture pH of microbial isolates

All bacterial isolates except *Acinetobacter* sp. (B2) and *Pseudomonas* sp. (B4 and B6) showed acidic pH range (Fig. 3.1). Out of eight different diazotrophic isolates, *Rhizobium* sp. showed significantly the lowest mean inoculum pH ($P < 0.05$, $5.1 \pm 0.02$). Further, acidic pHs were recorded for all the isolated fungi.

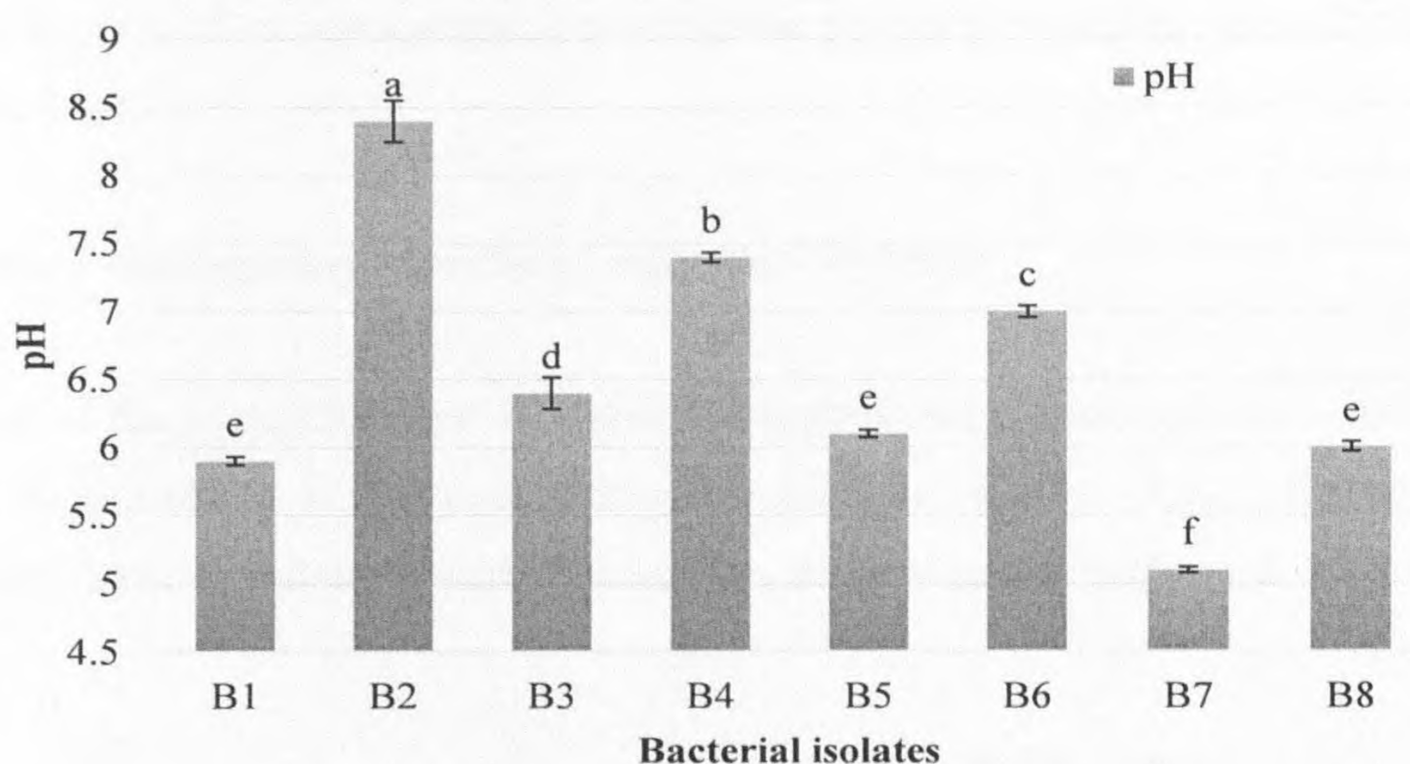



Figure 3.1- Mean pH of different bacterial broth cultures. B1 to B8- different bacterial isolates (B1- *Bacillus* sp., B2- *Acinetobacter* sp., B3- *Acidomonas* sp., B4- *Pseudomonas* sp., B5- *Serratia* sp., B6- *Pseudomonas* sp., B7- *Rhizobium* sp., B8- *Bacillus* sp.). Different letters on the columns show significant differences at 5% probability level. Vertical bars show standard deviations.

It has been reported that the beneficial soil bacteria can produce plant growth promoting hormones such as IAA and other complex organic acids (Rodriguez and Fraga, 1999), which can create acidic environment around the rhizosphere. Further, the production of organic acids by phosphate solubilizing bacteria has also been well documented and the principal organic acid producers, such as *Pseudomonas* sp., *Erwinia herbicola*, *Pseudomonas cepacia* and *Burkholderia cepacia* have been reported (Illmer and Schinner, 1992; Rodriguez *et al.,* 1999). Strains of *Bacillus liqueniformis* and *Bacillus amyloliquefaciens* have also been found to produce mixtures of lactic, isovaleric, isobutyric, and acetic acids. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among phosphate solubilizers (Banik and Dey, 1982; Illmer and

Schinner, 1992).Other mechanisms have been considered, such as the production of inorganic acids like sulphidric acid, nitric, and carbonic acids (Sperberg, 1958; Rodriguez *et al.*, 1999). Another possibility for the reduction of medium pH is the acid production due to the accumulation of $CO_2$ released by the microorganisms (Johnson, Hill and Piddock, 1999). Another study has reported the ability of producing 2-ketogluconic acid by *Rhizobium* (e.g., *Rhizobium/Bradyrhizobium*) species which reduce pH of the medium (Halder and Chakrabarty 1993; Hayat *et al.*, 2010). Therefore in the current study, low pH of *Bacillus* sp. (B1), *Acidomonas* sp., *Serratia* sp., *Rhizobium* sp. and *Bacillus* sp. (B8) isolates can be considered as a beneficial character of the isolates which might reflect the production of complex organic acids or the production of plant growth promoting hormones like IAA.

### 3.4.1.2 Nitrogenase activity of microbial isolates

Out of the eight different isolates, the highest significant ethylene production ($P < 0.05$, 2.88 nmol/hr ± 0.03) was shown by *Rhizobium* (Fig. 3.2). Except *Bacillus* sp. and *Rhizobium* sp. all other bacterial isolates showed low nitrogenase activity.

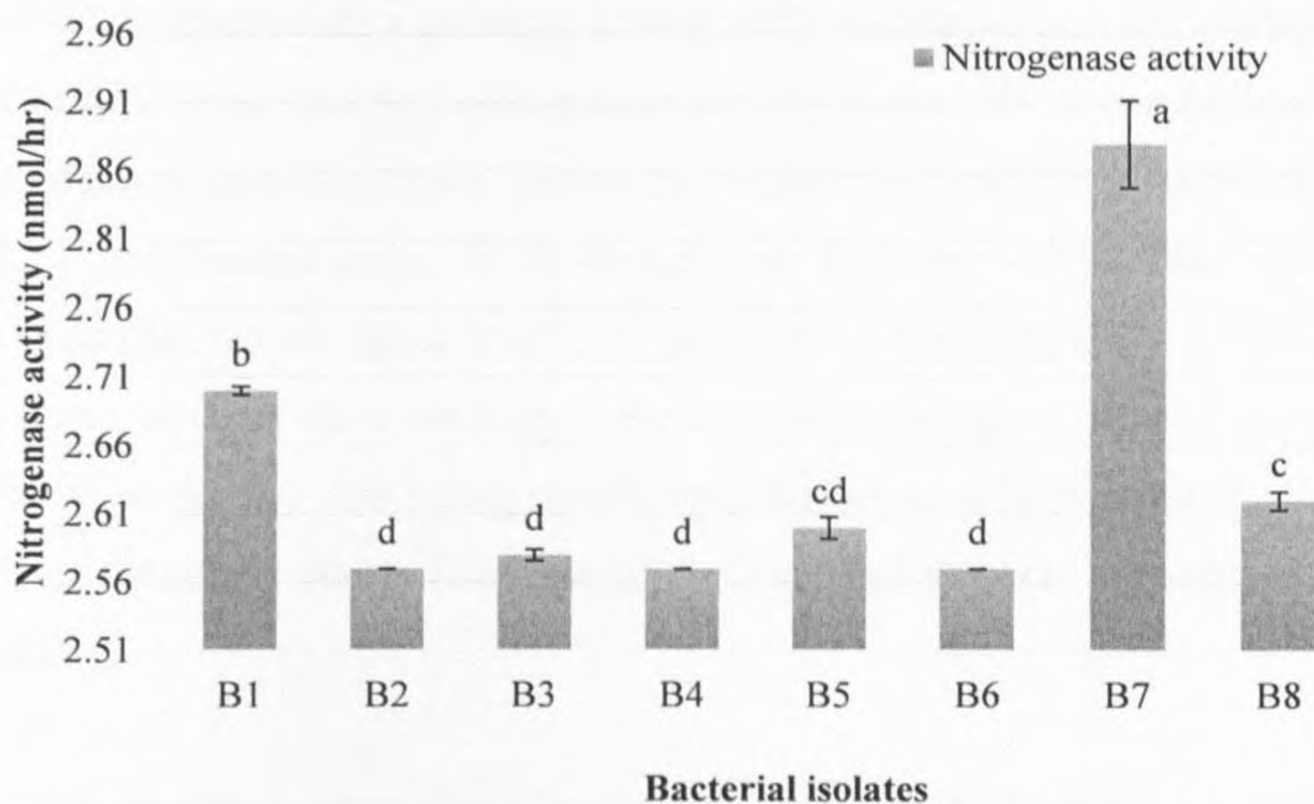



Figure 3.2- Mean ethylene production from Acetylene Reduction Assay (ARA) for different bacterial isolates. B1 to B8- different bacterial isolates (B1- *Bacillus* sp., B2- *Acinetobacter* sp., B3- *Acidomonas* sp., B4- *Pseudomonas* sp., B5- *Serratia* sp., B6- *Pseudomonas* sp., B7- *Rhizobium* sp., B8- *Bacillus* sp.). Different letters on the columns show significant differences at 5% probability level. Vertical bars show standard deviation.

According to the literature, the concentration of ethylene produced from the ARA, is an indirect measure of BNF used extensively to screen species for nitrogenase activity of prokaryotes. Nitrogenase enzyme responsible for $N_2$ fixation and reducing $C_2H_2$ (acetylene), an inhibitor for $N_2$ fixation, to $C_2H_4$ (ethylene) provides a useful assay for the quantification of $N_2$ fixation (Dilworth, 1966). The basis of the assay is that in the presence of atmospheres of acetylene, virtually all electron flow through nitrogenase is used to reduce $C_2H_2$ to $C_2H_4$. The ethylene produced in the assay can be measured by GC. The amount of reduced ethylene can be used to estimate the amount of fixed $N_2$ (Staal *et al.*, 2001). The assay was quickly adopted as standard practice in laboratories measuring nitrogenase activity because it was fast, inexpensive, widely applicable, and easy to perform. Several studies have proven that PGPRs may simultaneously use more than one mechanisms including biological $N_2$ fixation and producing phytohormones like IAA, cytokinins and GA to enhance plant growth stimulation (Zahir *et al.*, 2004; Bhattacharyya and Jha, 2012). Another study has reported high nitrogenase activity by PGPRs isolated from rice rhizosphere (Park *et al.*, 2005). The N2 fixers play a key role in the growth and persistence of effective microbial communities by supplying N through BNF (Seneviratne *et al.*, 2011). Asymbiotic nitrogenase activity in free-living cultures under appropriate conditions, has been demonstrated primarily in several strains belonging to the genus *Bradyrhhizobium*. A limited number of slow growing strains of *R. japonicum* and *Rhizobium* sp. 'cow pea group' are known to express high nitrogenase activity under culture conditions. It has been reported that the nitrogenase activity shown by microorganisms varies between 0. 25 – 76 nmol/hr (Gara and Shanmugam, 1978; Sloger and Berkum, 1988). However, the current study showed that the nitrogenase activity only varied between 2.5 – 2.9 nmol/hr. Further, none of these workers have correlated the $N_2$ fixing ability of the strains with the nitrogenase activity under the free living conditions. However, it is possible that *in vitro* nitrogenase activity may not be an indicator of *in vivo* performance. (Dadarwal, Kundu and Tauro, 1981).

### 3.4.1.3 Seedling vigor after application of bacterial isolates

Figure 3.3 shows the responses of different bacterial isolates on seedling vigor for lettuce seed germination assay. It was clearly shown that all bacterial isolates enhanced the seedling vigor compared to un-inoculated control. Out of eight bacterial isolates, the highest significant seedling vigor value (510.8 ± 20.4) was observed in *Rhizobium* sp. compared to

the other bacterial isolates ($P < 0.05$). *Rhizobium* sp. (B7), *Bacillus* sp. (B1and B8) and *Serratia* sp. were considered as the best responsive bacterial isolates in terms of seedling vigor. Further, it was clear that *Rhizobium* sp. enhanced the seedling vigor by approximately 35% compared to the un-inoculated control. However, it has been reported that a seed germination study which had been conducted using tomato seeds significantly enhanced the seed germination rate, root length shoot length and seedling vigor by 90% compared to the un-inoculated control by the application of rhizobacterial strains (Agrawal and Agrawal, 2013). Another study has reported that the inoculation *Bacillus* sp. enhanced the root length by 13-15% compared to the control group (Mena-Voilante and Olalde-Portugal, 2007).

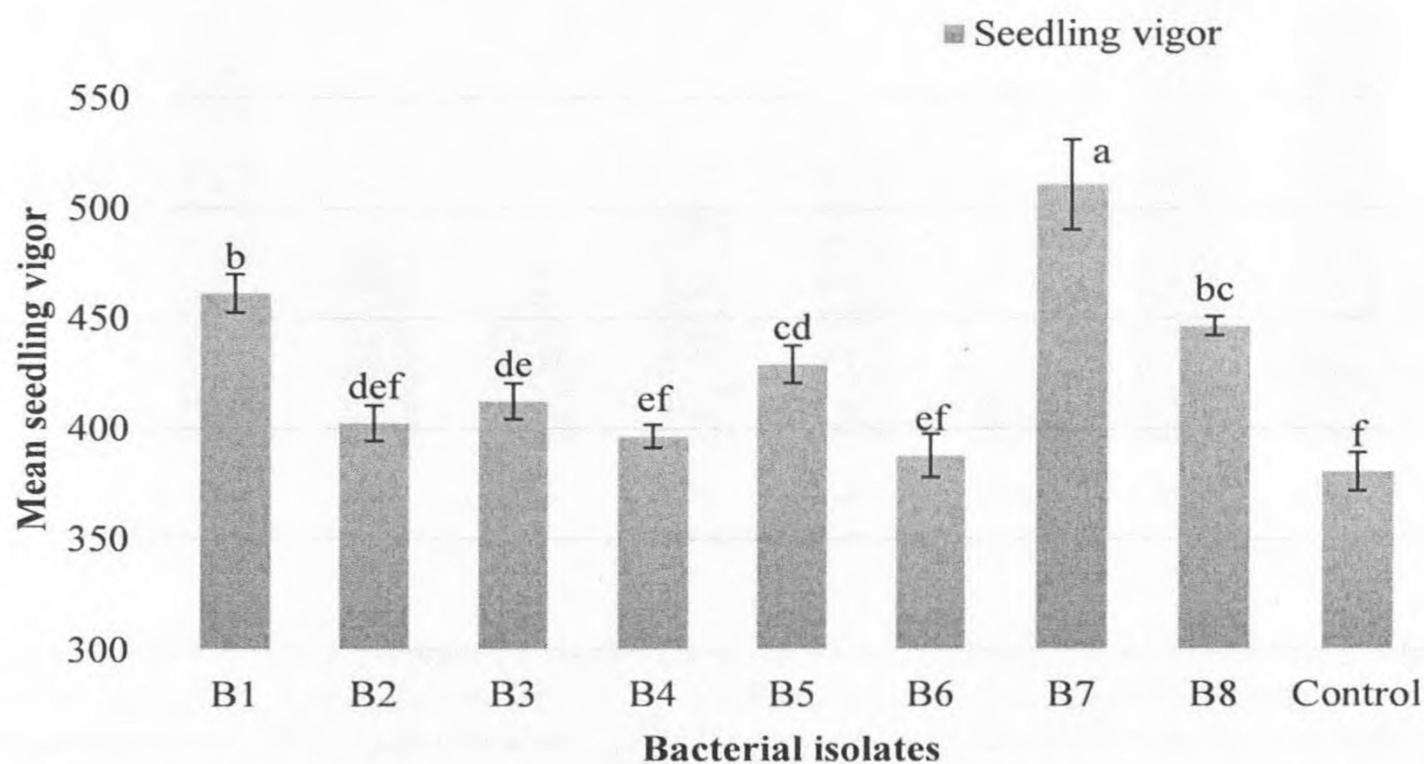



Figure 3.3- Mean seedling vigor for lettuce seeds treated with different bacterial isolates (control – only ML medium). B1 to B8- different bacterial isolates (B1- *Bacillus* sp., B2- *Acinetobacter* sp., B3- *Acidomonas* sp., B4- *Pseudomonas* sp., B5- *Serratia* sp., B6- *Pseudomonas* sp., B7- *Rhizobium* sp., B8- *Bacillus* sp.). Different letters on the columns show significant differences at 5% probability level. Vertical bars show standard deviations.

### 3.4.1.4 Quantification of IAA production

According to the current study, *Rhizobium* sp. (B7) showed the highest significant IAA production ($P < 0.05$, 21.54 μg/ml ± 0.9) compared to all other bacterial strains (Fig. 3.4) whereas *Pseudomonas* sp. (B6) recorded very low IAA production (2.2 μg/ml ± 0.6). Similar IAA productions have been reported by several other studies. For instance, *Pseudomonas* and *Acinetobacter sp* isolated from wheat and rye rhizosphere have been reported to produce IAA ranging from 3.98 μg/ml to 13.33 μg/ml (Leinho and Vacek, 1994;

Yasmin *et al.*, 2009). Another study has shown higher IAA production by *Pseudomonas fluorescens* and *Stenotrophomonas maltophilia* with the concentrations of 100.5 μg/ml for fluorescens and 112.8 μg/ml for *S. maltophilia* (Part *et al.*, 2005). However, in comparison with previous recorded data, moderate IAA production was recorded by *Rhizobium* sp. (21.54 μg/ml ± 0.9) and *Bacillus* sp. (B1-15. 58 μg/ml ± 0.9, B8- 16.16 μg/ml ± 0.7) in the current study.

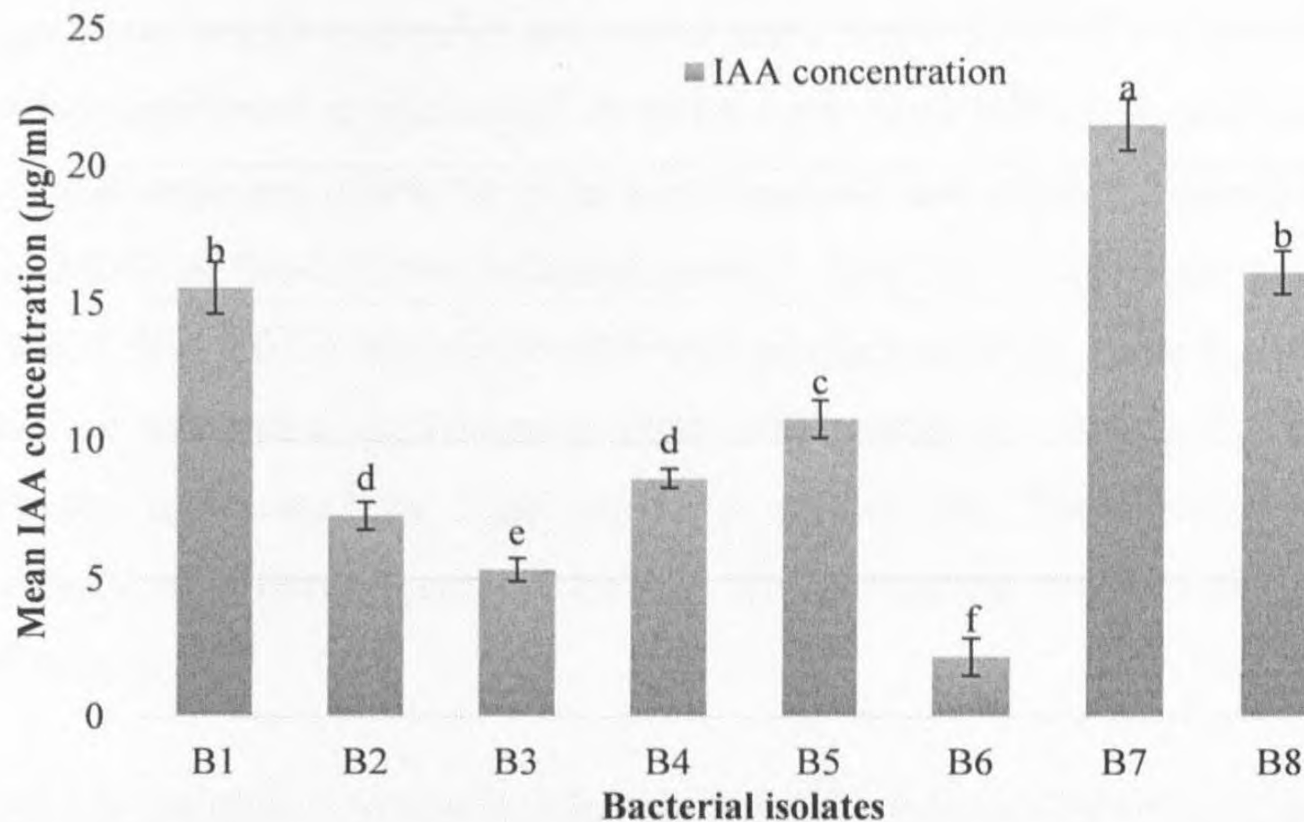



Figure 3.4- Mean IAA production by different bacterial isolates. B1 to B8- different bacterial isolates (B1- *Bacillus* sp., B2- *Acinetobacter* sp., B3- *Acidomonas* sp., B4- *Pseudomonas* sp., B5- *Serratia* sp., B6- *Pseudomonas* sp., B7- *Rhizobium* sp., B8- *Bacillus* sp.). Different letters on the columns show significant differences at 5% probability level. Vertical bars show standard deviations.

IAA is one of the most important phytohormones and functioned as important signal molecule in the regulation of plant development. IAA produced by bacteria, improve plant growth by increasing number of root hairs and lateral roots. It has been well documented that many species of PGPRs are able to synthesize phytohormones like IAA, cytokinin and GA (Frankenberger and Arshad, 1995) and thereby promote plant growth and increase germination rate of seeds (Kloepper *et al.*, 2007; Bhattacharyya and Jha, 2012). Further, the inductive role of IAA on stolon initiation and tuber induction in potato was documented in earlier studies (Dragicevic *et al.*, 2008; Nookaraju *et al.*, 2011). A diverse group of microbes, including soil, epiphytic and tissue colonizing bacteria have been found to synthesize IAA (Patten and Glick, 1996; Park *et al.*, 2005). The species of bacteria capable of producing IAA include *Pseudomonas* sp. *Bacillus* sp. *Klebsiella* sp. *Azospirillum* sp. *Enterobacter* and *Serratia* sp. (Martens and Frankenberger, 1991; Yasmin *et al.*, 2009).

Strains of *Bacillus amyloliquefaciens* and *B. subtilus* have been observed to produce higher concentrations of IAA and have significantly increased crop growth and development. Further, it has been reported that the IAA production is varied between bacterial strains (Frankenberger and Arshad, 1995).

### 3.4.2 Correlations between different screening parameters

Significant negative correlations were observed (r = 0.715, P = 0.046) between mean pH of the bacterial broth medium and mean IAA production by different bacterial isolates (table 3.3) and mean pH of the bacterial broth medium and mean nitrogenase activity (r = 0.734, P = 0.038) of the different bacterial isolates . Further, a significant negative relationship (r = 0.821, P = 0.013) was observed between mean seedling vigor and mean pH of the broth medium whereas a significant positive relationship (r = 0.943, P = 0.000) was observed between mean seedling vigor and IAA production. Therefore, it was clear that IAA produced by different bacterial isolates effected for the medium pH and seed germination efficiency.

Table 3.3- Correlation coefficients (r) between different screening parameters for bacterial monocultures

| | Inoculum pH | Nitrogenase activity | Seedling vigor |
|---|---|---|---|
| Nitrogenase activity | -0.734 (0.038) | | |
| Seedling vigor | -0.821 (0.013) | 0.947 (0.000) | |
| IAA production | -0.715 (0.046) | 0.845 (0.008) | 0.943 (0.000) |

Values within parentheses are probability levels.

A relationship has been reported by another *in vitro* study with some bacterial species that improved $N_2$ fixation ability, over produced IAA, thereby decreasing the pH (Bianco and Defez, 2010). It has been estimated that 80% of bacteria isolated from rhizosphere can produce plant growth regulator IAA (Patten and Glick 1996; Hayat *et al.*, 2010). *Bacillus*

sp. and *Paenibacillus* sp. have the ability to produce higher concentrations of IAA (448 μg $ml^{-1}$) which creates a low pH environment around the rhizosphere leading to P solubilization (Acuna *et al.*, 2012). This negative correlation between pH and IAA production by rhizobacteria like *Bacillus sp.* while promoting plant growth has been recorded by several studies (Trivedi and Pandey, 2008; Banerjee *et al.*, 2010).

It has been reported that beneficial soil microorganisms have the ability of producing different phytohormones like IAA and GA; thereby promote plant growth and increase germination rate of seeds (Bhattacharyya and Jha, 2012). Evidences can be found for the enhancement of growth performances and seedling vigor by the inoculation of beneficial rhizobacterial strains like *Rhizobium* due to the ability of producing phytohormones like IAA, vitamins, siderophores (Hussain *et al.*, 2009; Bhattacharyya and Jha, 2012). Further, inoculated PGPRs have been produced IAA in the presence of seed exudates that might have triggered faster germination (Bashan, 1998; Mia *et al.*, 2012). Rice inoculation with *Rhizobium leguminosarum* has shown significant growth promoting effects on rice seedlings due to the activity of IAA (Biswas *et al.*, 2000; Hayat *et al.*, 2010). It has been confirmed that the exogenous IAA produced by PGPRs controls a wide variety of processes in plant development and plant growth: low concentrations of IAA can stimulate primary root elongation, whereas high IAA levels stimulate the formation of lateral roots (Dobbelaere *et al.*, 1999; Remans *et al.*, 2008).

### 3.4.3 Pot experiment for monocultures

#### 3.4.3.1 Evaluation of pathogenicity using a pot experiment

After six weeks from seed sowing, black spotted yellow colour leaves were observed in both treatment and control plants and it was identified as the white fly pest attack (plate 3.1b). In addition, dark lesions were observed at the leaf edges after fifth week from seed sowing in both treatments and non-treated control and the symptoms were similar to potato late blight disease. Lesions started to develop at the leaf edges, where dew is retained for longest period of time (plate 3.1.a). Lesions appeared first on the lower leaves and after eight weeks they occurred on upper leaves as well. Disease was confirmed by observing the infected leaf surfaces and sections through a light microscope. Further *Alternaria* sp. was identified from both treated plants and untreated control plants by observing leaf sections through light microscope. It was confirmed that the *Alternaria* sp. and late blight were not

arisen due to the inoculation of bacterial or fungal strains. Further, it was noted that none of the diseases were in severe stage. Wilted plats were not observed even after four weeks and eight weeks from seed sowing. Also the pest attack was well behind the threshold level.

(a)

(b)

Plate 3.1a- External appearance of infected potato leaves with potato late blight disease (initial stage). Plate 3.1.b- Infected potato leaf with white fly damage.

According to the literature, late blight, caused by the water mold *Phytophthora infestans*, has the potential to be a very destructive disease of potato. It has been reported that the first symptoms of late blight in the field are small, dark, circular to irregularly shaped lesions, which appear three to five days after infection. During cool, moist weather, lesions expand rapidly into large, dark brown or black spots, often surrounded by a pale green to yellow border. As new infections occur and existing lesions coalesce, entire leaves may become blighted and destroyed within a few days (Andrivon, 1995).

Figure 3.5 represents the mean number of affected potato leaves by late blight disease during the entire growth period of the potato plant. Since bacteria and fungi were grown in two different media, two control experiments were conducted as control1 (un-inoculated CCM broth) and control 2 (un- inoculated CZ broth). The results revealed that the number of late blight infected leaves was not significantly different between un-inoculated control and treatments ($P > 0.05$). Further, all bacterial treated plants showed less number of infected leaves compared to un- inoculated control 1 though they were not significantly different. Therefore, it was confirmed that potato late blight disease and bacterial wilt disease were not due to the inoculation of microbial isolates.

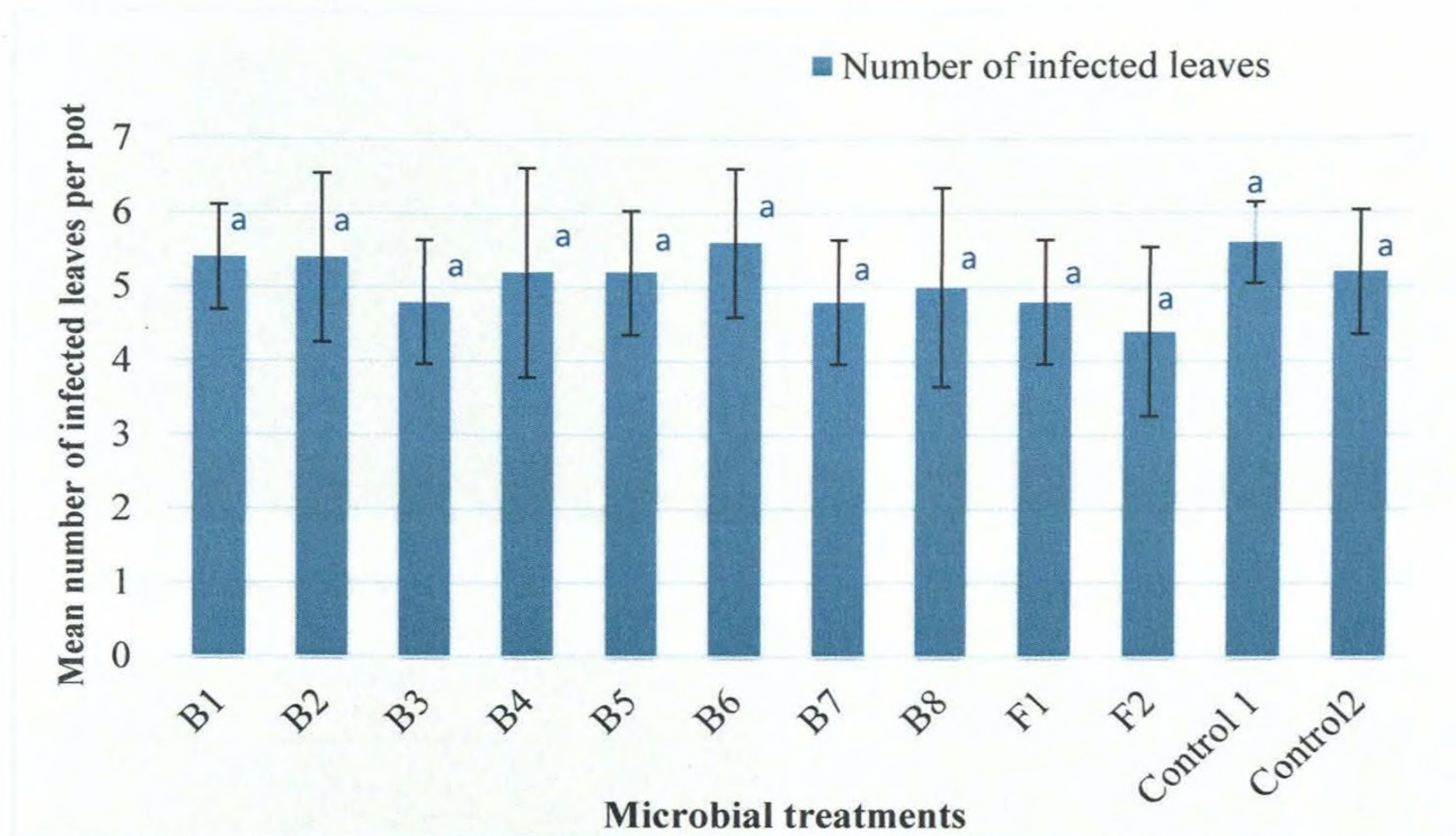



Figure 3.5- Mean number of late blight disease infected leaves for different microbial isolates. B1 to B8- different bacterial isolates (B1- *Bacillus* sp., B2- *Acinetobacter* sp., B3- *Acidomonas* sp., B4- *Pseudomonas* sp., B5- *Serratia* sp., B6- *Pseudomonas* sp., B7- *Rhizobium* sp., B8- *Bacillus* sp.). F1 and F2- fungal isolates (*Aspergillus* sp. and *Trichoderma* sp.). Control 1- un-inoculated CCM broth. Control 2- un-inoculated CZ broth. Different letters on the columns show significant differences at 5% probability level. Vertical bars show standard deviations.

### 3.4.3.2 Growth responses of potato to microbial inoculations in soil

Positive vegetative plant growth responses (size of the shoot, number of shoots) were observed by the inoculation of *Rhizobium* sp. compared to the non- treated control after seven weeks from seed sowing Bacterial treated plants appeared as healthy and greenish, which the number of leaves were higher than that of non- treated control (plate 3.2.a and plate 3.2b). It was clear that the number of tubers and the size of the tubers were high in the plants treated with *Rhizobium* sp. (B7) compared to non- treated control (plate 3.2 c- plate 3.2 f).

(a) Magnification x 1/4

(b) Magnification x ¼

(c) Magnification x1/5

(d) Magnification x 1/5

(e) Magnification x 1/2

(f) Magnification x 1/2

Plate 3.2- Growth responses of potato plants grown in pots for bacterial treatments (before and after harvesting). (a)- Plant response after seven weeks from seed sowing for *Rhizobium* sp. (b) Plant response after seven weeks from seed sowing for non- treated control. (c)- Shoot after harvesting for *Rhizobium* sp. (d) Shoot after harvesting for non- treated control. (e)- Tubers after harvest for *Rhizobium* sp. (f)- Tubers after harvesting for non- treated control (control- only CCM).

In comparison with all other treatments, treatment with *Rhizobium* sp. (B7) significantly enhanced the mean tuber weight ($P < 0.05$, 56.67 g ± 1.36) of potato (table 3.4). All bacterial isolates except *Acinetobacter* sp. and *Pseudomonas* sp. (B6), enhanced tuber weight of potato compared to non- treated control 1. Both fungal treatments did not affect to enhance the tuber weight compared to non- treated control 2. Further, all bacterial treatments did not significantly enhance ($P > 0.05$) the tuber number of potato compared to the non- treated control 1. The highest mean tuber number (4.2 ± 0.45) and the highest mean stolon number (3.2 ± 0.84) were observed by the treatment with *Rhizobium* sp. (B7). Two sample t-test confirmed that *Rhizobium* sp. (B7) significantly enhanced the tuber number ($P < 0.05$) and stolon number ($P < 0.05$) compared to the non- treated control 1. Both fungal treatments did not enhance the mean tuber weight, mean tuber number and the mean stolon number compared to the non- treated control 2.

Table 3.4- The effect of different bacterial isolates on tuberization of potato grown in pots.

| Treatments | Tuber weight (g/plant) Mean±SD | Tuber number (per plant) Mean±SD | Stolon number (per plant) Mean±SD |
|---|---|---|---|
| B1 | $50.42 \pm 1.29^{b}$ | $3.8 \pm 0.45^{ab}$ | $3.0 \pm 0.71^{ab}$ |
| B2 | $38.75 \pm 0.73^{e}$ | $3.0 \pm 1.23^{ab}$ | $1.8 \pm 0.84^{ab}$ |
| B3 | $46.89 \pm 0.94^{cd}$ | $3.6 \pm 1.19^{ab}$ | $2.8 \pm 1.09^{ab}$ |
| B4 | $46.60 \pm 1.01^{d}$ | $2.4 \pm 1.14^{ab}$ | $2.2 \pm 0.45^{ab}$ |
| B5 | $49.35 \pm 0.99^{bc}$ | $3.2 \pm 0.55^{ab}$ | $2.4 \pm 0.89^{ab}$ |
| B6 | $37.97 \pm 0.96^{e}$ | $2.8 \pm 0.45^{ab}$ | $1.8 \pm 0.84^{ab}$ |
| B7 | $56.67 \pm 1.36^{a}$ | $4.2 \pm 0.45^{a}$ | $3.2 \pm 0.84^{a}$ |
| B8 | $49.20 \pm 0.61^{bc}$ | $3.4 \pm 0.89^{ab}$ | $2.2 \pm 0.84^{ab}$ |
| F1 | $20.88 \pm 1.85^{f}$ | $1.8 \pm 0.84^{b}$ | $1.4 \pm 0.54^{b}$ |
| F2 | $22.30 \pm 1.08^{f}$ | $1.8 \pm 0.84^{b}$ | $1.4 \pm 0.54^{b}$ |
| Control 1 | $37.35 \pm 1.01^{e}$ | $2.6 \pm 0.89^{ab}$ | $1.4 \pm 0.54^{b}$ |
| Control 2 | $21.86 \pm 1.55^{f}$ | $1.8 \pm 0.45^{b}$ | $1.4 \pm 0.54^{b}$ |

Control 1– only CCM. Control 2- only CZ broth. B1 to B8- different bacterial isolates (B1- *Bacillus* sp., B2- *Acinetobacter* sp., B3- *Acidomonas* sp., B4- *Pseudomonas* sp., B5- *Serratia* sp., B6- *Pseudomonas* sp., B7- *Rhizobium* sp., B8- *Bacillus* sp.). F1 and F2- fungal isolates (*Aspergillus* sp. and *Trichoderma* sp.). Different letters show significant differences at 5% probability level.

According to screening experiments of the current study, *Rhizobium* sp. (B7) showed the lowest pH and the highest $N_2$ fixing ability (Fig. 3.1 and Fig. 3.2). Therefore, the tuber enhancement by *Rhizobium* sp. might be due to the multiple effect of growth promoting phytohormones like IAA and nitrogenase activity. It has been reported that PGPRs can be used as biofertilizers, or phyto-stimulators and can promote plant growth directly through $N_2$ fixation, phyto-hormone production and phosphate solubilization. Further, it has been explained that this may benefit plants by providing utilizable N through fixation of atmospheric $N_2$ or by the production of plant growth stimulators like IAA and GA (Jha *et al.*, 2013).

Some strains of rhizobacteria have been reported to influence tuber number and tuber yield in potato (Burr, Schroth and Suslow, 1978; Oswald *et al.*, 2010). It has been reported that a significant increment in growth and yield of potato plants after the treatment of *Pseudomonas* sp. with seed tubers (Burr *et al.*, 1978; Nookaraju *et al.*, 2011). Vrany and Fiker (1984) has reported a 4 to 30% increment in plant growth and tuber yield in potato after application of PGPRs. Similarly, Sturz (1995) found an increased tuber number and average tuber weight with the application of PGPRs. Apart from tuberization, plant growth, root number and lignin content in potato have been reported to enhance by the inoculation

of PGPRs (Frommel, Nowak and Lazarovits, 1991; Nookaraju *et al.*, 2011). Screened PGPRs in potato have been reported to increase tuber yields in PGPRs treated plants to early tuber induction, fast leaf area development and greater photosynthetic rates (Oswald *et al.*, 2010; Nookaraju *et al.*, 2011). Similar results have been recorded from greenhouse studies with positive effects of the three selected isolates on potato tuber number, average tuber size and total yield (Nookaraju *et al.*, 2011).

#### 3.4.3.3 Correlations between growth parameters

Significant negative relationships were observed between mean pH of microbial inoculant and mean tuber weight ($r = 0.861$, $P = 0.006$), mean pH and mean tuber number ($r = 0.780$, $P = 0.023$) and mean pH and mean stolon number ($r = 0.801$, $P = 0.017$). Further, it was observed (table 3.5) that there were significant positive relationship between IAA concentration and mean tuber weight ($r = 0.870$, $P = 0.005$). However significant correlation was not observed between IAA concentration and mean tuber number ($r = 0.706$, $P = 0.051$).

Table 3.5- Correlation coefficients (r) between different plant parameters of potato and the properties of microbial inoculants

| | Tuber weight | Tuber number | Stolon number | IAA production |
|---|---|---|---|---|
| Tuber number | 0.714<br>(0.047) | | | |
| Stolon number | 0.867<br>(0.005) | 0.836<br>(0.010) | | |
| IAA production | 0.870<br>(0.005) | 0.706<br>(0.051) | 0.669<br>(0.070) | |
| pH | -0.861<br>(0.006) | -0.780<br>(0.023) | -0.801<br>(0.017) | -0.715<br>(0.046) |

Values within parentheses are probability levels.

This implies that the inoculated microbial inocula colonize the rhizosphere, producing high acidity and IAA which enhanced the tuber number and tuber weight. It has been reported by another study that beneficial microbial inocula like PGPRs in the soil solution near root

hairs, helps to increase the plant growth promotion through the secretion of plant growth promoters like IAA (Acuna *et al.*, 2011).

*Bacillus* sp. (B1 and B8), *Acidomonas* sp., *Serratia* sp. and *Rhizobium* sp. responded positively to all the growth parameters of potato plants grown in soil medium under greenhouse conditions whereas both fungal strains did not respond.

### 3.4.4 Selection of efficient beneficial bacterial isolates from screening experiments

*Rhizobium* sp. (B7) showed the highest score for the statistical ranking of different screening experiments (table 3.6). All bacterial isolates except *Acinetobacter* sp. and *Pseudomonas* sp. (B4 and B6) showed higher performances for all the screening experiments including greenhouse pot experiment. Therefore, *Bacillus* sp. (B1 and B8), *Acidomonas* sp. (B3), *Serratia* sp. (B5) and *Rhizobium* sp. (B7) were selected as the best performing beneficial bacterial isolates since they showed the highest ranking for the screening experiments and for the greenhouse evaluations. Therefore, these bacterial isolates were selected to establish biofilms in combination with *Aspergillus* sp. and *Trichoderma* sp.

Table 3.6- Statistical ranking of different bacterial isolates on different screening parameters

| Bacterial isolate | Selection parameter | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | pH | ARA | Seedling vigor | IAA production | Pot experiment | | | Total |
| | | | | | Tuber weight | Tuber number | Stolon number | |
| B1 | 7 | 7 | 7 | 6 | 7 | 7 | 7 | 48** |
| B2 | 1 | 2 | 3 | 3 | 2 | 3 | 3 | 17 |
| B3 | 4 | 4 | 4 | 2 | 4 | 6 | 6 | 30** |
| B4 | 2 | 2 | 2 | 4 | 3 | 1 | 4 | 18 |
| B5 | 5 | 5 | 5 | 5 | 6 | 4 | 5 | 35** |
| B6 | 3 | 2 | 1 | 1 | 1 | 2 | 3 | 13 |
| B7 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 56** |
| B8 | 6 | 6 | 6 | 7 | 5 | 5 | 4 | 39** |

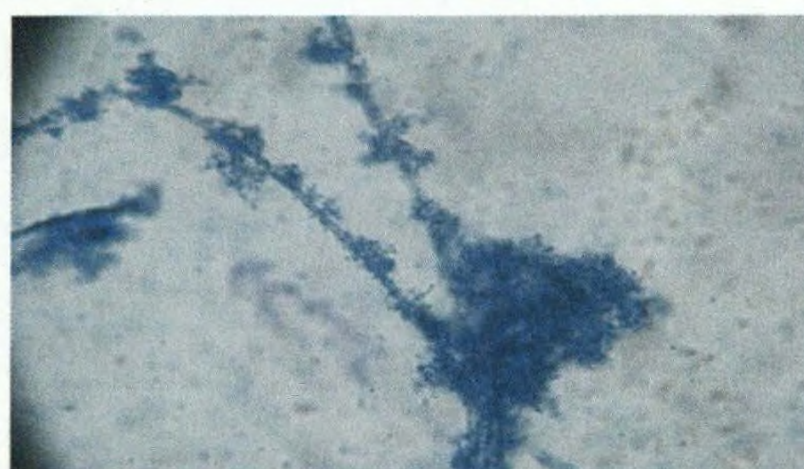
(**- higher scores for the statistical ranking. Ranking was performed using MINITAB 16 statistical software. B1- *Bacillus* sp., B2- *Acinetobacter* sp., B3- *Acidomonas* sp., B4- *Pseudomonas* sp., B5- *Serratia* sp., B6- *Pseudomonas* sp., B7- *Rhizobium* sp., B8- *Bacillus* sp.)

### 3.4.5 Establishment and screening of biofilms

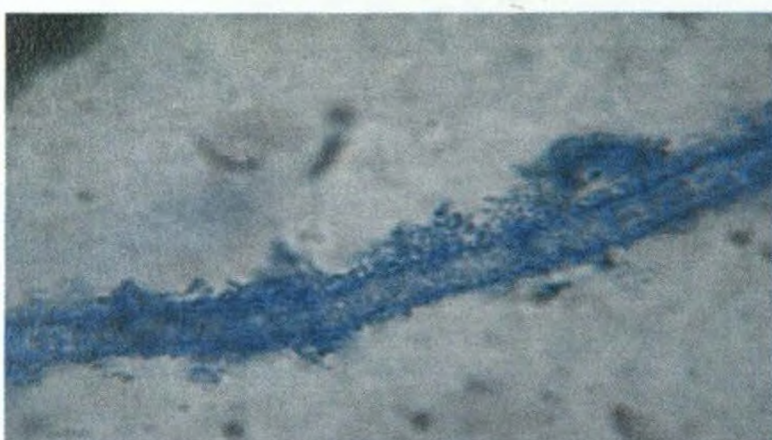
#### 3.4.5.1 Establishment of simple biofilms

All bacterial and fungal combinations showed successful biofilm formation since the fungi acts as the biotic surface to which the bacteria adhere to consume fungal exudates (Toole, Kaplan and Kolter, 2000). These biofilms are characterized by high bacterial cell densities and bacterial cells in the biofilms are protected against adverse environmental conditions (Seneviratne *et al.*, 2008). Microscopic observations revealed that the attachment process was initiated from five days onwards and detachment of cells from biofilms was recorded after 18 days of co-culturing. Bacteria used in this study were mainly beneficial bacteria which were able to fix $N_2$. When they were co-cultured *in vitro* with fungi, the bacteria attached and colonized on fungal mycelia to form the biofilms, known as FBBs (Khan *et al.*, 2009). In the current study, it was clearly observed that the bacterial isolates were attached with fungal mycelia through a slimy mass, which is known as EPS (plate 3.3). Dark blue colour was due to the absorption of stain in to the cell materials as well as to EPS.

(a)



(b)



(c)

Plate 3.3 -Microscopic views of simple biofilm combinations produced from isolated fungal and bacterial isolates. (a)- biofilm formation from a fungi and a rod shape bacteria (Magnification 400 X). (b)- biofilm formation from a fungi and a cocci shape bacteria (Magnification 400X). (c)- Fungal- bacterial biofilm showing the attachments of the coccus shape bacteria to the fungal hyphae. Darkness is due to cotton blue stain absorbed by EPS produced by the biofilms. Stain, lactophenol cotton blue. Magnification, X 1000.

Fungi-associated bacterial communities appear to be different from the microflora in bulk soil in terms of species composition and relative abundance (Frey-Klett *et al.*, 2005; Rangel-Castro, Levenfors and Danell, 2002; Timonen and Hurek, 2006). Bacterial colonization on fungal surface may enable the bacteria to exploit the fungus as a source of nutrients. Bacteria may utilize nutrients from the fungal cell wall and secreted products from the fungi (Hogan and Kolter, 2002). These bacterial–fungal interactions play very important roles in microbial community ecology. For instance, bacterial attachment to the hyphal surfaces may enhance synergistic actions of bacteria and fungi required to breakdown complex substrates (Hogan and Kolter, 2002). Some bacterial species can serve as biocontrol agents that protect plants from pathogenic fungi (Whipps, 2001). It has been reported that bacterial strain *Pseudomonas fluorescens* can effectively protect tomato plants against infection by *Fusarium oxysporum* and its ability to colonize both the roots and fungal cells likely aids in this interaction (Bolwerk *et al.*, 2003).

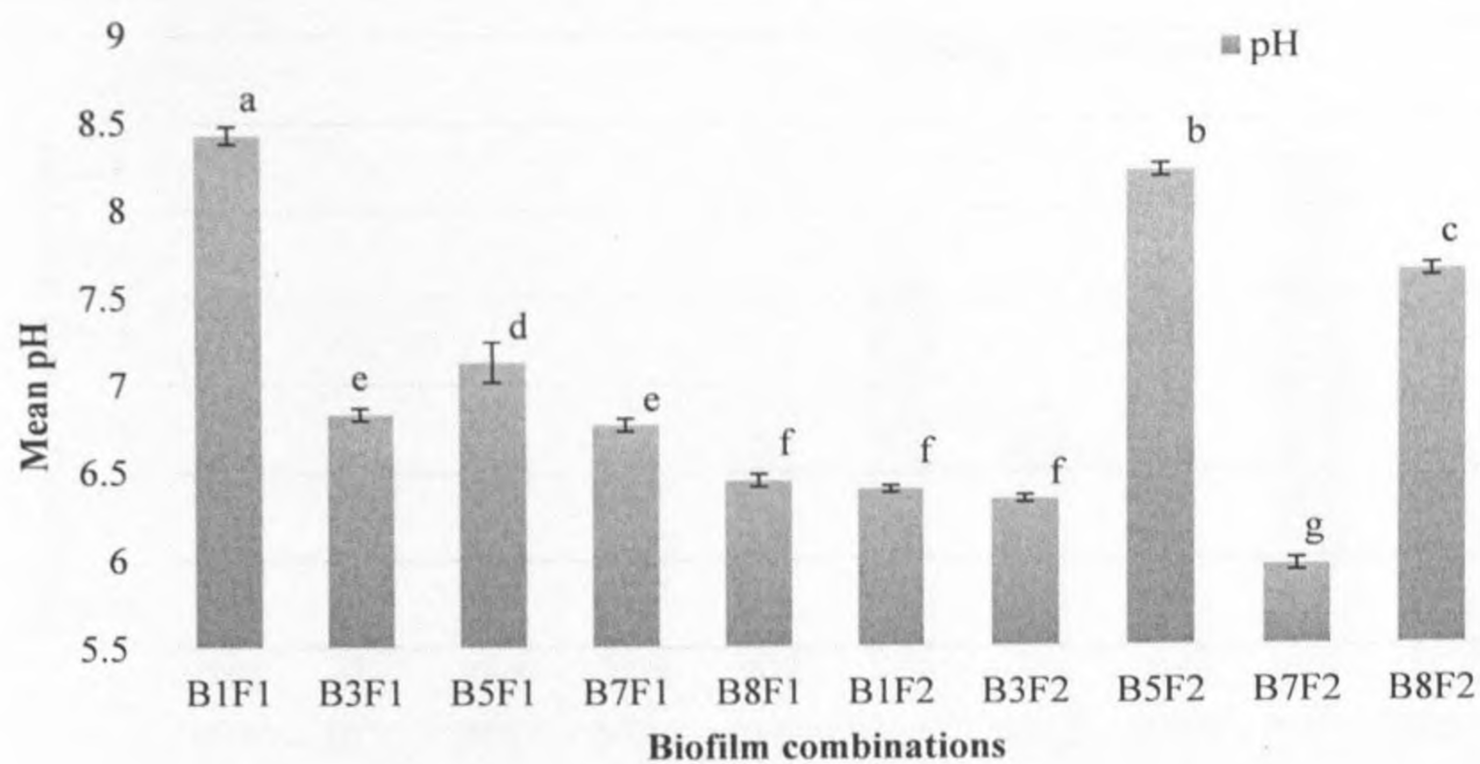
It has been reported that the EPS is primarily responsible for the morphology and function of biofilms, and it is considered to be key components in determining their chemical and biological properties. The EPS is composed of polysaccharides, proteins, nucleic acids and other substances which help to protect the biofilm organisms from various environmental stress factors, such as UV radiation, extreme pH conditions, osmotic shock, dehydration, antimicrobial substances, predators, etc. (Costerton *et al.*, 1987; Romanova *et al.*, 2006). It is recorded that this may be the reason why microorganisms prefer to exist in the biofilm mode rather than the planktonic stage (Seneviratne *et al.*, 2009).

### 3.4.5.2 Screening experiments for simple biofilms

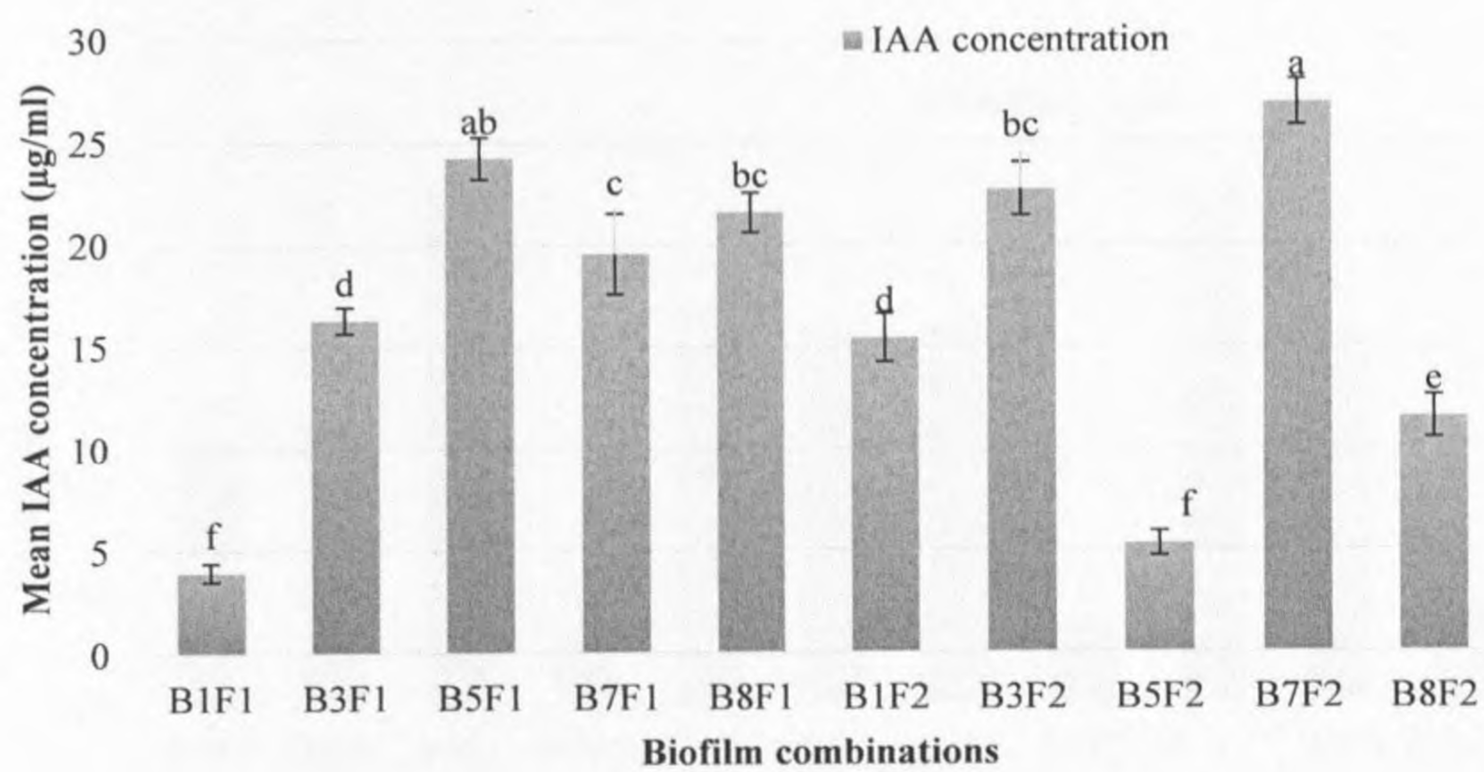
All biofilm combinations except B1F1, B5F2, B8F2 and B5F1 showed acidic pH range (Fig. 3.6 a). Biofilm combination, B7F2 showed the lowest significant mean pH ($P < 0.05$, 5.96 ± 0.03) compared to all other combinations. Further, B7F2 showed the highest significant mean IAA production ($P < 0.05$, 26.86 μg/ml ± 1.1) compared to all other combinations except B5F1 combination (Fig.3.6 b). This low pH and high IAA production might be due to the effect of *Rhizobium* sp. which showed the lowest pH ( 5.1 ± 0.02) and highest IAA production (21.54 μg/ml ± 0.9) in the screening experiments for bacterial monocultures (Fig. 3.1 and Fig. 3.4). The lowest IAA productions were recorded by biofilm combinations BF5 (3.98 μg/ml ± 0.4) and B5F2 (5.3 μg/ml ± 0.6).

The highest significant ethylene production ($P < 0.05$, 2.91 nmol/hr ± 0.03) was shown by the biofilm combination B7F2 compared to all other treatments (Fig. 3.6 c). Biofilm combination B7F2 was a combination of *Trichoderma* sp. and *Rhizobium* sp. which had shown the highest ethylene formation during screening of bacterial isolates (Fig. 3.2). Further, higher ethylene productions were recorded by most of the biofilm cultures over their monocultures (Fig. 3.2 and Fig. 3.6 c). It has been reported that microbial biofilms of beneficial microorganisms show higher nitrogenase activity than their monocultures with no biofilm formation (Jayasinghearachchi and Seneviratne, 2004a; Seneviratne and Jayasinghearachchi, 2005).

Further, biofilm combination B7F2 (634.45 ± 1.1) and B5F1 (631.9 ± 2.6) showed high seedling vigor compared to all other combinations (Fig. 3.6 d) whereas biofilm combinations B7F1 (434.71 ± 9.3) and B5F2 (435.02 ± 1.5) showed the lowest vigor values. All the combinations except B7F1 and B5F2 showed high vigor values compared to control (446.77 ± 4.9). It was clearly observed that the seedling vigor values for the biofilm cultures were much higher than that of their monocultures (Fig. 3.3 and Fig. 3.6 d). Similar results have been recorded from other studies conducted for FBBs. For instance, high seed germination percentages with high root lengths (close or equals to 100%) have been recorded by FBBs compared to their bacterial monocultures (Buddhika *et al.*, 2014).




(a)

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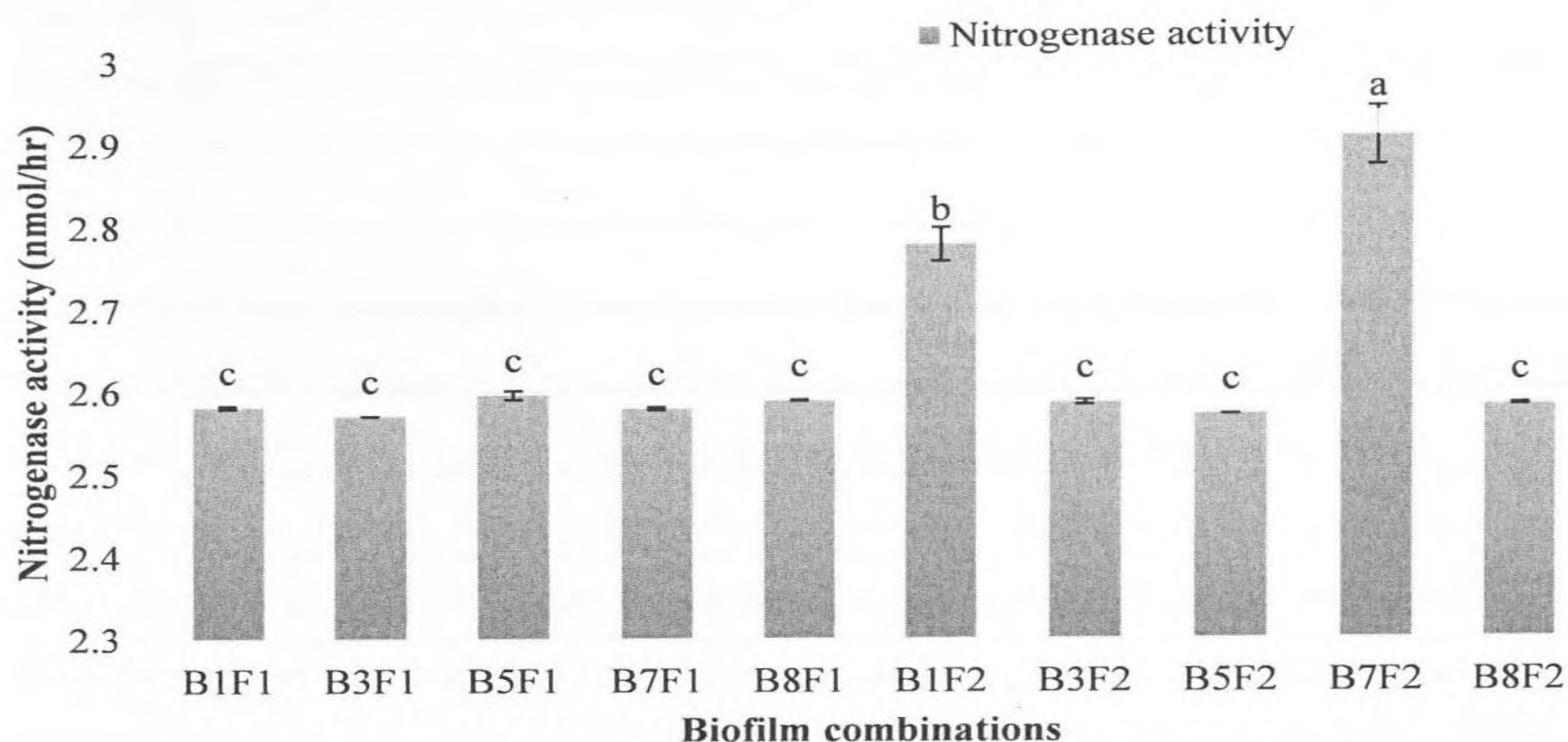
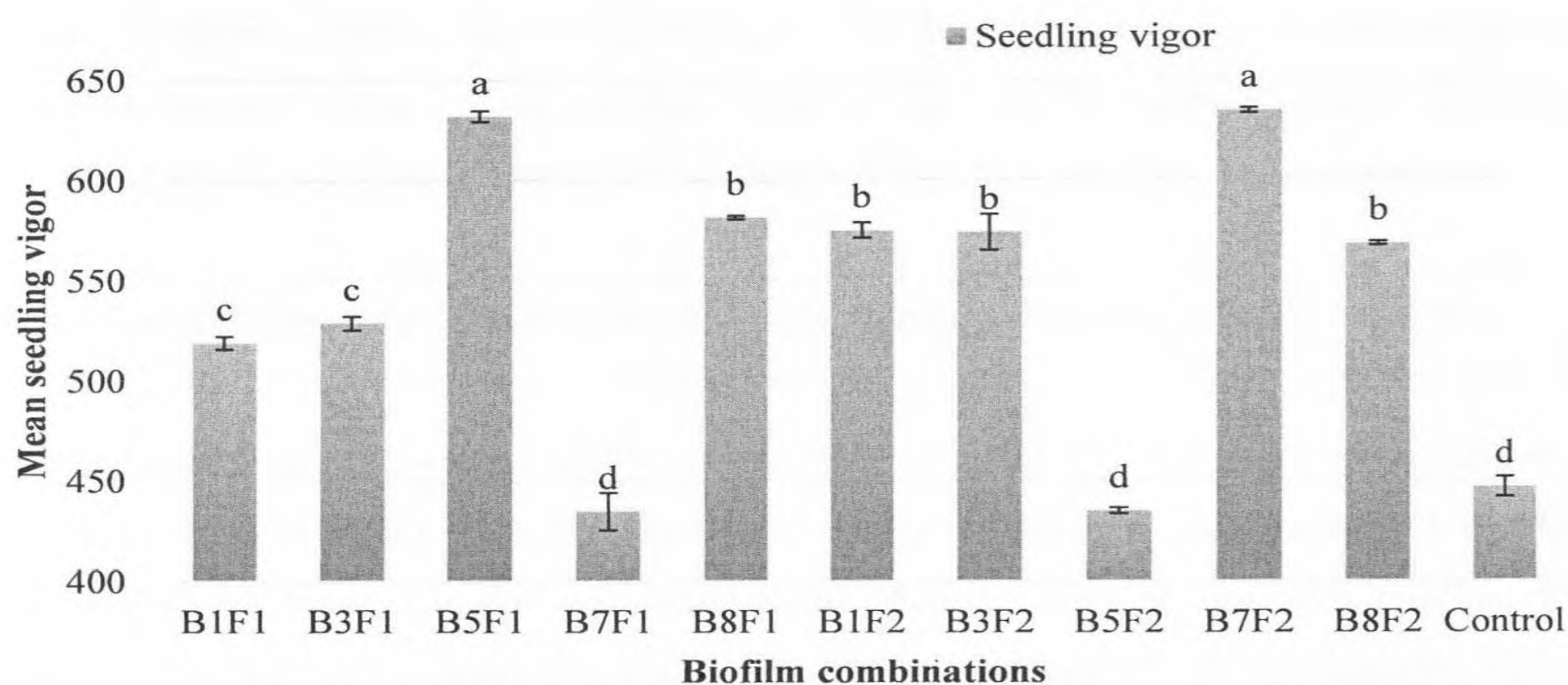



(c)




(d)

Figure 3.6- The responses of different simple biofilm combinations on screening experiments. (a)- The responses on mean pH of broth cultures. (b)- The responses on mean IAA production. (c)- The responses on mean ethylene production. (d)- The responses on mean seedling vigor (control – only ML medium). B1F1 to B8F2- simple biofilm combinations. (B1- *Bacillus* sp., B2- *Acinetobacter* sp., B3- *Acidomonas* sp., B4- *Pseudomonas* sp., B5- *Serratia* sp., B6- *Pseudomonas* sp., B7- *Rhizobium* sp., B8- *Bacillus* sp.). F1 and F2- fungal isolates (*Aspergillus* sp. and *Trichoderma* sp.). Different letters on the columns show significant differences at 5% probability level. Vertical bars show standard deviations.

High IAA productions were recorded by most of the biofilm cultures over their monocultures (Fig. 3.6b and Fig. 3.4). Similar results have been recorded by a different study conducted on FBBs (Buddhika *et al.*, 2014). Further, a previous study conducted with lichen fungi and a diazotroph has shown a higher $H^+$ secretion in biofilms than their

monocultures (Seneviratne and Indrasena 2006). Moreover, elevated production of IAA by the biofilms has been reported by another recent study (Jayasinghearachchi and Seneviratne 2005).

Seedling vigor is a parameter to assess seed germination efficiency. Another study worked on cereal crops in Sri Lanka has reported that the application of FBBs, enhanced the seed germination efficiency or the vigor values of cereals (Weerathne *et al.*, 2012). Further evidences can be found for the beneficial soil microbial biofilm activities on producing different phytohormones like IAA and thereby promoting plant growth and germination of seeds (Kloepper *et al.*, 2007; Buddhika *et al.*, 2014). It has also been reported that the root growth and seed vigor are attributable to the action of IAA (Sachdev *et al.*, 2009; Budhhika *et al.*, 2014). According to the current study, the seeds inoculated with biofilm combinations showed higher root lengths than the seed inoculated with their bacterial monocultures. Further, biofilm combination B7F2 enhanced the seedling vigor up to approximately 140% over non treated control. In general, most of the biofilm combinations showed higher performances for all the screening experiments than that of their monocultures.

### 3.4.5.3 Correlations between different screening parameters for biofilms

A significant negative relationship (r = 0.881, P = 0.001) was observed between mean inoculum pH and mean IAA production by different biofilm combinations (table 3.7). It was clearly observed that the negative relationship between mean IAA concentration and mean pH for the biofilm combinations (r = 0.881, P = 0.001) was higher than that of their bacterial monocultures (r= 0.715, P = 0.046). This implies that the biofilms produce higher IAA than that of their monocultures which contribute more to reduce the medium pH.

Table 3.7- Correlation coefficient (r) between properties of simple biofilms

| | IAA production | Biofilm pH |
|---|---|---|
| Biofilm pH | -0.881<br>(0.001) | |
| Seedling vigor | 0.617<br>(0.057) | -0.512<br>(0.130) |

Values within parentheses are probability levels.

Further, it has been proved that there is a regulation of the production of IAA like substances in FBBs, which is related to culture medium pH. When bacteria are in biofilms, it seems that there is a possibility of manipulating IAA production at optimum level for higher plant growth benefits for effective formulations of biofertilizers (Seneviratne *et al.*, 2008; Buddhika *et al.*, 2014). As such, the highest microbial effect may not be achieved by the conventional practice of plant inoculation with monocultures or mixed cultures of effective microbes, but rather by biofilmed inocula (Seneviratne *et al.*, 2008). This relationship has been reported by another study expressing that FBBs of beneficial microorganisms produce higher acidity and plant growth promoting hormones like IAA than their mono or mixed cultures with no biofilm formation (Bandara *et al.*, 2006). Another study has shown the significant negative relationship between pH and IAA like substances production in liquid culture media of the biofilms (Seneviratne *et al.*, 2008b).

A significant relationship was not observed (r = 0.617, P = 0.057) between mean seedling vigor and mean IAA production by different biofilm combinations (table 3.7). It was clearly observed that the relationship between mean seedling vigor and mean IAA production for bacterial monoculture (r = 0.943, P = 0.000) was much higher than that of the biofilm cultures. However, IAA production and seedling vigor values showed by the biofilm cultures were much higher than that of the monocultures (Fig. 3.6 b, Fig. 3.6 d, Fig. 3.4 and Fig. 3.3). This implies that there are factors other than IAA that lead to increase seedling vigor in the case of biofilms. Thus, developed biofilms can be suggested as a natural biological formulation to increase seedling vigor through the creation of favorable environment required for breaking seed dormancy which is not yet understood fully in the application of biofertilizers.

Contribution of developed FBBs in making such environment for higher seedling vigor was confirmed by the increasing availability of diverse organic compounds (Herath *et al.*, 2013). A wide range of beneficial biochemical exudates in a developed FBBs in comparison to its bacterial monocultures have been observed. In support of this, interactions among microbes for diverse release of organic compounds were observed by Saini *et al* (1986) and De Boer *et al* (2005), which cannot be seen in planktonic forms of them due to lack of coordinated biological functions.

### 3.4.5.4 Selection of beneficial biofilm combinations from screening experiments

Biofilm combination B7F2 showed the highest score for the statistical ranking of different screening experiments (table 3.8). Biofilm combinations B7F2, B5F1, B3F2 and B1F2 showed higher performances for all the screening experiments and were selected as the best performing biofilm combinations. Therefore, microbial isolates in those biofilm combinations were selected to establish higher order biofilms.

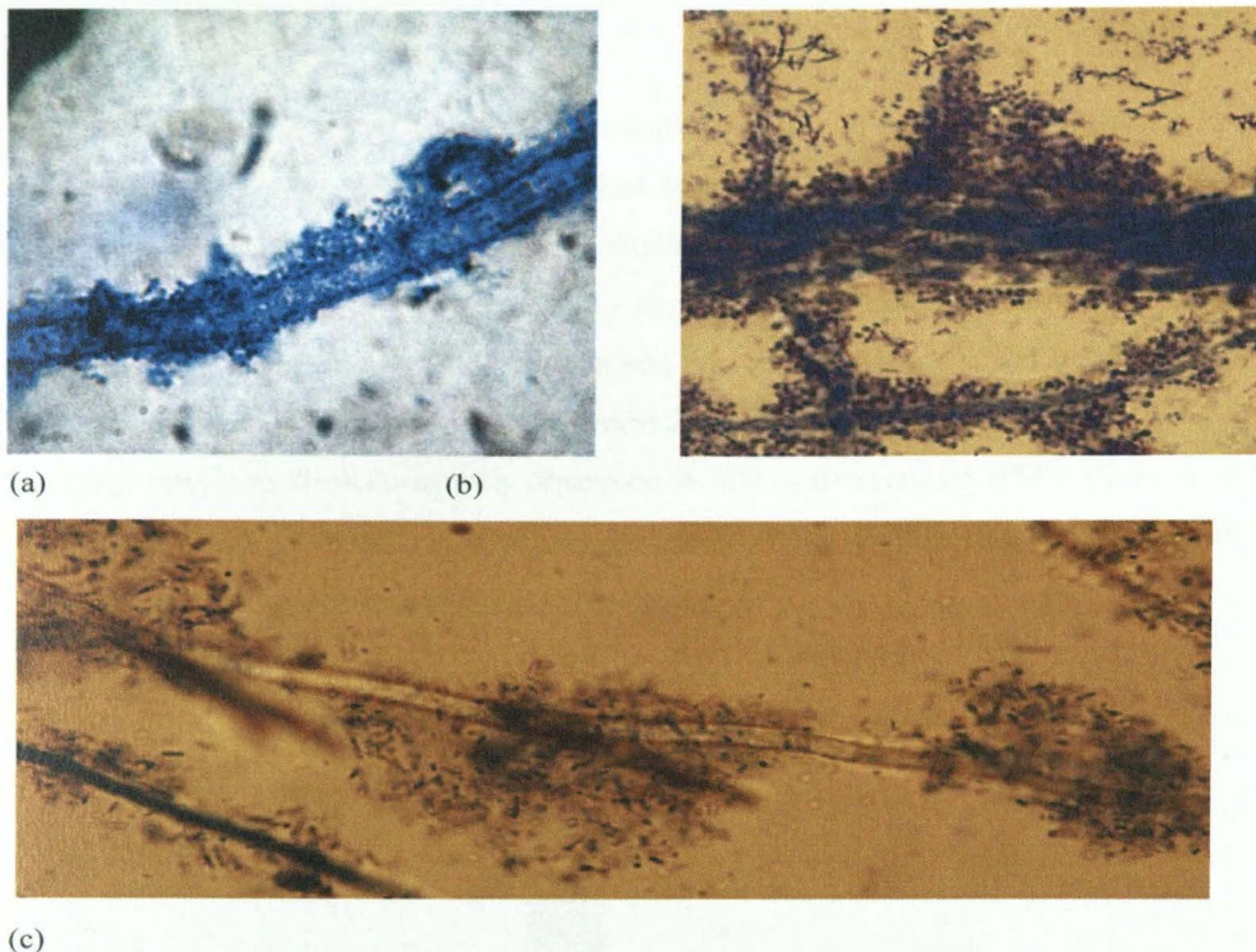
Table 3.8- Statistical ranking of simple biofilm combinations on different screening parameters

| Biofilm combination | Selection parameter | | | | Total score |
|---|---|---|---|---|---|
| | pH | ARA | IAA | Seedling vigor | |
| B1F1 | 1 | 5 | 1 | 3 | 10 |
| B3F1 | 5 | 1.5 | 5 | 4 | 15.5 |
| B5F1 | 4 | 8 | 9 | 9 | 30** |
| B7F1 | 6 | 3 | 6 | 1 | 16 |
| B8F1 | 7 | 7 | 7 | 8 | 29** |
| B1F2 | 9 | 9 | 4 | 7 | 29** |
| B3F2 | 8 | 6 | 8 | 6 | 28 |
| B5F2 | 2 | 1.5 | 2 | 2 | 7.5 |
| B7F2 | 10 | 10 | 10 | 10 | 40** |
| B8F2 | 3 | 4 | 3 | 5 | 15 |

(**- higher scores for the statistical ranking. Ranking was performed using MINITAB 16 statistical software. B1- *Bacillus* sp., B2- *Acinetobacter* sp., B3- *Acidomonas* sp., B4- *Pseudomonas* sp., B5- *Serratia* sp., B6- *Pseudomonas* sp., B7- *Rhizobium* sp., B8- *Bacillus* sp. F1- *Aspergillus* sp. and F2- *Trichoderma* sp.)

### 3.4.5.5 Establishment of higher order biofilms from selected bacteria and fungi

All the bacterial isolates showed successful biofilm formation by attaching with the fungal biomass through EPS (plate 3.4). Higher order biofilm HBF1 was developed by the combination of two bacterial isolates (B1- *Bacillus* sp. and B7- *Rhizobium* sp.) with one fungal species (F2- *Trichoderma* sp.) whereas higher order biofilm HBF2 was developed by the combination of *Serratia* sp. (B5) and *Bacillus* sp. (B8) with *Aspergillus* sp. (F1).



(a) (b)

(c)

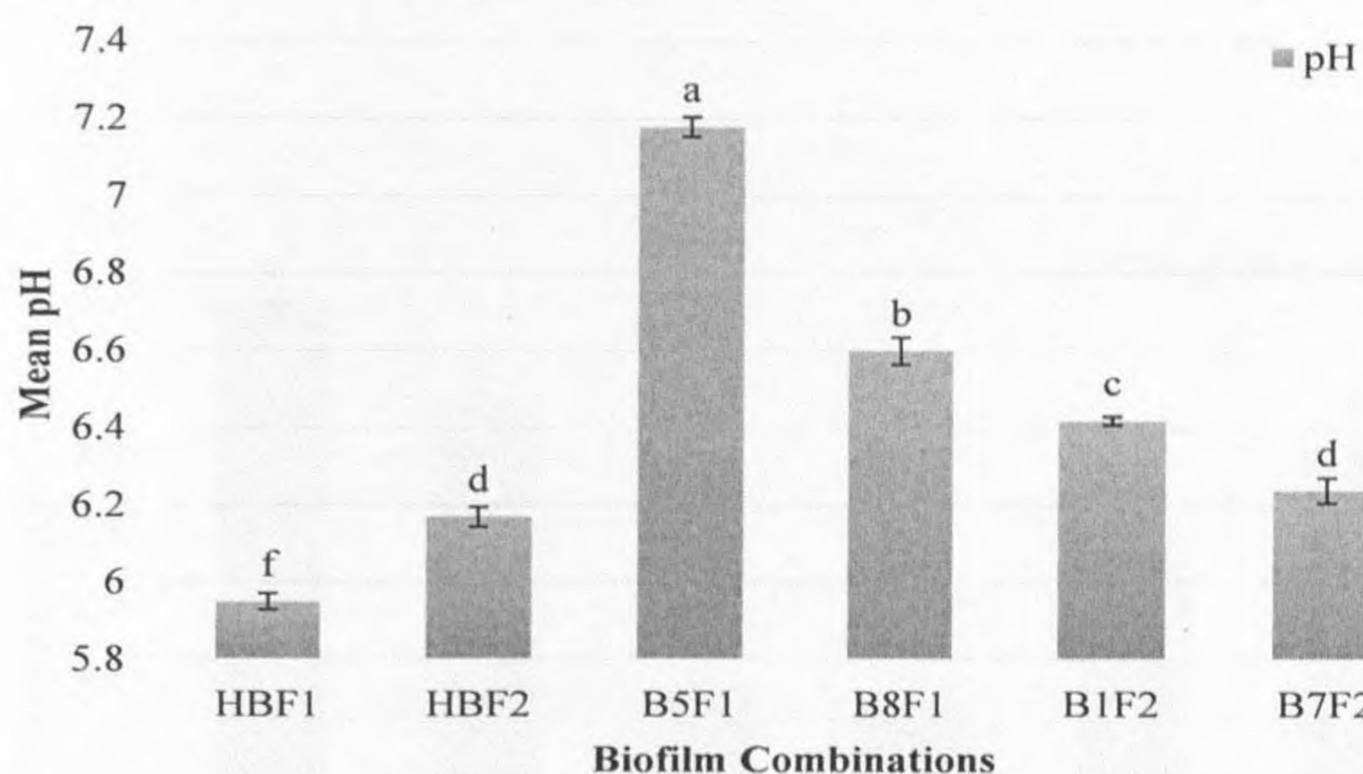
Plate 3.4 - Bacterial colonization on *Trichoderma* mycelium in FBBs. (a)- Microscopic view of higher order biofilm combination HBF1 (b) and (c)- Microscopic views of higher order biofilm HBF1 (Olympus X51 microscope at University of Sydney, Australia). Darkness is due to cotton blue stain absorbed by EPS produced by the biofilms. Stain, lactophenol cotton blue. (Magnification 1000X)

According to the literature, *Trichoderma* sp. represents the most widely employed biocontrol agents, besides their role as plant growth promoters and potential as valuable sources of secondary metabolites/ enzymes which find use in pharmaceuticals, industry and agriculture. Further, it has been proved that the potential formation of fungal-bacterial biofilms with *Trichoderma* spp under *in-vitro* static conditions (Harman, 2006; Triveni, Prasanna and Saxena, 2012).

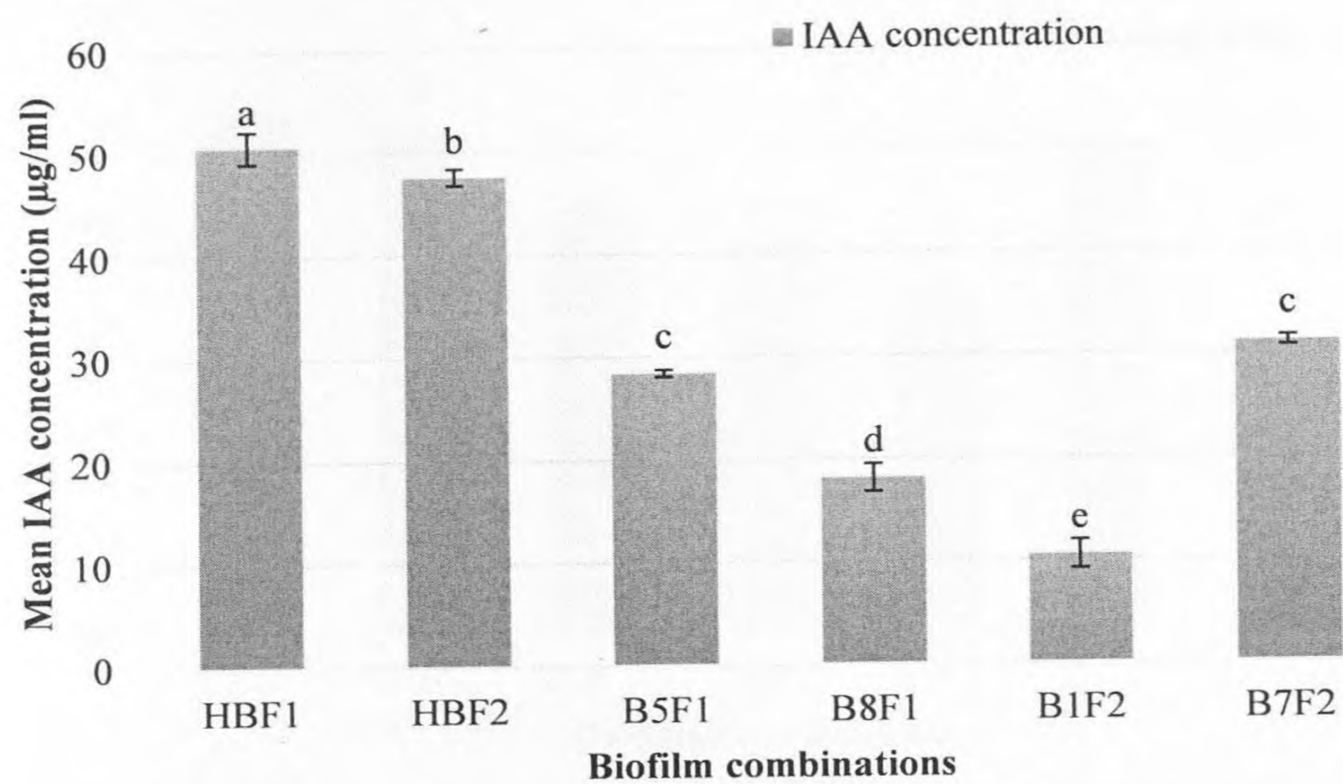
### 3.4.5.6 Screening experiments for higher order biofilms

Higher order biofilm combination HBF1 showed the lowest significant mean pH ($P < 0.05$, $5.95 \pm 0.02$) and the highest significant IAA production ($P < 0.05$, 50.68 μg/ml $\pm$ 1.5) compared to all other biofilm combinations (Fig. 3.7 a and b). It can be considered that the low pH and high IAA production of HBF1 combination might be due to the effect of its bacterial monocultures which showed the lowest pH and the highest IAA production (Fig. 3.1 and Fig. 3.4).

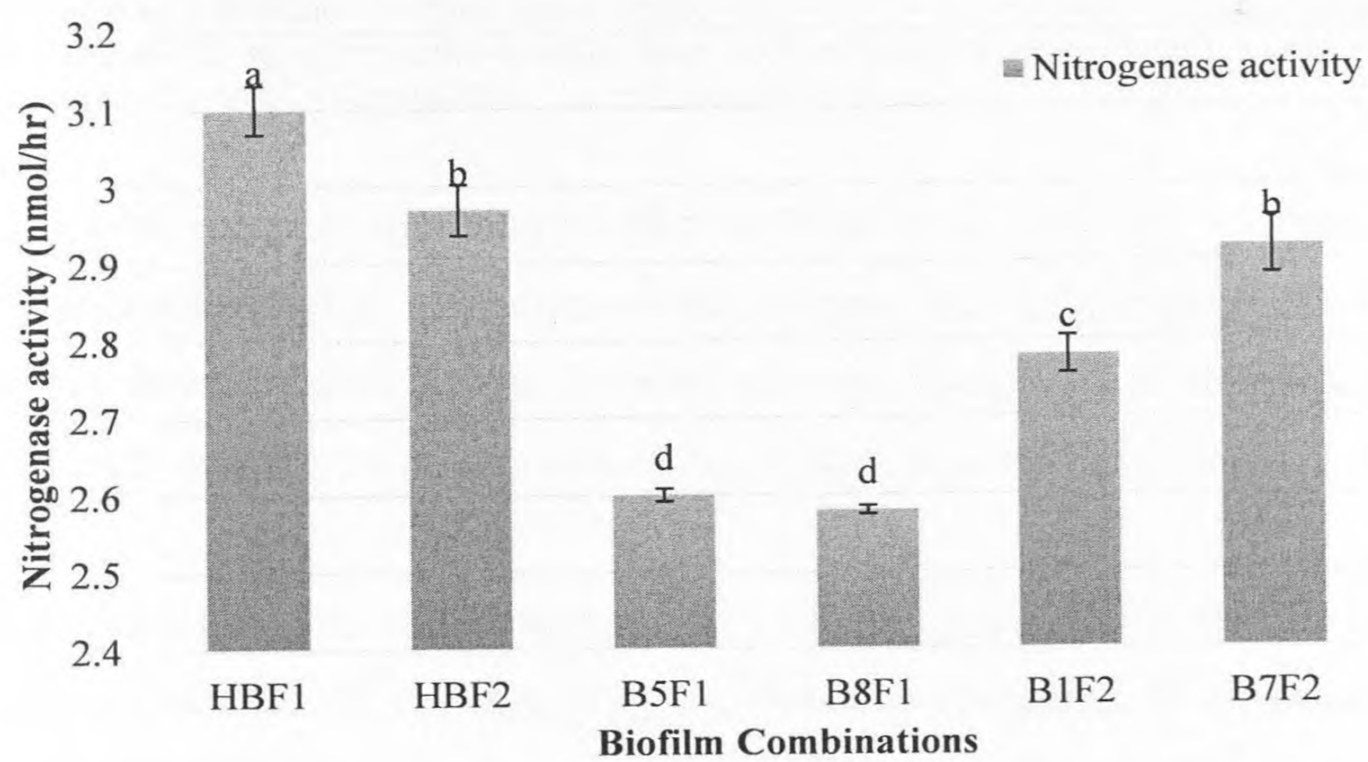
Out of the biofilm cultures, all higher order biofilm combinations showed low pH values and high IAA concentrations compared to other simple biofilm combinations. Ethylene formation from ARA ($P < 0.05$, 3.1 nmol/hr ± 0.03) and seedling vigor ($P < 0.1$, 658.57 ± 4.8) were significantly high in higher order biofilm HBF1 compared to simple biofilm cultures (Fig. 3.7 c and d). It was observed that all the higher order biofilm cultures showed high ethylene production and high seedling vigor compared with simple biofilm cultures. Lowest ethylene formation was observed in the combination B5F1 (2.6 nmol/hr ± 0.008) and B8F1 (2.58 nmol/hr ± 0.005). Further, all biofilm combination enhanced seedling vigor compared to the non- treated control (440.4 ± 7.6).



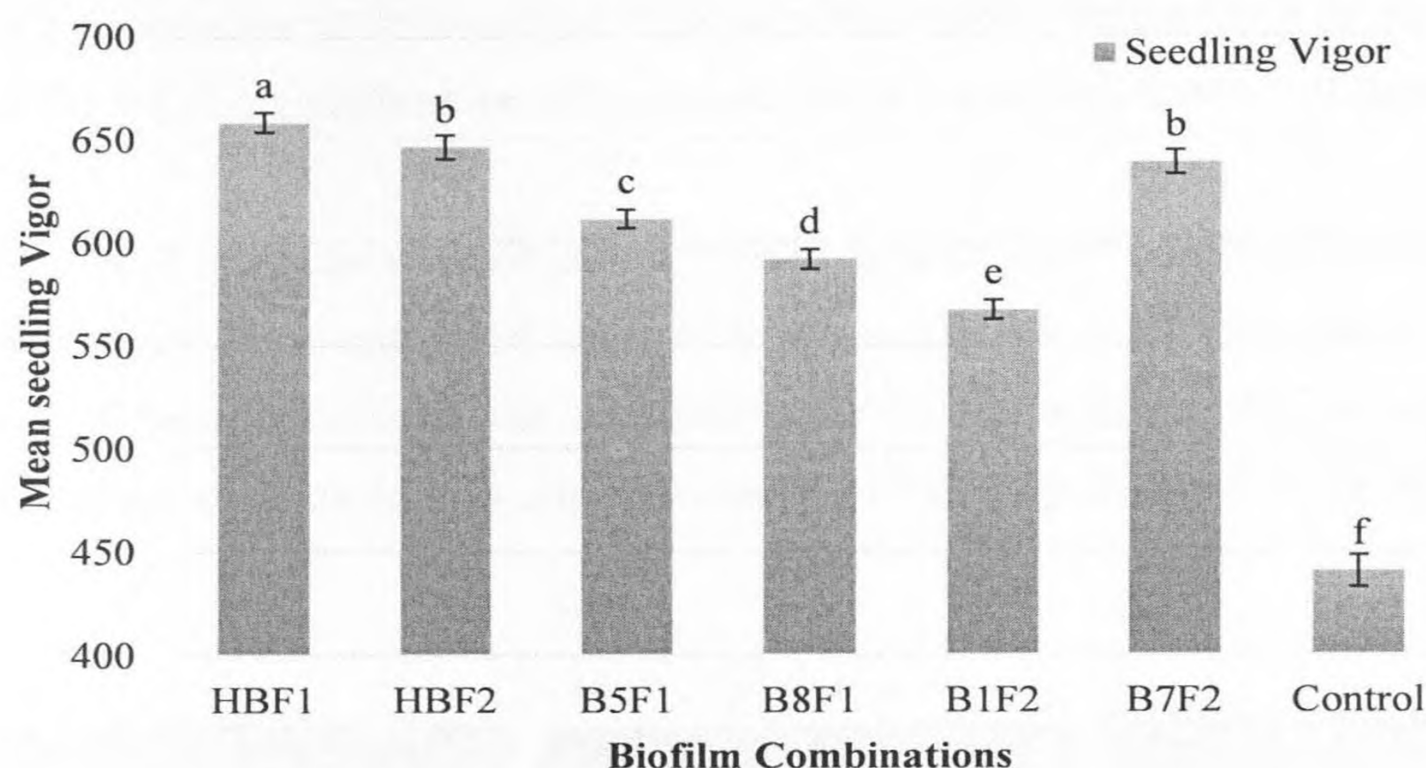

(a)




(b)




(c)




(d)

Figure 3.7- The responses of different biofilm combinations on screening experiments. (a)- The responses on mean pH. (b)- The responses on mean IAA production. (c)- The responses on mean ethylene production. (d)- The responses on mean seedling vigor. HBF1 and HBF2- higher order biofilm combinations. B5F1, B8F1, B1F2 and B7F2- simple biofilm combinations. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

It has been recorded that higher order biofilms have the ability to enhance the dry weights of plants compared to monoculture inoculations. In a field experiment for tea, higher order biofilm (two bacteria + one fungus) showed higher shoot dry weight compared with monoculture or a biofilm consists of one fungus and one bacteria (Seneviratne *et al.*, 2009).

Higher order biofilm combinations HBF1 (BFBF1) and HBF2 (BFBF2) showed the highest performances for all the screening experiments compared to all selected simple biofilm combinations. Therefore, those were selected as beneficial biofilm combinations and were subjected for direct pot and field experiments with potato seed tubers. Positive effects of higher order biofilms on plant growth and developments have been well documented. It has been reported that the inoculation of higher order biofilm including *Trichoderma harzianum* significantly enhanced shoot and root biomass of plants (Srinath *et al.,* 2003).

### 3.4.6 GA production by the higher order biofilms

GA production was not detected by the higher order biofilm combinations.

**3.4.7 Molecular identification of microorganisms responsible for biofilm formation**

**3.4.7.1 PCR amplification of genomic DNA extracted from soil microorganisms**

Clear PCR products were obtained for all bacterial DNA amplified using 16s rRNA primers and Fungal DNA amplified using ITS primers (plate 3.5). The size of the extracted DNA from all bacterial strains was approximately 1500bp whereas the size of the extracted DNA from the fungal strain was approximately 550-600 bp (extraction of DNA- appendix 4).

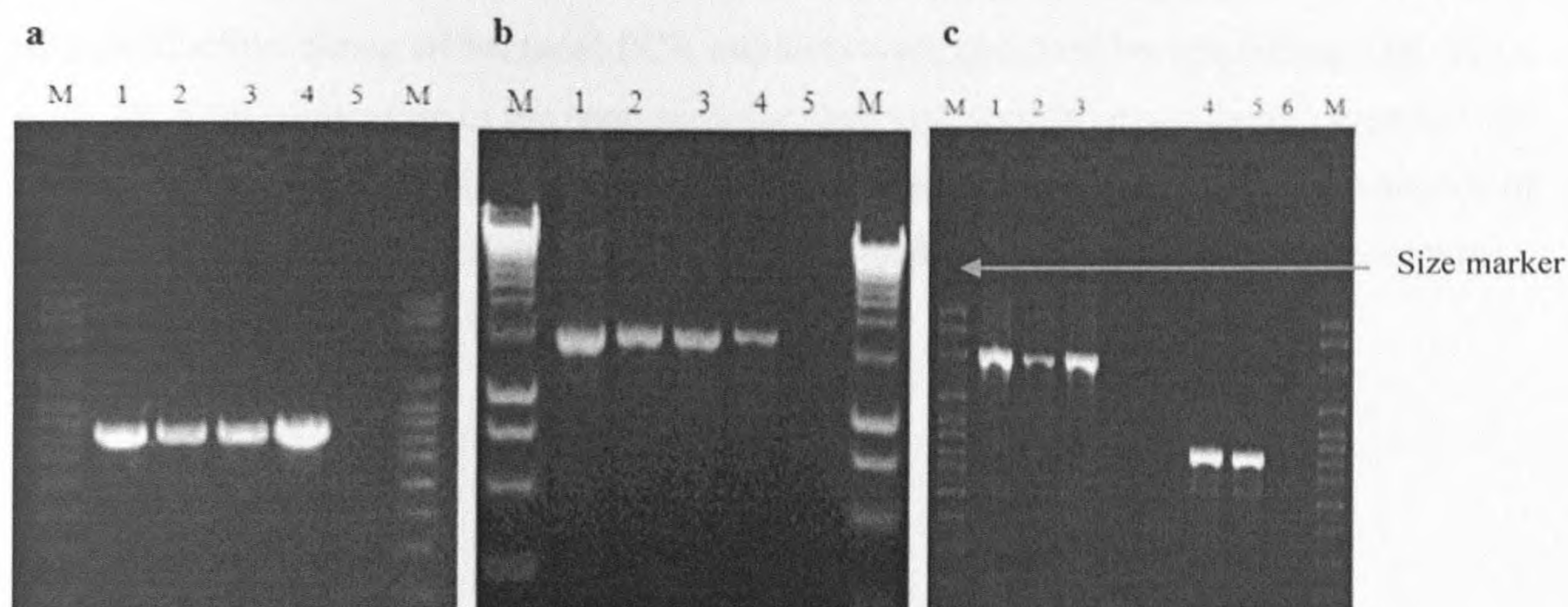



Plate 3.5- PCR products of different bacterial and fungal genomic DNA. (a)- PCR products of fungal DNA amplified using ITS specific primers (Lane M, A 100bp Plus DNA size marker. Lane 1 and 4, undiluted DNA from *Trichoderma* sp. Lane2 and 3, 1;10 diluted DNA from *Trichoderma* sp.). (b)- PCR products of bacterial DNA amplified using 16Sr RNA specific primers (Lane M, A 100bp Plus DNA size marker. Lane 1-2, DNA from *Bacillus* sp. Lane 3, DNA from *Rhizobium* sp. Lane 4 and 5, positive and negative control). (c)- PCR amplification products of different bacterial and fungal genomic DNA (Lane 1-2, DNA from *Bacillus* sp. Lane 3, DNA from *Rhizobium* sp.). Lane 4, undiluted DNA from *Trichoderma* sp. Lane 5, 1: 10 diluted DNA from *Trichoderma* sp. Lane 6, negative control).

**3.4.7.2 Determination of the concentration of purified DNA samples**

Spectra obtained for the PCR products of all bacterial and fungal strains confirmed that all DNA samples contained a high range of DNA concentrations (appendix 5). Maximum absorbance was obtained at 260 nm for all PCR products. The absorbance ratio A260/A280 obtained for all samples (>1.8) confirmed that the extracted DNA were in high purity and quality (appendix 5).

DNA quantification is the key to good molecular biology results, and failure to produce high-quality evidence can sometimes be directly attributed to an incorrect estimate of the concentration of DNA template used. The latest micro volume quantification Nano Drop

2000 spectrophotometer can be implemented to minimize consumption of precious samples and provide fast assessment of nucleic acid concentration and purity. The A260/A280 ratio is used as indicator for DNA purity. A ratio higher than 1.8 is assumed suitable for gene expression measurements (Siqueira *et al.,* 2005).

### 3.4.7.3 Restriction digestion and RFLP analysis

Unique RFLP patters were observed for all microbial samples and patterns were found to be reproducible. Since all bacterial PCR products were obtained by amplifiing 16S rRNA gene, the total summation of the band sizes for each lane should equals or less than to 1500 bp (Lanes with different digestion patterns represent different PCR products). Analysis of RFLP banding patterns for fungal sample F2 confirmed their high purity since the total band size was less than 1500 bp (plate 3.6).

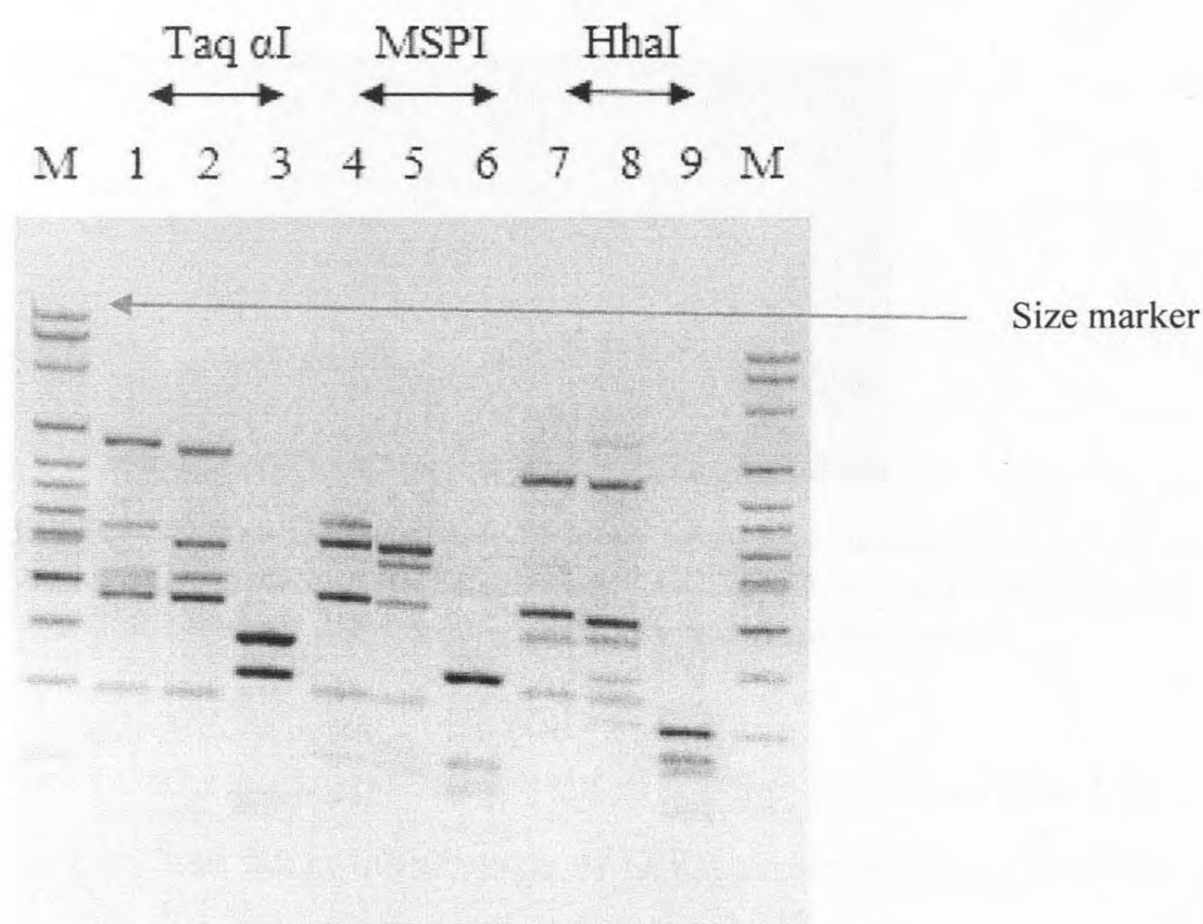



Plate 3.6- RFLP profile obtained for the PCR products from *Bacillus* sp., *Rhizobium* sp. and *Trichoderma* sp. digested with three different restriction enzymes. Lane M, A 100bp Plus DNA size marker. Lane 1-3, PCR products digested with TaqαI. Lane 4-6, PCR products digested with MSPI. Lane 7-9, PCR products digested with HhaI. ( Lane1, 4, 7- PCR products from *Rhizobium* sp. sample. Lane 2, 5, 8- PCR products from *Bacillus* sp sample. Lane 3, 6, 9- PCR products from *Trichoderma* sp. sample).

However, RFLP analysis confirmed that the total sumation of the band sizes for each bacterial strains (*Bacillus* sp. and *Rhizobium* sp.) were exceeded the the size of 16S rRNA gene and this was considered due to a contamination. Therefore, sequence analysis for those contaminated bacterial samples were performed after developing PCR products

through a new DNA extraction protocol. The PCR products of *Trichoderma* sp. was subjected for direct sequence analysis since it was considered as un- contaminated pure DNA sample.

### 3.4.7.4 PCR amplification of DNA extracted from rapid method

Mother culture *Bacillus* sp. and *Rhizobium* sp.were found to be contaminated/accompanied with another bacterial isolate (B9). Therefore, PCR amplification was performed again for all the bacterial isolates (*Bacillus* sp., *Rhizobium* sp. and B9) after extracting DNA using rapid DNA extraction method. Clear PCR products were obtained for all bacterial DNA amplified using 16S rRNA primers. The size of the extracted DNA from all bacterial strains was approximately 1500bp (plate 3.7).

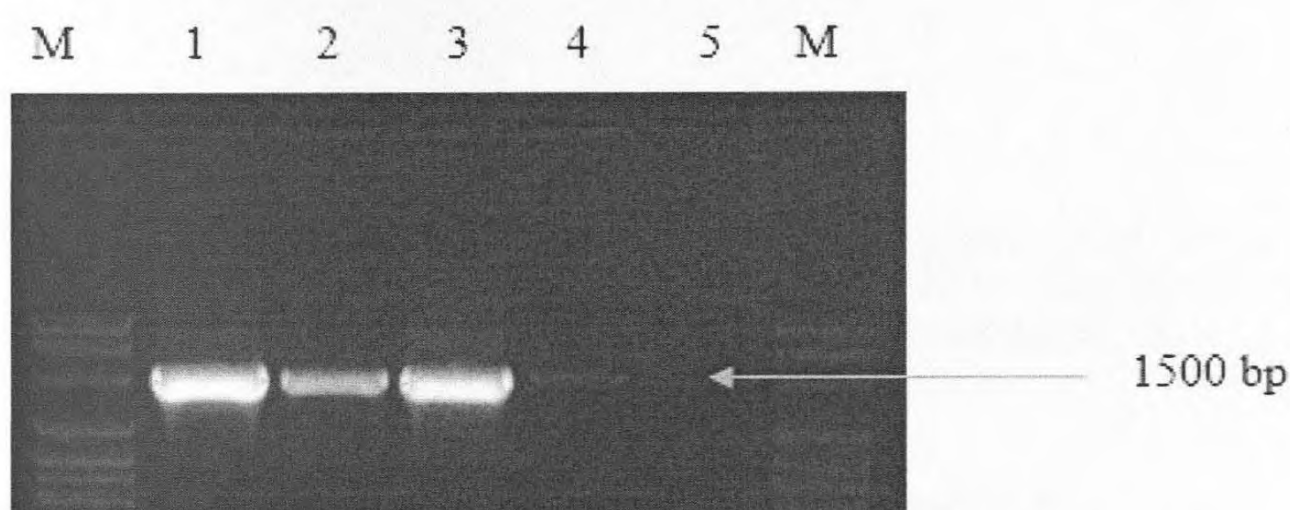



Plate 3.7- PCR products of three different bacterial genomic DNA. (Lane M, A 100bp Plus DNA size marker. Lane 1, PCR product from *Bacillus* sp. Lane 2, PCR product from bacterial sample B9. Lane 3, PCR product from *Rhizobium* sp. Lane 4 and 5, positive and negative control.

Nano Drop 2000 spectra obtained for the amplified new DNA samples confirmed that all samples had acceptable range of DNA concentration (*Bacillus* sp.- 237.5 ng/µl, *Rhizobium* sp.-287.9 ng/µl and B9- 203.8 ng/µl). Maximum absorbance was obtained at 260 nm for all PCR products. The absorbance ratio A260/A280 obtained for all samples (>1.8) confirmed that the extracted DNA were in high purity and quality.

### 3.4.7.5 Restriction digestion and RFLP analysis for new DNA samples

RFLP analysis successfully differentiated the PCR amplified products of all three bacterial strains (plate 3.8). A different unique RFLP banding pattern was obtained for *Rhizobium*

sp. whereas very closely related banding patterns were observed in *Bacillus* sp. and B9. Each PCR product was confimed as un-contaminated products since the total size of bands from each PCR productdid not exceed the total size of 16S rRNA gene (1500bp).Therefore, the DNA samples from all bacterial strains were subjected to the sequence analysis to confirm their identities. All banding patterns obtained by gel electrophoresis were found to be reproducible.

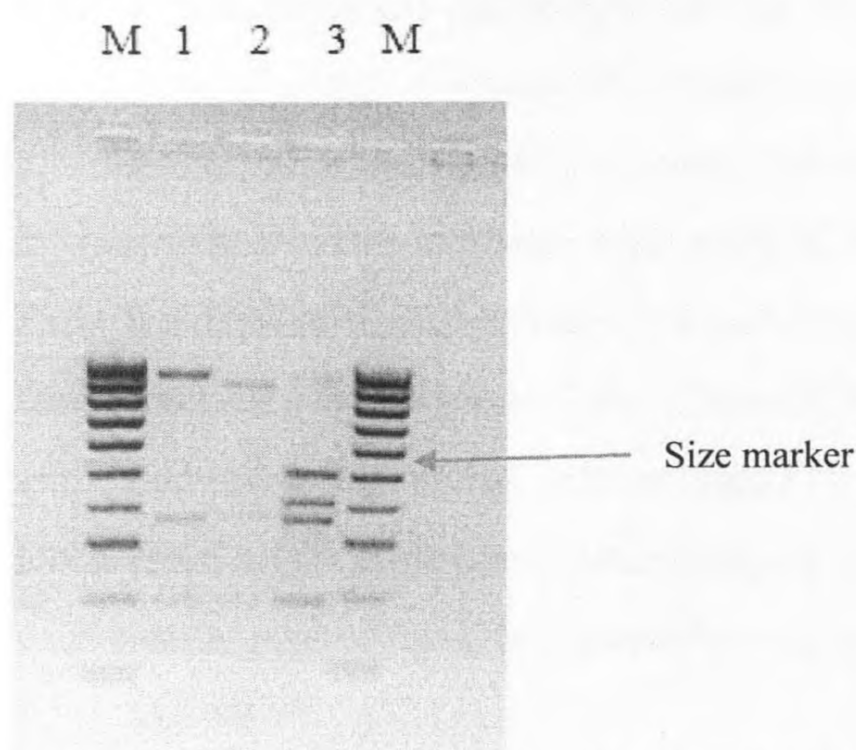



Plate 3.8- RFLP profile obtained for the PCR products of *Bacillus* sp., B9 and *Rhizobium* sp. digested with TaqαI restriction enzyme. Lane M, A 100bp Plus DNA size marker. Lane 1, PCR product from *Bacillus* sp. Lane 2, PCR product from B9. Lane 3, PCR product from *Rhizobium* sp.

### 3.4.7.6 Sequence analysis using an online databases

The sequence alignment using GenBank and RDP online databases confirmed the identities of all bacterial and fungal samples. DNA sequences of bacterial samples B1 and B9 showed 100% similarity with *Bacillus pumilus* (ACC No. KJ 801622.1) and *Bacillus subtilis* (ACC No. KM 269071.1), respectively. The bacterial isolate B7 had initially been identified as *Rhizobium* sp. through biochemical tests. Interestingly, the molecular analysis and sequence alignment revealed that it was similar to *Bradyrhizobium japonicum* (ACC No. KF 995114.1) and not similar to *Rhizobium* sp. The query sequence of fungal strain F2 showed 100 % similarity with the *Trichoderma harzianum* (ACC No. KC 330218.1) in NCBI BLAST. It has been recorded that the strains in *Bacillus* are divided into 5 groups based on phylogenetic analysis and *B. pumilus* belong to the *B. subtilis* group (Liu *et al.*, 2013). Strains representing these species share many common properties and show relatively few characteristics by which they can be separated (Donnell *et al.*, 1980).

**3.4.8 Community analysis of fungal-bacterial biofilm through TRFLP**

**3.4.8.1 Extraction of genomic DNA from biofilm combinations**

The purest DNA was extracted using the commercial Mo Bio extraction kit, which was the only method that incorporated a post-extraction purification, however the kit results in less DNA and smaller fragments. According to the current study, all bands were visualized as smears on agarose gel rather than having clear sharp bands plate 3.9 a). This was due to the generation of series of small DNA fragments after beat beating method of DNA extraction kit. Further, all banding patterns except lane 3, 4 and 7 confirmed that the DNA extraction kit performed better to obtain high yield of DNA from all microbial biofilm combinations. Faint bands obtained from lane 3, 4 and 7 might be due to the degradation of DNA during the extraction process (plate 3.9a). Clear PCR products were obtained from all biofilm DNA amplified using 16S rRNA primers and ITS primers (plate 3.9b). The size of the extracted DNA from all biofilm combinations using 16S rRNA primers was around 1500 bp in size whereas the size of the DNA amplified using ITS primers were around 500- 600 bp in size.

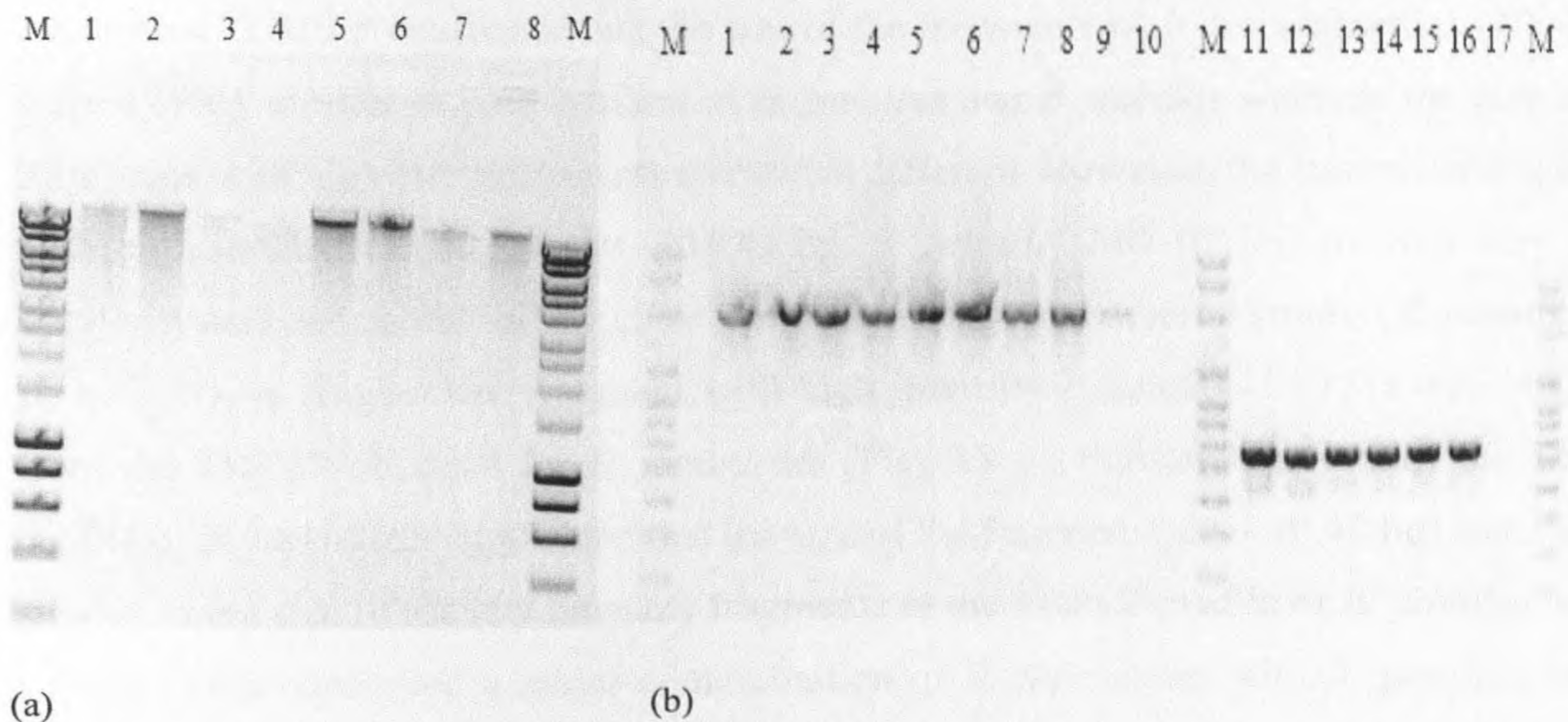



Plate 3.9 (a)-Agarose gel electrophoresis of the DNA extracted using MO BIO PowerSoil DNA isolation kit from the biofilm combinations. Lanes M, banding pattern of 1Kb (Hyperladder 1Kb, BioLine). Lanes 1-8 and lane 10-11, DNA extracted from the different replicates of a biofilm combination (b)- PCR products of DNA extracted from biofilm combinations. Lanes M, banding pattern of 1Kb (Hyperladder 1Kb, BioLine). Lane 1-8, PCR products amplified using 16S rRNA primers. Lane 11-16, PCR products amplified using ITS primers. Lane 9, positive control. Lane 10 and 17, negative control.

Nano Drop 2000 spectra obtained for the amplified DNA samples confirmed that all the samples have acceptable range of DNA concentrations (24.8 ng/µl -72.8 ng/µl). Maximum absorbance was obtained at 260 nm for all PCR products. The absorbance A260/A280

obtained for all samples (>1.8) confirmed that the extracted DNA were in high purity and quality.

### 3.4.8.2 Community analysis using TRFLP method

Figure 3.8 shows TRFLP profiles obtained for different biofilm forming microbial strains. Similar fragment sizes of TRFLP profile represent closely related microbial strains whereas different fragment sizes represent different strains. Peaks with orange colour (due to liz 500) represent the fragments generated from DNA ladder (marker) whereas peaks with black and blue colour represent the fragments generated from bacterial and fungal strains respectively. The abundance of each fragment is determined based on fluorescence intensity and expressed as either peak height or peak area (Schutte *et al.*, 2008).

Two different clear fragments, one with high intensity (*B. pumilus*-5468, *B. subtilis*-7764) and the other one with low intensity (*B. pumilus*- 2257, *B. subtilis*-5135), were observed in each TRFLP profiles obtained for *B. pumilus* and *B. subtilis* (Fig.3.8 a and Fig. 3.8b). Analysis of TRFLP confirmed that the size of the fragments with low intensities (40.48 bp) were exactly similar in both profiles of *B. pumilus* and *B. subtilis* whereas the size of the fragments with high intensities were somewhat different. However, the size of the fragments with high intensities (*B. pumilus*- 239.49 bp, *B. subtilis*- 240. 07 bp) showed very close similarity and this confirmed the close similarity of the two bacterial strains (*B. pumilus and B. subtilis*). A single clear fragment with high intensity (intensity- 17771) was observed from the TRFLP obtained for *B. japonicum* (Fig. 3.8 c). Further, analysis of the TRFLP profile of *B. japonicum* confirmed that the size of the fragment (size- 40.48 bp) was exactly similar to the size of the low intensity fragments in the TRFLP profile of *B. pumilus and B. subtilis*. This confirmed a minor contamination of *B. japonicum* with *B. pumilus and B. subtilis* cultures. Moreover, a clear single fragment was observed in the TRFLP obtained for *T. harzianum* (Fig. 3.8 d). The analysis confirmed that the size of the fragment in the TRFLP profile of *T. harzianum* (intensity- 6706, size-254.95 bp) was completely different from other TRFLP fragments and this confirmed that the strain was not closely related with the other isolates.

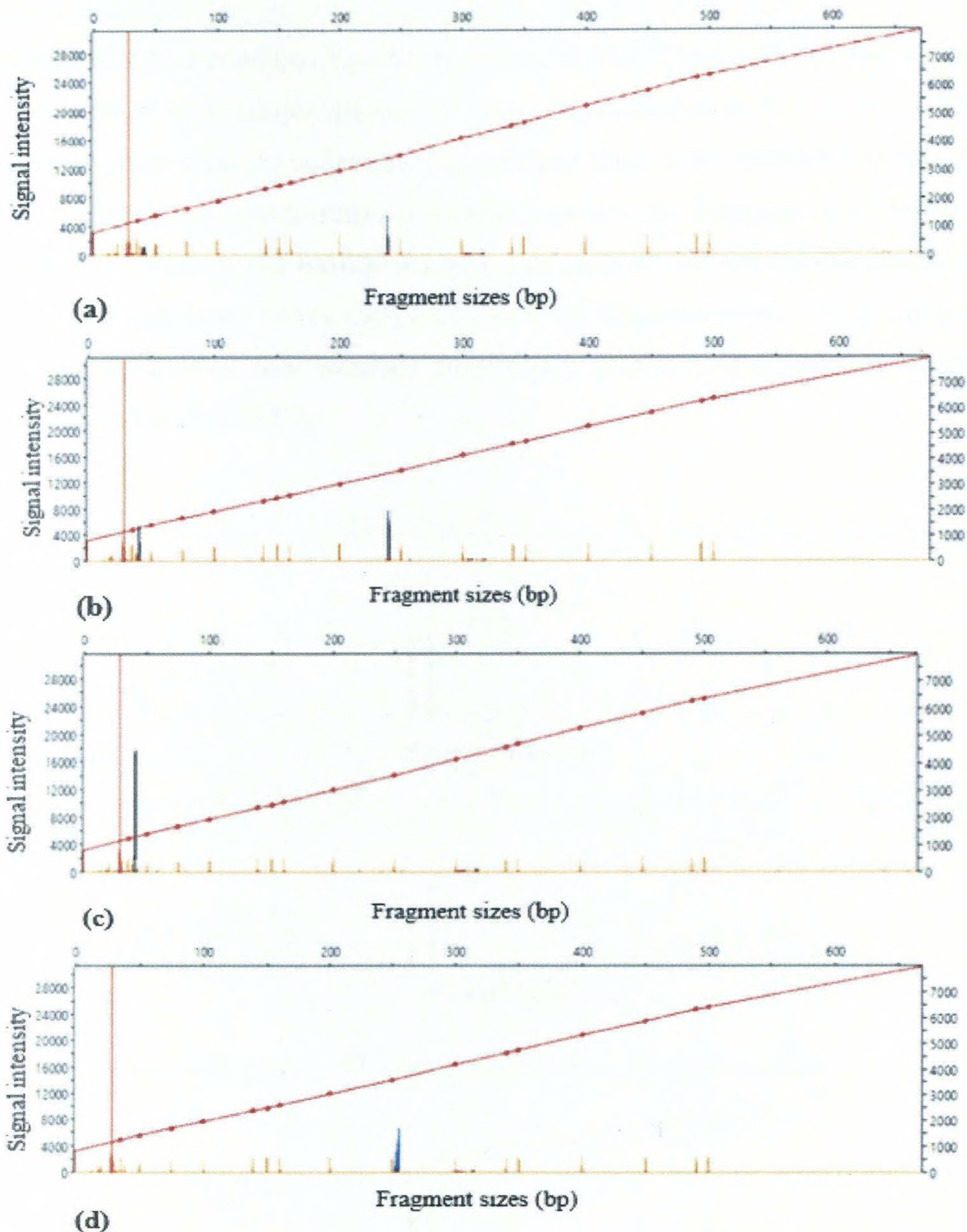



Figure 3.8- Electropherograms showing TRFLP profiles obtained for the PCR products amplified using 16S rRNA genes and ITS regions of different biofilm forming microbial strains. (a)- TRFLP profile obtained for the bacterial strain *B. pumilus*. (b)- TRFLP profile obtained for the bacterial strain *B. subtilis*. (c)- TRFLP profile obtained for the bacterial strain *B. japonicum*. (d)- TRFLP profile obtained for the fungal strain *T. harzianum*.

Figure 3.9 a,b,c show TRFLP profiles obtained for three replicates of biofilm combination BFBF1 and figure 3.9d shows the TRFLP profiles obtained for contaminated biofilm mother culture. The interaction of individual microbial strains in developing the biofilm BFBF1 can be explained by analyzing each of the TRFLP profiles. According to the figure 3.9, three different fragments with different intensities were observed in each TRFLP profiles of biofilm BFBF1. A clear fragment with higher intensity (intensity- 9689) was

observed than that of the other fragments in all TRFLP profiles and the size of this fragment (size- 254.95 bp) was exactly similar to the fragment size of *T. harzianum*. This implies that *T. harzianum* strongly contributed to produce the biofilm BFBF1. Further, closely located two different fragments were detected in between the fragment size 200 bp- 250 bp in the TRFLP profiles of the biofilm BFBF1. Analysis of the profile confirmed that the size of those two fragments were exactly similar to the fragment sizes of *B. pumilus* and *B. subtilis*. Moreover, another low intensity fragment was observed in all four replicates with the fragment size of 40.48 bp.

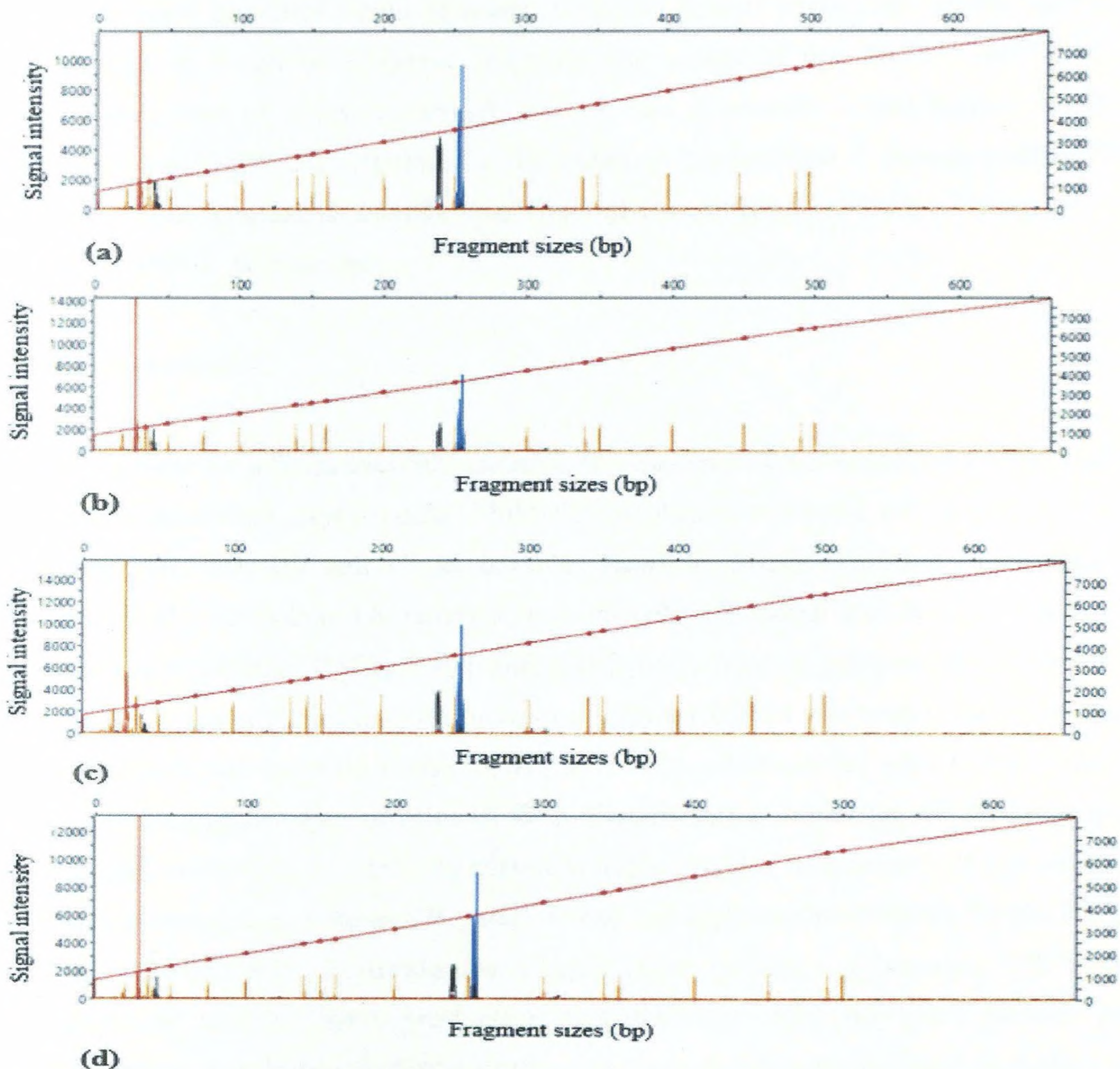



Figure 3.9- Electropherogram showing the TRFLP profiles obtained for the PCR products amplified using 16S rRNA genes and ITS regions of biofilm communities. a, b, c and d are the replicates of TRFLP profiles for the biofilm combination BFBF1.

It was noticed that the size of the fragment with low intensity (40.48 bp) in biofilm TRFLP profile was exactly similar to the size of the fragment obtained for TRFLP profile of *B. japonicum*. Therefore, it was obvious that all three bacterial strains contributed to develop the biofilm BFBF1 with *T. harzianum*. Based on the fragment intensities, the degree of the interaction of all three bacterial strains in terms of developing the biofilm was entirely different. It was noticed that the ratio of the fragment intensities of *B. japonicum*, *B. subtilis* and *B. pumilus* in the TRFLP profile obtained for the biofilm community, was 1: 1.9: 2.3, respectively. Different fragment intensities of the TRFLP profile were attributed to different cell numbers of each microbial strains residing in the biofilm. Therefore, the number of cells of each bacterial strain attached with the fungal mycelium of the biofilm can be expressed in terms of different fragment intensities of the TRFLP profile. Thus, cell abundance ratio of *B. japonicum*, *B. subtilis* and *B. pumilus* in the biofilm BFBF1 can be expressed as 10:19:23, respectively. This clearly implied that *B. japonicum* had the lowest contribution, whereas *B. pumilus* had the highest contribution for the formation of biofilm BFBF1 with *T. harzianum.*

## 3.5 Conclusions

Out of all the beneficial bacterial isolates, B7 was considered as the most responsive isolate of tested screening experiments. Molecular analysis confirmed the identities of microbial samples B1, B7, B9 and F2 as *Bacillus pumilus*, *Bradyrhizobium japonicum, Bacillus subtilis* and *Trichoderma harzianum*, respectively. Bacterial and fungal isolates in biofilm combinations B7F2, B5F1, B8F1 and B1F2 were used to produce higher order biofilms since these simple biofilm combinations showed higher responses for all the screening experiments. All bacterial samples (B1, B7, B9) were interacted with *T. harzianum* in order to form the higher order biofilm BFBF1. However, the degree of the bacterial attachment on fungal mycelium was entirely different in the biofilm community. *B. japonicum* had the lowest contribution, whereas *B. pumilus* had the highest contribution for the formation of biofilm BFBF1 with *T. harzianum.* Higher order biofilm combination HBF1 had higher nitrogenase activity, IAA production and seedling vigor for plant growth promotion compared to the simple biofilm cultures and those combinations could be further evaluated for their growth promotion of plants by direct application to crop plants.

# CHAPTER 4

# EVALUATION OF RESPONSIVE FUNGAL-BACTERIAL BIOFILMS AS BIO-FERTILIZERS THROUGH GREENHOUSE AND FIELD EXPERIMENTS

## 4.1 Introduction

Fertilizer is widely used to supply essential nutrient to increase plant yield. Yields of most crops increase linearly with the amount of fertilizer that they absorb (Loomis and Conner, 1992). Due to this fact, the agricultural sector strongly depends on fertilization with mineral nutrients. When crops are grown under modern high-production conditions, substantial amounts of nutrients are lost from the soil due to the leaching and soil erosion (Taiz and Zeiger, 2002). To prevent deficiencies, nutrients are added back to the soil in the form of fertilizers. This is very serious issue in short term cultivation crops and cash crops like potato. As in most agricultural crops, large amounts of N and P inputs are usually used in potato cropping systems in order to enhance the yield. Potato crop is a heavy remover of soil nutrients and removes 1.5 times the amount of nitrogen and 4 to 5 times the amount of phosphate compared to other vegetable crops (Bansal and Trehan, 2011). Therefore, extensive application of CF is required annually in order to enhance yield responses and therefore the cost of production is fairly high.

However, in conventional agriculture, high cost inputs, particularly CF and agrochemicals have been reported to affect adversely to the soil fertility and crop productivity in the long term, mainly due to the depletion of soil fauna and microbes (Seneviratne, 2009). It is accepted that the N fertilizers suppress the beneficial actions of microbes, particularly $N_2$ fixers. This tends to produce N poor soil microbial communities with low biomass, due to diminished N supply by the $N_2$ fixers (Seneviratne *et al.*, 2011). Thus, the reduced use of CF with increased application of biofertilizers is considered a promising approach to rectify the pressure on the environment derived from agricultural practices. Though bio-fertilizers are not exact fertilizers, giving nutrition to crop plants, they can enhance soil nutrient content through the activity of microbial biomass in the biofertilizer itself. Several bio-fertilizers have been introduced in the recent years, which also acts as natural stimulators of plant growth and development.

Application of microbial inoculants as bio-fertilizers has been realized as an alternative option due to their promising role in substantially, reducing the excessive use of CF, nutrients and pesticides (Malusa, Sas-Paszt and Ciesielska, 2012). Microbial bio-fertilizers include selective organisms like bacteria, fungi and algae. These are capable of fixing atmospheric N and solublization of native and added nutrients (for example: P) in the soil and turn them into available forms to plants. The microbial bio-fertilizers trap some amount of the N available in soil and fix it in the soil which benefits the plant. Since bio-fertilizer consist of many beneficial microbes, the nutrients availability in soil are improved after their application due to many dimensions (Boraste *et al.*, 2009).

Recently, the use of biofilms has also been proposed as a possible means to produce effective plant inocula. The soil application of beneficial biofilms in the form of bio-fertilizers has been identified to be a promising approach to replenish depleted microbes as well as to enhance productivity in the soil (Kulasooriya, 2008). The majority of plant-associated beneficial bacteria like PGPRs found on roots and in soil form biofilms. Therefore, using PGPRs strains that form biofilms could be a strategy to ease the formulation and production of inocula. Biofilm-based inocula could also facilitate the production of bio-fertilizers considering the biofilm as a carrier. The carrier is the major portion of the biofilm inoculant, delivering the beneficial microbial strains in good physiological condition (Malus *et al.*, 2012). The carrier should be designed to provide a suitable microenvironment for the PGPRs, should assure a sufficient shelf life of the product and the formulation should allow an easy dispersion or dissolution in the volume of soil near the root system. The biofilms developed in the current study is one such beneficial microbial combination with a proper carrier material that can be used as bio-fertilizers for non-legumes, which is now known as biofilmed biofertilizers (BFBFs).

Beneficial microbial communities in such BFBFs attaché to the plant roots of some crops help in the cycling of nutrients as well as the bio-control of pests and diseases, resulting in improved agricultural productivity (Seneviratne, 2003). It has been reported that similar BFBFs, using *Bradyrhizobium–Penicillium* combination have been observed to exhibit better growth, $N_2$ fixing ability and colonization abilities than their monocultures (Jayasinghearachchi and Seneviratne, 2004; Seneviratne and Jayasinghearachchi, 2003). Interestingly, the BFBFs have shown that they can produce equal or even relatively high yields with only 50% of recommended CF of several crops like tea and maize, in

comparison to 100% of the fertilizers, from the first year of their application (Seneviratne *et al.*, 2009). It has been reported that the application of BFBFs containing two bacteria and one fungus with only 50% of the recommended rates of CF, increased the dry matter accumulation in rice shoots substantially by 55% compared to those observed for 100% application of the recommended fertilizers alone. Moreover, moderate application of 50% of the recommended fertilizers with the two-bacterial BFBFs helped increase leaf growth of tea compared to even 100% of the recommended fertilizer application. Anthurium plantlets treated with four-microbe biofilm and 50% of recommended CF in an inert particle medium showed a higher relative growth rate of plant dry weight than that of the 100% of the recommended fertilizers alone (Seneviratne *et al.*, 2009). Further, it has been well documented that the enhancement of soil basic nutrients by the application of BFBFs couple with CF. When applied directly to the soil, BFBFs increased N and P availabilities compared to the monocultures (Seneviratne and Jayasinghearachchi, 2005). Further, it has been reported that the biofilmed inocula can be effectively used in biosolubilisation of rock phosphate. This has been demonstrated by developing biofilms from *Penicillium* sp., *Pleurotus ostreatus* and *Xanthoparmelia mexicana*, a lichen fungus which increased the P solubilisation up to approximately 230% compared to the fungus-only cultures (Jayasinghearachchi and Seneviratne, 2006a; Seneviratne and Indrasena, 2006). As such, diverse forms of the biofilmed inocula may satisfy the future demand of augmented crop productivity with increased $N_2$ fixation, nutrient uptake and biocontrol of diseases.

These findings suggested that the input of CF can be reduced by 50% during the vegetative growth phase and possibly for the entire crop of such non-legumes, which could be a huge economic gain in terms of fertilizer saving. The moderate application (50%) of the CF nutrients helps increase the microbial biomass of the biofilm, which in turn tends to increase the microbial efficiency or the functionality, as the concentrations of the fertilizer nutrients, particularly N depletes. The biofilm acts as a nodule-like structure or a pseudonodule-fixing $N_2$ in terms of non- legumes. This fixed $N_2$ may be transferred to the root, and in return the root may supply carbon sources to the biofilm, the processes of which need future investigations. The release of organic acids by the biofilm helps suppress microbial pathogens (Browning *et al.*, 2006) as well as increase mineralization of soil nutrients in the rhizosphere (Seneviratne and Jayasinghearachchi, 2005). Moreover, plant growth hormones, such as IAA produced by the biofilms (Bandara *et al.*, 2006), should increase the growth of roots and mycorrhizal fungi. In this manner, this association constitutes an

excellent metabolic cooperation that helps the healthy growth of the plant. In addition, the BFBFs are also important in replenishing beneficial microbial communities in deteriorated soils due to heavy use of chemical inputs and intensive cropping (Seneviratne, 2009). Further, this benefit can be maximized for the short term cultivations and cash crops like potato since those crops need extensive rate of CF annually. Therefore, this study was focused to evaluate the effect of FBBs as a bio-fertilizer on growth responses of potato which is an intensively used non-legume crop, while reducing the CF application.

## 4.2 Objectives

1. To evaluate the developed BFBFs on the growth enhancement of potato.
2. To analyze the effect of developed BFBFs on soil nutrient enhancement.
3. To investigate whether tuberization may be induced in tuberization non- inducing locations using BFBFs.

## 4.3 Methodology

### 4.3.1 Evaluation of the responsive biofilms for potato seed tuber growth under greenhouse conditions

Responsive biofilm combinations (HBF1 and HBF2) from the screening experiments (3.3.6.1) were evaluated for their growth responses on potato seed tubers under greenhouse conditions at the Regional Agriculture Research and Development Center, Bandarawela, Sri Lanka. The experiment was conducted from November 2012 to March 2013. Growth enhancement was evaluated using tuber fresh weight, tuber number, stolon number, shoot dry weight and root dry weight of potato plants grown in pots as follows.

#### 4.3.1.1 Soil preparation and planting

Soil collected from 0 - 10 cm depth of a loamy soil in Regional Agriculture Research and Development Center, Bandarawela was used as the medium for the pot experiment. The soil sample was sieved to obtain uniform soil particles (particle size < 2 mm). Pots were prepared with gauge 200 black polythene and filled (3/4 of the pot) with the soil samples obtained. Disease free seed tubers ('Granola' variety) were obtained from government

certified Regional Agriculture Research and Development Center, Bandarawela. Two seeds were grown in each pot with the obtained soil medium.

#### 4.3.1.2 Application of biofilm cultures

Fresh biofilm cultures were prepared on biofilm forming medium and incubated for five days under the continuous mixing. Low cost medium (Patented) was used as the carrier material to inoculate the biofilm to the plant. Before inoculation, low cost medium was heat sterilized for 15 min to minimize contamination. 10 ml of each biofilm broth culture was inoculated separately with 200 ml of heat sterilized low cost medium and incubated for five days under normal room temperature with continuous mixing. Microscopic observations were made for the inoculated medium (with low cost medium) for the confirmation of further bacterial attachment on fungal mycelia. Biofilm mixture with low cost medium was diluted by 250 times with clean water before the application. After two weeks from seed sowing, each prepared biofilm combinations with the carrier material was applied using a spray tank directly around the root zone of the potato plants grown in pots. Biofilm inoculation was repeated again after five weeks from seed sowing simultaneously with CF application. All the pots were arranged according to CRD inside the greenhouse till harvesting. Five replicates were maintained for each treatment (HBF1 BFBF1 and HBF2 BFBF2). Treatment combinations are as follows.

TR1-100% CF
TR2- 50% CF
TR3- 50% CF + BFBF1
TR4-50% CF + BFBF2
TR5- BFBF1 alone
TR6- BFBF2 alone
TR7- No amendments

#### 4.3.1.3 Fertilizer application

Rates of the CF application were calculated per plant basis according to DOA recommendations. A mixture of urea (2.0 g/kg), TSP (3.33 g/kg), MOP (1.33 g/kg) was mixed with the soil as a basal fertilizer mixture before filling soil in to pots (TR1). 50% of the recommended fertilizer was initially mixed with soil for the treatment TR2, TR3 and TR4 as the basal mixture. Fertilizer application (2.0 g/kg of urea and 1.33 g/kg of MOP) was repeated after five weeks from seed sowing simultaneously with biofertilizer application. Moisture level of the medium was maintained constantly by applying 250 ml of water for each pot every day. Plants were grown with a daily minimum-maximum temperature range of 20 °C – 30 °C.

#### 4.3.1.4 Harvesting

After 90 days from seed sowing, plants were uprooted carefully without damaging the root system and were washed carefully with water to remove unwanted materials attached to the root system. Subsequently, the individual plants and the soil samples were collected in to black polythene bags accordingly with different treatments and were brought in to the laboratory to analyze the soil samples and underground and above ground biomass of the plants. Fresh weights of the tubers, number of stolons and tubers, dry weights of roots and shoots were measured to evaluate the growth responses for the treatments.

#### 4.3.1.5 Analysis of soil parameters

##### 4.3.1.5.a Soil pH

Twenty grams of soil samples from each treatments were mixed with 50 ml of distilled water and the mixtures were stirred for 10 minutes. Subsequently, the soil mixtures were kept for 30 min without stirring, followed by 2 min stirring again. After mixing, pH of the supernatants were measured (Anderson and Ingram, 1998). Three replicates were maintained for each treatment.

#### 4.3.1.5.b Total soil C

Dried and ground one gram (<0.15 mm) of soil sample was added to a 100 ml conical flask followed by adding 10 ml of 5% potassium dichromate solution and allowed to wet completely. Standard solution series was prepared using a pure sucrose stock solution. 20 ml of conc. $H_2SO_4$ was added from a burette and mixed gently. After cooling 50 ml of 0.4% barium chloride was added and kept overnight. The absorbance of the supernatant was measured at 600 nm using a spectrophotometer and total organic Carbon was estimated using the following equation (Anderson and Ingram, 1998).

Percentage Organic Carbon = (K X0.1)/ (W X 0.74)

K= Corrected concentration (difference between concentration for blank and the concentration for unknown)

#### 4.3.1.5.c Total soil N - Kjeldahl method

4.4 ml of digestion mixture (0.42 g selenium powder, 14 g lithium sulphate, 30% $H_2O_2$, 420 ml of conc. $H_2SO_4$) was mixed with finely ground 1 g (>0.15 mm) of soil sample (W) in a digestion tube followed by digestion at 360 °C for 2 hours (blank- only.digestion mixture). About 50 ml of water was added after cooling the mixture and mixed well to dissolve the remaining sediments. Subsequently, the mixture was topped up to 100 ml with water and was kept for 30 min to settle down the sediments. The clear supernatant was used for N analysis using distillation and titration methods. About 50 ml of distillate was obtained from the blank solution using steam distillation apparatus of the Kjeldahl instrument (Model No. VELP Scientifica UDK 127) followed by titration with HCl acid solution. Subsequently, 15 ml (A) of sample solution and 12 ml of alkali mixture (25 g NaOH + 1.25g $Na_2S_2O_3$ in 50 ml of water) were transferred to the reaction chamber of the apparatus. About 5 ml of boric acid indicator solution was added to 25 ml of distillate obtained after the distillation of sample solution. Then the mixture was titrated using HCl and the volume (T) of the HCl was recorded (Anderson and Ingram, 1998).

Percentage of total N= (T x S x 0.01)/ (A x W)

#### 4.3.1.5.d Total soil P

The digestion was followed as explained in 4.3.1.5 c for the soil samples. Subsequently, 1 ml of sample solution was pipetted to a test tube followed by the addition of 4 ml of 1% ascorbic acid solution. The mixture was kept for 1 hour for the colour development after adding 3 ml of molybdate reagent (4.3 g of ammonium molybdate was dissolved in 400 ml of water. Subsequently, 400 ml of 0.1% antimony sodium tartrate solution was added to the solution and mixed well while acidifying the mixture with $H_2SO_4$). Absorbance of the mixture was measured at 880 nm using an UV visible spectrophotometer (Model No. Genetics 6). Concentration of the sample was determined using calibration plot drawn with standard $KH_2PO_4$ solution (Anderson and Ingram, 1998).

### 4.3.2 Evaluation of the effect of responsive biofilms on potato seed tubers under different field conditions

Responsive biofilm combinations selected from screening experiments (HBF1= BFBF1 and HBF2= BFBF2) were evaluated for their growth responses on potato seed tubers under different field conditions in Sri Lanka. The experiment was conducted from November 2013 to April 2014. Growth enhancement was evaluated using tuber fresh weight, tuber number, stolon number, shoot dry weight and root dry weight of potato plant grown in different field conditions.

#### 4.3.2.1 Justification for the section of suitable locations

Temperature plays a key role in regulating growth and yield in potatoes (Bodlaender, 1963; Ewing, 1981; Gregory, 1965; Tibbitts *et al.*, 1989). High temperatures regimes (30 °C–35 °C) are inhibitory for tuberization whereas low temperature regimes (17 °C–27 °C) induce potato tuberization. Also, a day/ night temperature fluctuation (thermoperiod) is considered to be necessary for maximum tuber yields (Benoit, Grant and DeVine, 1986; Ewing, 1981). Day/night atmospheric temperature difference should be at least 8-10 °C, in order to induce tuberization. Therefore, the selection of field locations was considered as one of the critical factors in this study.

Three different locations were selected based on day-night temperature differences for the field experiment as follows. Soil characters were also considered for the selection of field locations.

- Bandarawela (6° 48' 0" N, 80° 58' 0" E) [up country intermediate zone ($IU_3$) 1506 m amsl], Sri Lanka.– tuberization induced condition (average day Tem. 30 °C, average night Tem. 19 °C).
- Horana (6°46'59.3"N, 80°05'10.0"E) [low country wet zone, 93 m amsl], Sri Lanka - tuberization non-induced condition (average day Tem. 29 °C, average night Tem. 24 °C)
- Bibile (7°09'20.5"N, 81°13'26.4"E) [low country intermediate zone, 305 m amsl], Sri Lanka – tuberization non-induced condition (average day Tem. 30 °C, average night Tem. 23 °C).

Since the field location at Horana failed due to water logging, a different field location at Padukka (6°50'32.0"N, 80°05'44.8"E) [low country wet zone, 155 m amsl], Sri Lanka was selected for the third field trial (Colombo district- similar day/night temperature difference as of Horana location).

At present potato is extensively cultivated in the district of Nuwara Eliya (Up country wet zone >1000m amsl) in two major seasons, "Yala" (Feb - July) and "Maha" (Aug - Dec.) and Badulla district specifically in Bandarawela and Welimada (Up Country intermediate zone- 1000 to 1500 m amsl). The climatic conditions of those districts are ideally suited for seed potato production. Therefore, Bandarawela was selected as one of major field locations for this study. Though Bibile is located in Badulla district, the climatic condition is not favorable for potato mass cultivation. However, according the previous data available at the DOA, Sri Lanka there is a possibility of cultivating potato at Bibile area. Colombo doesn't have any favorable atmospheric conditions for potato tuber induction. Therefore, Colombo was selected as a control field site to determine whether there is any effect of BFBFs on potato tuberization, irrespective of climatic conditions.

#### 4.3.2.2 Bed preparation and planting

Selected each field location was marked according to the standard dimensions of the beds and the land was ploughed and prepared according to the standard practices. Drainage system was established around the field location to create favorable soil conditions for potato cultivation. A net was established around the whole field location in order to minimize rodents and wild boar attacks. Bed preparation was performed according to the standard dimensions for potato (treatment plot size – 2.4 m x 1.25 m, between two treatment plots – 1 m) and treatment plots were arranged according to randomized complete block design (RCBD) method. Government certified disease free potato seed tubers ('Granola' variety) were obtained from Regional Agriculture Research and Training Center, Bandarawela and were sown in the prepared locations. Each treatment plot contained 20 seed potatoes (between rows – 60 cm, between two plants – 25 cm) and three replicates and seven different treatments were maintained in each different locations (between replicates – 1 m).

#### 4.3.2.3 Application of biofilm cultures

Biofilm culture preparation and application were performed as explained in section 4.3.1.2.

#### 4.3.2.4 Fertilizer and agrochemical applications

A mixture of urea (17 g/m$^2$), TSP (27 g/m$^2$), and MOP (12.5 g/m$^2$) was mixed with soil as a basal fertilizer mixture for the treatment TR1 plot (100% CF). Half (50%) of the recommended fertilizer was applied as a basal mixture to the 50% CF treatment plots (TR2, TR3 and TR4). Fertilizer application (17 g/m$^2$ of urea and 12.5 g/m$^2$ of MOP) was repeated after five weeks from seed sowing, simultaneously with the BFBFs applications. Since potato crop is highly susceptible to pest and disease attacks, agrochemicals were applied starting two weeks from the seed sowing till harvesting. Two types of fungicides (Mancozeb and Cabriotop) were applied once in three weeks to prevent the emergence of potato late and early blight diseases. All fungicides were applied using a hand spray only for the leaves within the DOA recommended limits. Watering was carried out twice a day (morning and the evening) starting from seed sowing till harvesting. Since Bibile field location had sandy soil, the amount of water applied during the morning period was increased. Plant growth

and development was facilitated by maintaining and cleaning (removing of weeds) the treatment beds every three weeks. Frequent observations were made on the fields to check the emergence of any disorders or pest attacks and diseases.

#### 4.3.2.5 Harvesting

After 90 days from seed sowing, plants from selected locations were uprooted carefully without damaging the root system and were washed carefully with water to remove unwanted materials attached to the root system. Subsequently, the individual plants of different treatments were collected into black polythene bags and were brought into the laboratory to analyze underground and above ground biomass of the plants. Soil samples were also collected from selected locations into black polythene bags separately to analyze the soil parameters.

#### 4.3.2.6 Statistical analysis

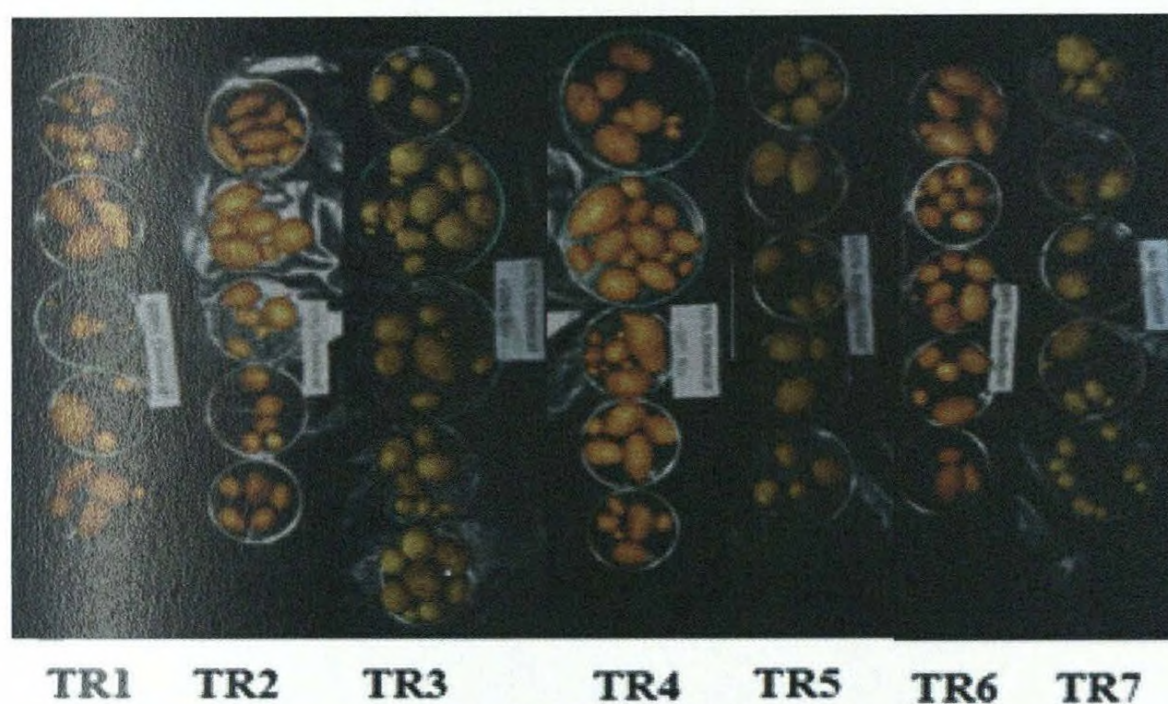
Normality of the data and constancy of residuals were confirmed. Statistical data analyses were performed on all data collected using the one way Analysis of Variance (ANOVA) Model in MINITAB 16 Statistical Software. The mean values of dry and fresh weights of the tubers, roots and shoots were compared on treatment basis using the tukey's simultaneous test at 5% significance level. Further, mean values of soil parameters such as soil pH, organic C, total N and total soil P were compared on treatment basis using the tukey's simultaneous test at 5% significance level.

### 4.4 Results and Discussion

### 4.4.1 Evaluation of the biofilm effect on potato tubers under greenhouse conditions

#### 4.4.1.1 Analysis of plant biomass

Plate 4.1 shows the effect of different BFBFs and CF combinations on potato vegetative growth and tuber development. According to the photographs, it was observed that CF treatments enhanced the vegetative growth compared to other treatment combinations.



TR1 TR2 TR3 TR4 TR5 TR6 TR7

Plate 4.1- Tuber yield of potato obtained by treating with different BFBFs and CF combinations under greenhouse conditions. Treatments TR1-TR7 are 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone No amendments treatments respectively.

BFBFs treatments greatly enhanced the tuber number and size of potato (plate 4.1). Similar effects have been reported by another study that the additions of soil microorganisms improved quality and the size of the potato tubers. (Sugiarto *et al*., 2013).

Mean fresh weight of the tubers for the treatment TR3 (131.19 g ± 4.33) was significantly higher ($P < 0.05$) than that of the other treatments (table 4.1). Treatment TR3 enhanced the tuber weight of potato by approximately 92% compared to the treatment TR2. Out of the seven treatments, the highest mean tuber number (8 ± 1.15) was observed in the treatment TR3. Another study has been reported that integrated application of CF and non-CF, improved potato tuber yield by 39.9% as compared to the CF alone (Frommel, Nowak and Lazarovits, 1993). Mean stolon number for the treatment TR3 (10.2 ± 1.67) was significantly higher ($P < 0.05$) than that of the other treatments. Both treatment TR3 and TR4 enhanced the mean tube number, mean stolon number and mean fresh weight of tubers

even compared to the treatment TR1 (100% CF). However, a significant difference was not observed (P > 0.05) between treatment TR3 and TR4 in terms of tuber number. Further, treatment TR5 which was only the BFBFs without any CF, enhanced the tuberization (tuber weight and tuber number) compared to the treatment TR1. This might be due the effect of the beneficial activity of the developed microbial biofilm. It has been reported that the tuberization is a complex phenomenon influenced by a variety of intrinsic and extrinsic factors (Vreugdenhil and Struik, 1999) including microorganisms in the soil.

Further, treatment TR1 showed the highest mean shoot dry weight and mean root dry weight of potato compared to the other treatments. Further, T-test confirmed that the mean shoot dry weight ($P < 0.005$, 4.99 g ± 0.56) and mean root dry weight ($P < 0.005$, 0.48 g ± 0.07) for the treatment TR1 were significantly higher than that of treatment TR2. However, it was clearly observed that treatment TR1 reduced the tuberization (mean tuber weight and meant tuber number) compared to treatment TR2. The main reason of this might be due to the stress factors with high accumulation of CF including N fertilizers inside the pot, thereby creating high N concentration around the rhizosphere.

Table 4.1- The effect of different CF and BFBFs treatments on different parameters of potato.

| Treatment | Tuber weight (g/plant) Mean ± SD | Tuber number (Per plant) Mean ± SD | Stolon number (Per plant) Mean ± SD | Shoot weight (g/plant) Mean ± SD | Root Weight (g/plant) Mean ± SD |
|---|---|---|---|---|---|
| TR1 | $61.03 \pm 3.75^{cd}$ | $5.00 \pm 0.94^{ab}$ | $5.40 \pm 0.89^{bc}$ | $4.99 \pm 0.56^{a}$ | $0.48 \pm 0.07^{a}$ |
| TR2 | $68.15 \pm 2.99^{bc}$ | $6.40 \pm 1.43^{ab}$ | $7.20 \pm 1.09^{b}$ | $3.95 \pm 0.52^{bc}$ | $0.31 \pm 0.05^{cd}$ |
| TR3 | $131.19 \pm 4.33^{a}$ | $8.00 \pm 1.15^{a}$ | $10.20 \pm 1.67^{a}$ | $4.19 \pm 0.62^{ab}$ | $0.32 \pm 0.05^{cd}$ |
| TR4 | $80.94 \pm 11.31^{b}$ | $7.80 \pm 2.94^{a}$ | $7.00 \pm 1.58^{b}$ | $4.60 \pm 0.47^{ab}$ | $0.19 \pm 0.02^{e}$ |
| TR5 | $70.45 \pm 8.16^{bc}$ | $5.40 \pm 1.95^{ab}$ | $5.40 \pm 2.07^{bc}$ | $3.11 \pm 0.59^{cd}$ | $0.43 \pm 0.04^{ab}$ |
| TR6 | $46.28 \pm 8.25^{de}$ | $3.60 \pm 1.07^{b}$ | $3.60 \pm 1.14^{c}$ | $2.13 \pm 0.29^{d}$ | $0.38 \pm 0.03^{bc}$ |
| TR7 | $41.72 \pm 11.95^{e}$ | $3.80 \pm 1.55^{b}$ | $3.80 \pm 1.64^{c}$ | $2.15 \pm 0.31^{d}$ | $0.24 \pm 0.04^{de}$ |

Treatments; TR1- 100% CF, TR2- 50% CF, TR3- 50% CF + BFBF1, TR4- 50% CF + BFBF2, TR5- BFBF1 alone, TR6- BFBF2 alone, TR7- no amendments. Same letter are not significantly different at 5% probability level.

It has been well documented that the plant growth regulators have been suggested to play a prominent role in the control of tuberization in potato (Ewing, 1987; Vreugdenhil and Struik, 1989; Xu *et al.*, 1998). Plant growth promotion as a result of IAA has been

documented in several plants in recent years (Spaepen, Vanderleyden and Remans, 2007). It was observed that exogenous IAA treatment induced larger tubers at an earlier stage of the growth of potato (Harmey, Crowle and Clinch, 1966). Vrany and Fiker (1984) reported a 4-30% increase in plant growth and tuber yield when potato seed tubers were inoculated with PGPRs before planting. Few other studies have suggested a positive role of some of these soil bacteria on plant growth (Frommel *et al.*, 1989) and tuber yield (Sturz, 1995) in potato. Also it has been proven that in the absence of GA, addition of IAA resulted in much shorter stolons thereby enhancement of tuber initiation (Torrent *et al.*, 2011). This implies that the enhancement of the tuberization by the treatment TR3 might be due the effect of overall beneficial activity shown by the BFBFs.

Further, it has been reported that the high or continuous supply of N fertilizers inhibit the tuberization even if there is tuber inducing atmospheric conditions and it promotes production of the inhibiting hormone GA and depresses the promoting hormone Absisic acid (ABA) (Jackson, 1999; Krauss, 2013). The corresponding low ABA: GA ratio increases shoot growth and delays tuber production, tending to decrease tuber yields of early potatoes. Further, it has been well documented that the application of GA promotes stolon elongation and inhibits tuber formation (Smith and Rappaport, 1969; Kumar and Wareing, 1972). It was also reported that a decline of GA activity in potato (*Solanum tuberosum* L.) plants is associated with tuberization (Railton and Wareing, 1973; Krauss and Marschner, 1982).

#### 4.4.1.2 Evaluation of soil characters

#### 4.4.1.2.a Soil pH

Treatments TR3 showed the lowest soil pH (4.11 ± 0.07) compared to the other treatments (Fig. 4.1). All treatments showed low soil pH (pH below 5) compared to treatment TR7 (control). However, the pH reduction recorded by all the CF and BFBFs combine treatments (TR1- TR4) was an unusual and this might be due to the high accumulation of CF inside the pot throughout the growth period.

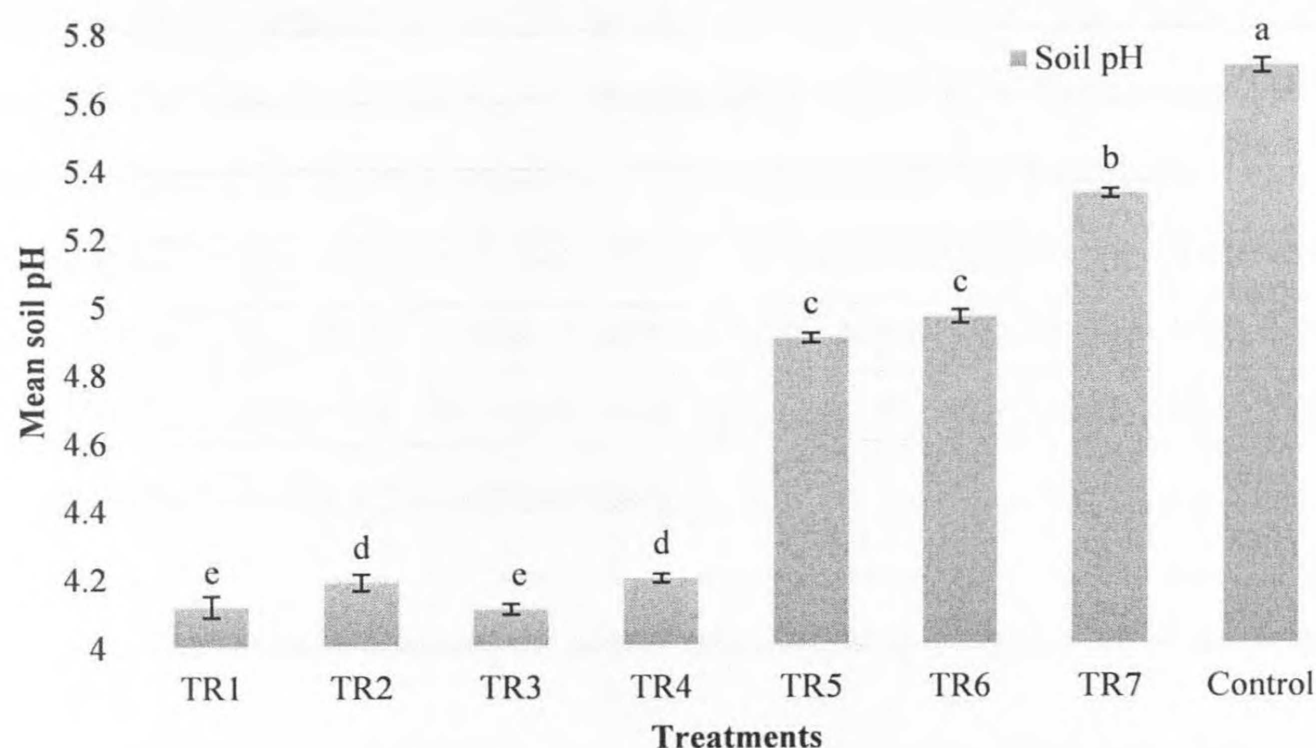



Figure 4.1– Mean soil pH after different CF and BFBFs treatments. Treatments; TR1- 100% CF, TR2- 50% CF, TR3- 50% CF + BFBF1, TR4- 50% CF + BFBF2, TR5- BFBF1 alone, TR6- BFBF2 alone, TR7- no amendments. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

Further, acidic secretions of the microbial communities in the developed BFBFs might also have an effect on reducing the soil pH. Similar results showing the reduction of soil pH by the inoculation of microbial biofilms have been reported by another study (Seneviratne and Indrasena, 2006).

#### 4.4.1.2.b Soil nutrients

It has been well documented that the quantity and composition of microbial biomass is sensitive to changes in the soil chemical and physical environment and organic matter quality changes (Jaiswal *et al.*, 2010). Therefore, their inclusion in biofertilizer/biocontrol consortia can improve the nutrient mobilization and improve the nutrient status of soils and crops. The current study showed similar results, to what others have found in terms of nutrient enhancement in soil by the soil microbial inoculation. According to the current study, treatments TR3 (11.15 mg/g ± 01.63) showed the highest total SOC content compared to all other treatments (table 4.2). Treatment TR3 enhanced the SOC content by approximately 14% than that of the treatment TR2 and by 80% than that of the treatment TR7. All treatments enhanced the soil C content compared to the treatment TR7. Further, the current study showed the enhancement of other soil nutrients such as N and P by the application of BFBFs. Out of the eight treatments, the highest soil total N (241.7 mg/kg ±

11.33) content was recorded by treatment TR1 whereas the highest total P content (39.4 ppm ± 4.71) was recorded by treatment TR3 (table 4.2). Treatment TR3 enhanced the soil total N content by approximately 24% compared to the treatment TR2. Further, treatment TR3 significantly enhanced the soil P content compared to treatment TR2 and it was approximately by 100%. Thus, it was clearly shown that the biofilm combinations BFBF1 and BFBF2 enhanced the total soil nutrient content with 50% recommended CF in comparison with the other treatments.

Table 4.2 – The effect of different CF and BFBFs treatments on different soil nutrient contents in potato pot experiment.

| Treatment | SOC (mg/g) Mean ± SD | Total N (mg/kg) Mean ± SD | Total P (ppm) Mean ± SD |
|---|---|---|---|
| TR1 | $10.83 \pm 0.61^{a}$ | $241.7 \pm 11.33^{a}$ | $35.50 \pm 4.69^{a}$ |
| TR2 | $9.74 \pm 0.97^{ab}$ | $187.18 \pm 4.31^{b}$ | $19.70 \pm 0.91^{c}$ |
| TR3 | $11.15 \pm 1.63^{a}$ | $232.64 \pm 5.86^{a}$ | $39.40 \pm 4.71^{a}$ |
| TR4 | $9.92 \pm 0.46^{ab}$ | $177.58 \pm 4.65^{b}$ | $22.1 \pm 1.71^{bc}$ |
| TR5 | $8.71 \pm 0.63^{b}$ | $161.98 \pm 3.11^{bc}$ | $27.1 \pm 2.86^{b}$ |
| TR6 | $8.87 \pm 0.77^{b}$ | $167.32 \pm 3.89^{bc}$ | $20.6 \pm 2.63^{c}$ |
| TR7 | $6.16 \pm 0.92^{c}$ | $117.82 \pm 1.81^{d}$ | $17.5 \pm 1.12^{c}$ |
| Initial | $6.15 \pm 0.55^{c}$ | $126.30 \pm 3.34^{cd}$ | $10.8 \pm 2.08^{d}$ |

Treatments; TR1- 100% CF, TR2- 50% CF, TR3- 50% CF + BFBF1, TR4- 50% CF + BFBF2, TR5- BFBF1 alone, TR6- BFBF2 alone, TR7- no amendments. Same letter are not significantly different at 5% probability level.

A significant enhancement in SOC has been recorded in microbe-inoculated soil which could be correlated with MBC values (Prasanna *et al.,* 2011). Further, the inclusion of beneficial microorganisms has been well known for their C sequestering ability (Jaiswal *et al.*, 2010) and may have served a significant role in the C enrichment of soil. Moreover, enhancement of soil nutrients including N and P by the application of PGPRs have been reported by several other studies (Prasanna *et al.*, 2011: Rodriguez and Fraga, 1999: Duarah *et al.,* 2011).

Table 4.3 shows the correlation matrix between potato plant parameters and SOC content for the pot experiment. A significant correlation was observed between shoot dry weight and SOC content ($r = 0.822$, $P = 0.023$). However, a significant correlation was not observed

between tuber weight and SOC content. Therefore, it can be assumed that the SOC content was directly affected to enhance the vegetative growth of potato plant.

Table 4.3- Correlation coefficients (r) between plant parameters and SOC content obtained from a potato pot experiment under greenhouse conditions

| | SOC | Shoot dry weight | Root dry weight |
|---|---|---|---|
| Tuber weight | 0.685 (0.089) | 0.535 (0.216) | -0.120 (0.798) |
| Shoot dry weight | 0.822 (0.023) | - | - |
| Root dry weight | 0.311 (0.498) | - | - |

Values within parentheses are probability levels.

SOC is important for all three aspects of soil fertility, namely chemical, physical and biological fertility (Chan, 2008). The maintenance or restoration of soil quality is highly dependent on organic matter and an array of beneficial macro organisms and microorganisms that it supports. (Perera and Weerasinghe, 2014). It has been reported that the SOC is directly affected for the agricultural production and the soil quality enhancement (Jobbagy and Jackson, 2000). Further, SOC content of soil contributes to enhance the health of soil microbial community and thereby enhances the soil nutrient flow. This directly contributes to enhance the vegetative growth of crop plants (Li and Feng, 2002).

### 4.4.2 Evaluation of the effect of biofilm on potato seed tubers under different field conditions

#### 4.4.2.1 Plant responses at different field locations

It was clearly observed that the plants treated with BFBF1 were healthier (bright green in colour) and the shoot sizes were much larger than that of the plants treated with 100% CF after six weeks from seed sowing. Further, the size of the leaves of BFBF1 treated plants were comparably higher than that of the plants treated with 100% CF (appendix 6). Any pest attack or blight conditions were not observed after six weeks from seed sowing in both BFBFs and CF treated plants.

Field location at Horana failed due to the water logging and unfavorable soil conditions. Plants did not grow even after eight weeks from seed sowing and most of the seed tubers were rotten in both BFBFs treated and CF treated beds including the control. Therefore, the site was given up and a different site at Padukka area (similar climatic condition to Horana site) was selected to continue potato field trial.

#### 4.4.2.2 Analysis of plant biomass

#### 4.4.2.2.a Plant analysis for field location- Bandarawela

It was observed that the treatment TR3 greatly enhanced the tuber number and the size of the tubers. Vegetative growth of the plants was enhanced by both treatment TR1 and TR3 (plate 4.2).

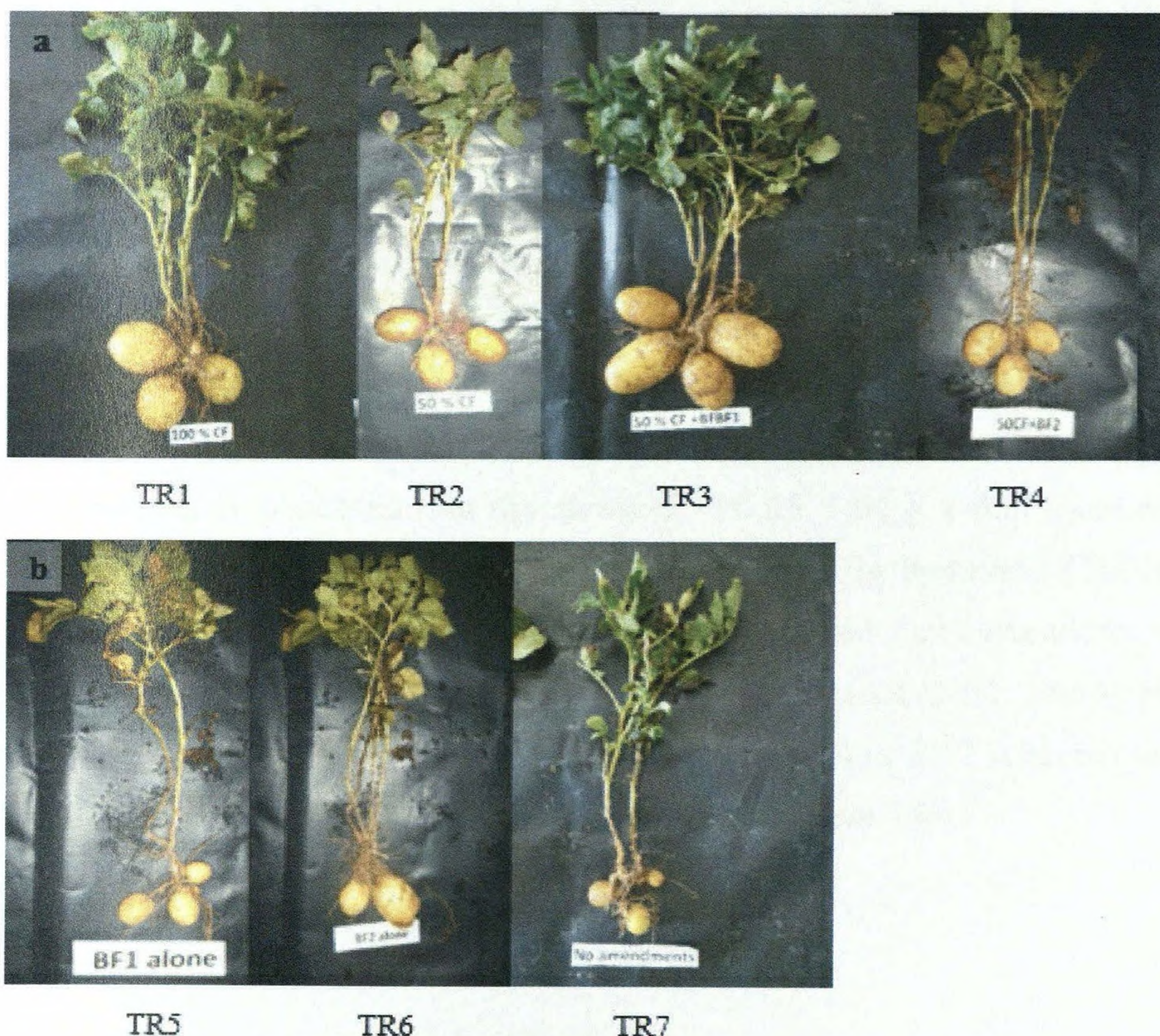



Plate 4.2- Potato tuber yield for different BFBFs and CF treatments at Bandarawela field site. (a) and (b)- Tuber yield with vegetative biomass per seed tuber (seed tubers for the figure (a and b) were selected randomly in each treatment). TR1, TR2, TR3, TR4, TR5, TR6 and TR7 are the treatment of 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone No amendments treatments respectively.

Out of all treatments, the highest tuber weight (428.9 g ± 48.40) and the highest tuber number (7.25 ± 2.35) were recorded from the treatment TR3 (table 4.4). A T-test confirmed that the fresh weight of the tubers ($P < 0.05$) and the number of tubers ($P < 0.05$) for the treatment TR3 were significantly higher than that of the treatment TR2. Treatment TR3 enhanced the tuber fresh weight by approximately 68% compared to the treatment TR2 and approximately 7% even compared to the treatment TR1. Further, mean tuber weight was reduced by approximately 2.5% with the application of treatment TR4 compared to treatment TR2. Therefore, it was clearly observed that there was no effect of biofilm combination BFBF2 on the enhancement tuberization at Bandarawela field site.

All the treatments enhanced the mean tuber weight and mean tuber number compared to treatment TR7. Further, treatment TR3 enhanced the mean tuber number by approximately 43% compared to treatment TR2 and by approximately 4% even compared to the treatment TR1. Plate 4.3 clearly shows that the stolon number (20.1 ± 6.27) was enhanced by treatment TR3 and the stolons were enlarged to initiate tubers. This was confirmed by the table 4.4 which shows the mean stolon number was significantly enhanced by the treatment TR3 compared to all other treatments ($P < 0.05$). Treatment TR3 enhanced the stolon number by approximately 63% compared to the treatment TR2 and by approximately 28% compared to treatment TR1. Further, mean stolon number was reduced by approximately 24% by the application of treatment TR4 compared to the treatment TR2.

It was clearly observed that the shoot ($P < 0.05$, 6.51 g ± 0.88) and root dry weight ($P < 0.05$, 1.62 g ± 0.42) were significantly enhanced by the treatment TR1 compared to all other treatments (table 4.4). Further, treatment TR3 showed some effect on enhancement of shoot and root dry weight of potato compared to treatment TR2. The lowest mean shoot dry weight (3.11 g ± 1.06) was recorded by the treatment TR7 whereas the lowest mean root dry weight (0.86 g ± 0.38) was recorded by treatment TR5.

Table 4.4– The effect of CF and BFBFs combinations on tuberization of potato at Bandarawela field site.

| Treatment | Tuber weight Mean ± SD | Tuber number Mean ± SD | Stolon number Mean ± SD | Shoot weight Mean ± SD | Root Weight Mean ± SD |
|---|---|---|---|---|---|
| TR1 | $401.00 \pm 45.77^{a}$ | $6.96 \pm 1.93^{a}$ | $15.72 \pm 2.91^{b}$ | $6.51 \pm 0.88^{a}$ | $1.62 \pm 0.42^{a}$ |
| TR2 | $256.11 \pm 32.82^{b}$ | $5.08 \pm 1.26^{b}$ | $12.35 \pm 2.99^{c}$ | $4.29 \pm 1.14^{bc}$ | $1.06 \pm 0.31^{b}$ |
| TR3 | $428.90 \pm 48.40^{a}$ | $7.25 \pm 2.35^{a}$ | $20.10 \pm 6.27^{a}$ | $5.1 \pm 0.82^{b}$ | $1.08 \pm 0.27^{b}$ |
| TR4 | $249.60 \pm 46.60^{b}$ | $5.80 \pm 1.54^{ab}$ | $9.44 \pm 2.55^{cd}$ | $4.6 \pm 1.01^{b}$ | $0.99 \pm 0.30^{b}$ |
| TR5 | $145.31 \pm 45.80^{c}$ | $4.81 \pm 1.57^{b}$ | $7.69 \pm 3.22^{de}$ | $3.11 \pm 1.06^{d}$ | $0.86 \pm 0.38^{b}$ |
| TR6 | $214.55 \pm 31.56^{b}$ | $5.45 \pm 1.81^{ab}$ | $8.19 \pm 2.01^{de}$ | $3.35 \pm 1.32^{cd}$ | $0.99 \pm 0.30^{b}$ |
| TR7 | $113.34 \pm 50.60^{c}$ | $3.94 \pm 1.45^{b}$ | $5.62 \pm 1.15^{e}$ | $2.55 \pm 0.55^{d}$ | $0.92 \pm 0.43^{b}$ |

Treatments TR1, TR2, TR3, TR4, TR5, TR6 and TR7 are 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone No amendments treatments respectively. Same letter are not significantly different at 5% probability level.

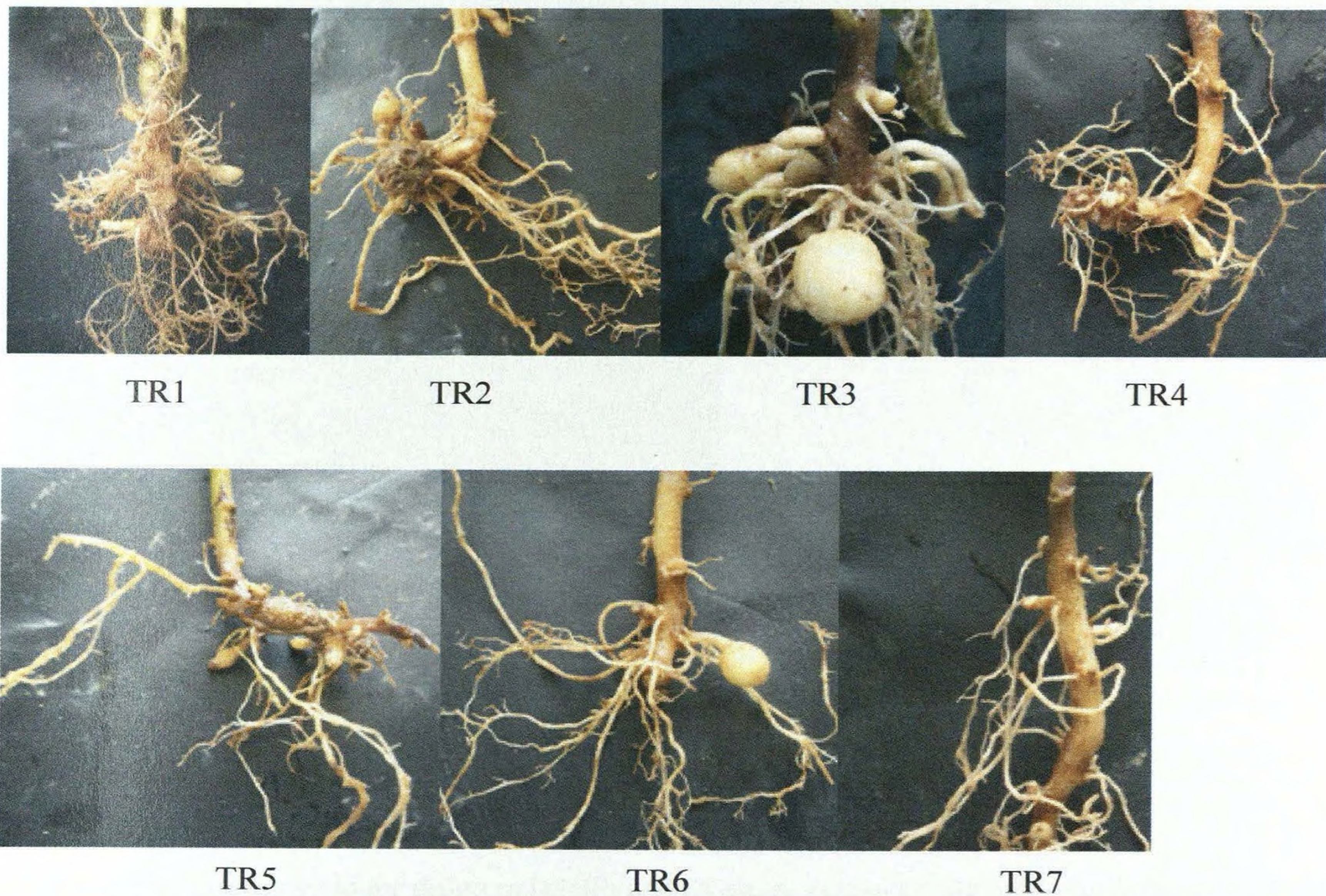



Plate 4.3- Development of stolon at different BFBFs and CF treatments at Bandarawela field site. One plant was selected randomly from each treatment for the photograph. TR1, TR2, TR3, TR4, TR5, TR6 and TR7 are the treatments of 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone No amendments treatments respectively.

It has been reported that shortage of N reduces the growth of vegetation and photosynthetic capacity necessary to support healthy roots and rapid tuber growth of potato. Previous studies have shown that increasing the concentration of CF up to an optimal level, can

increase the growth characteristics, such as; plant height, shoot dry matter, leaf expansion and stem branching capacity (Biemond and Vos, 1992; Sincik, Turan and Goksoy, 2008). Further, appropriate use of N fertilizer can lead to the achievement of optimum canopy development and subsequently increase crop yield (Kumar *et al.*, 2007; Najm *et al.*, 2013).

#### 4.4.2.2.b Plant analysis for field location- Bibile

It was observed that treatment TR3 greatly enhanced the tuber number and the size of the tubers. Further, treatments TR1 and TR3 enhanced the vegetative growth of potato compared to the other treatments (plate 4.4 a and b).

Plate 4.4- Potato tuber yield for different BFBFs and CF treatments at Bibile field site. (a) and (b)- Tuber yield with vegetative biomass per seed tuber (seed tubers for the figure (a and b) were selected randomly in each treatment). TR1, TR2, TR3, TR4, TR5, TR6 and TR7 are the treatment of 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone No amendments treatments respectively.

Out of all treatments, the highest significant tuber weight ($P < 0.05$, 98.23 g ± 12.25) was observed from the treatment TR3 (table 4.5). Interestingly treatment TR3 enhanced the tuber fresh weight by approximately 98% compared to treatment TR2 and approximately 42% even compared to treatment TR1. However, mean tuber weight of potato obtained at

Bibile field site was much lower than that of the potato tuber yield obtained at Bandarawela field site. Further, mean weight of tubers was reduced by approximately 11% by the application of treatment TR4 compared to treatment TR2. Therefore as in the Bandarawela site, it was clearly observed that there was no effect of biofilm combination BFBF2 on the enhancement tuber weight at Bibile field site. Further, the highest tuber number was recorded by the treatment TR3 (5.25 ± 1.12) compared to all other treatments at Bibile site. A T-test confirmed that the number of tubers for the treatment TR3 was significantly higher ($P < 0.05$) than that of the treatment TR2. All treatments enhanced the mean tuber weight and mean tuber number compared to the treatment TR7. Further, treatment TR3 enhanced the mean tuber number by approximately 86% compared to the treatment TR2 and approximately 26% even compared with treatment TR1. The highest mean stolon number (12.33 ± 2.26) was recorded by the treatment TR3 compared to all other treatments. Moreover, treatment TR3 enhanced the mean stolon number by approximately 56% compared to the treatment TR2 and approximately 23% even compared to the treatment TR1. All treatments enhanced the stolon number except treatment TR6 compared to TR7. Further, the highest mean shoot dry weight (4.45 g ± 0.57) and mean root dry weight (0.06 g ± 0.02) were observed from treatment TR1 compared to all other treatments. However, the vegetative growth of potato was considerably effected by the treatment TR3 compared to the other treatments except treatment TR1. Treatment TR5 (2.4 g ± 0.96) and TR6 (1.99 g ± 0.83) showed the lowest shoot dry weight even compared to treatment TR7 (2.44 g ± 0.92) which was the control.

Table 4.5– The effect of CF and BFBFs combinations on tuberization of potato at Bibile field site.

| Treatment | Tuber weight Mean ± SD | Tuber number Mean ± SD | Stolon number Mean ± SD | Shoot weight Mean ± SD | Root Weight Mean ± SD |
|---|---|---|---|---|---|
| TR1 | $69.38 \pm 8.98^{b}$ | $4.17 \pm 1.41^{ab}$ | $10.00 \pm 2.25^{ab}$ | $4.45 \pm 0.57^{a}$ | $0.70 \pm 0.06^{a}$ |
| TR2 | $49.62 \pm 6.82^{c}$ | $2.83 \pm 0.96^{bcd}$ | $7.92 \pm 2.22^{b}$ | $2.58 \pm 0.71^{b}$ | $0.43 \pm 0.07^{c}$ |
| TR3 | $98.23 \pm 12.25^{a}$ | $5.25 \pm 1.12^{a}$ | $12.33 \pm 2.26^{a}$ | $4.32 \pm 0.98^{a}$ | $0.62 \pm 0.04^{b}$ |
| TR4 | $44.37 \pm 11.96^{c}$ | $3.42 \pm 1.05^{bc}$ | $9.58 \pm 1.46^{b}$ | $2.77 \pm 0.84^{b}$ | $0.42 \pm 0.06^{c}$ |
| TR5 | $19.42 \pm 4.05^{d}$ | $2.08 \pm 0.72^{cd}$ | $4.83 \pm 1.92^{c}$ | $2.40 \pm 0.96^{b}$ | $0.31 \pm 0.04^{d}$ |
| TR6 | $23.96 \pm 5.62^{d}$ | $3.75 \pm 1.40^{b}$ | $3.37 \pm 1.40^{c}$ | $1.99 \pm 0.83^{b}$ | $0.36 \pm 0.05^{cd}$ |
| TR7 | $9.83 \pm 2.69^{e}$ | $1.92 \pm 0.85^{d}$ | $4.50 \pm 1.96^{c}$ | $2.44 \pm 0.92^{b}$ | $0.23 \pm 0.04^{de}$ |

Treatments TR1, TR2, TR3, TR4, TR5, TR6 and TR7 are 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone No amendments treatments respectively. Same letter are not significantly different at 5% probability level.

#### 4.4.2.2.c Plant analysis for field location- Padukka

As in the other field locations, it was observed that the treatment TR3 enhanced the tuber number and the size of the tubers compared to the other treatments at Padukka field site (plate 4.5). Vegetative growth of the plants was highly affected by the treatment TR1 and TR3. Size of the potato tuber were much smaller than that of the Bandarawela field site and Bibile field site.

Plate 4.5- Potato tuber yield for different BFBFs and CF treatments at Padukka field site. (a) and (b)- Tuber yield with vegetative biomass per seed tuber (seed tubers for the figure (a and b) were selected randomly in each treatment). TR1, TR2, TR3, TR4, TR5, TR6 and TR7 are the treatment of 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone No amendments treatments respectively.

Out of all treatments, the highest mean tuber weight (6.22 g ± 1.22), highest mean tuber number (2.33 ± 0.23) and highest mean stolon number (2.83 ± 0.37) were obtained from treatment TR3 (table 4.6). Treatment TR3 enhanced the tuber fresh weight by approximately 102% compared to treatment TR2 and approximately 33% even compared

to treatment TR1. However, mean tuber weight of potato obtained from Padukka field site was much lower than that of the potato tubers obtained from Bandarawela and Bibile field sites. Mean weight of tubers was reduced by approximately 52% by the application of treatment TR4 compared to treatment TR2. Moreover, mean tuber number was reduced by approximately 43% by the application of treatment TR4. Therefore as the other two sites, it was clearly observed that there was no effect of biofilm combination BFBF2 on the enhancement tuber weight at Padukka field site. Tuber development was not observed in treatment TR7. Treatment TR3 enhanced the mean stolon number by approximately 142% compared to treatment TR2 and approximately 22% even compared to treatment TR1. All treatments enhanced the stolon number except treatment TR6 compared to treatment TR7.

Out of all treatments, the highest mean shoot dry weight (3 g ± 0.62) and the highest significant root dry weight ($P < 0.05$, 0.86 g ± 0.10) were observed in the treatment TR3. However, shoot and root dry weights obtained from Padukka field site were much smaller than that of the dry weights obtained from Bandarawela and Bibile field sites.

Table 4.6– The effect of CF and BFBFs combinations on tuberization of potato at Padukka field site

| Treatment | Tuber weight Mean ± SD | Tuber number Mean ± SD | Stolon number Mean ± SD | Shoot weight Mean ± SD | Root Weight Mean ± SD |
|---|---|---|---|---|---|
| TR1 | $4.68 \pm 2.33^{ab}$ | $1.5 \pm 0.81^{ab}$ | $2.33 \pm 0.79^{ab}$ | $3.00 \pm 0.62^{a}$ | $0.86 \pm 0.10^{a}$ |
| TR2 | $3.08 \pm 0.34^{bc}$ | $1.17 \pm 0.66^{abc}$ | $1.17 \pm 0.84^{abc}$ | $1.81 \pm 0.72^{bc}$ | $0.54 \pm 0.08^{bc}$ |
| TR3 | $6.22 \pm 1.22^{a}$ | $2.33 \pm 0.23^{a}$ | $2.83 \pm 0.37^{a}$ | $2.74 \pm 0.41^{a}$ | $0.60 \pm 0.06^{b}$ |
| TR4 | $1.47 \pm 1.18^{cd}$ | $0.67 \pm 0.42^{bcd}$ | $2.17 \pm 0.31^{abc}$ | $2.51 \pm 0.22^{ab}$ | $0.49 \pm 0.09^{bcd}$ |
| TR5 | $0.60 \pm 0.47^{cd}$ | $0.33 \pm 0.20^{cd}$ | $0.83 \pm 0.60^{bc}$ | $1.95 \pm 0.51^{bc}$ | $0.38 \pm 0.04^{cde}$ |
| TR6 | $0.62 \pm 0.59^{cd}$ | $0.17 \pm 0.13^{cd}$ | $0.33 \pm 0.31^{c}$ | $1.94 \pm 0.26^{bc}$ | $0.33 \pm 0.04^{de}$ |
| TR7 | $0 \pm 0^{d}$ | $0 \pm 0^{d}$ | $0.50 \pm 0.34^{bc}$ | $1.52 \pm 0.64^{c}$ | $0.30 \pm 0.05^{e}$ |

Treatments TR1, TR2, TR3, TR4, TR5, TR6 and TR7 are 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone No amendments treatments respectively. Same letter are not significantly different at 5% probability level.

Greenhouse experiment and different field experiments confirmed that the biofilm combination BFBF1 was positively responded to all growth parameters of potato whereas BFBF2 combination failed to respond specially in field conditions. It was clearly observed that the tuber responses of potato at Bandarawela field site for the BFBF1 combination coupling with 50% CF was considerably higher than that of the other field sites. However, it was clear that the treatment TR3 (BFBF1+50% CF) enhanced the tuberization in all three

field sites whereas the full DOA recommended CF (100% CF) enhanced the vegetative growth of the potato crop. According to the previous DOA data, the average potato tuber yield obtained at Bandarawela cultivation area is approximately 15-18 MT/ha if the farmers follow the standard recommended fertilizers rates (Urea- 330 kg/ha + TSP- 270 kg/ha + MOP- 250 kg/ha). It was recorded that in some favorable seasonal periods the yield can be enhanced up to 22 MT/ha. However, our study showed that the maximum estimated tuber yield obtained for the 100% DOA recommended fertilizer rate was approximately 17 MT/ha. It is noteworthy that the result recorded at Bandarawela field site, indicated that the estimated tuber yield could be maximized up to 21 MT/ha only by using 50% of the DOA recommended CF with developed BFBF. These findings imply that the input of CF can be reduced by 50% with the use of developed BFBFs during the vegetative growth phase and possibly for the entire crop of potato, which could be a huge economic gain in terms of fertilizer saving.

Similar findings were recorded from Bibile and Padukka field locations in terms of potato crop responses for developed BFBFs. According to the previous climatic data, initially it was assumed that the Bibile field location has potential climatic conditions for potato cultivation during Maha season. According to the current study, the overall tuber yield for the 100% DOA recommended CF was much lower in Bibile field site (1.64 MT/ha) compared to Bandarawela location (17 MT/ha). It is noteworthy that the average potato tuber yield could be enhanced up to 2.67 MT/ha by using only 50% recommended CF with BFBF1 combination compared to 100% DOA recommended CF treatment at Bibile field location. However, the maximum tuber yield obtained from Bibile site is not enough to establish a profitable cultivation. This implies that even with the developed BFBFs it is impossible to establish a commercial potato cultivation at Bibile area. The unfavorable climatic factors at Bibile for potato tuber development could be the reason for the yield reduction. Further, tuber size was much smaller than that of Bandarawela.

As expected, our results further confirmed that the potato tuber yield cannot be enhanced up to a commercial level at Colombo area since the atmospheric conditions are highly unfavorable for potato tuberization. The tuber yield and the size of the tubers (resemble to micro tubers) were significantly lower than that of the other two field sites. However, another most interesting finding was that tuberization could be initiated by the application of BFBF1 with 50% CF compared with full DOA recommended CF. Therefore, it was

clearly observed that there was an inducing effect of the developed BFBFs on potato tuberization at Padukka field site though the climatic conditions are highly unfavorable. However, this implies that irrespective of the atmospheric conditions BFBFs has the potential of enhancing the tuberization of potato in all three field sites.

## 4.5 Conclusions

It was confirmed that the treatment TR3 (50% CF + BFBF1) responded positively to initiate tuberization, irrespective of the climatic conditions. According to the greenhouse experiment and different field experiments, treatment TR3 was considered as the best treatment for enhancing the crop productivity of potato whereas all BFBF2 combinations failed to respond specially under field conditions. It was clearly observed that the tuber responses of potato at Bandarawela field site for the treatment TR3 was considerably higher than that of the other field sites. Thus, it can be concluded that the responses of this treatment should be further evaluated and the mechanism of enhancing tuberization should be further studied.

# CHAPTER 5

# EVALUATION OF THE EFFECTS OF RESPONSIVE BIOFILM ON POTATO TUBERIZATION

## 5.1 Introduction

Potato tuberization is a complex process representing a transition of shoot to tuber involving several biochemical and molecular changes under complex external and internal physiological, environmental and nutritional regulation (Nookaraju *et al.,* 2012). Potato tuberization process can be divided in to four major phases (Fig. 5.1a). At the vegetative growth phase, plant absorbs nutrients from soil and enhances its vegetative growth. Photosynthesis provides the energy for the developing plant and rapidly expanding roots are active at this stage to acquire moisture and nutrients. Tuber initiation and the bulking phases are considered as the most critical phases of the tuberization process (Mikkelsen and Hopkins, 2009).

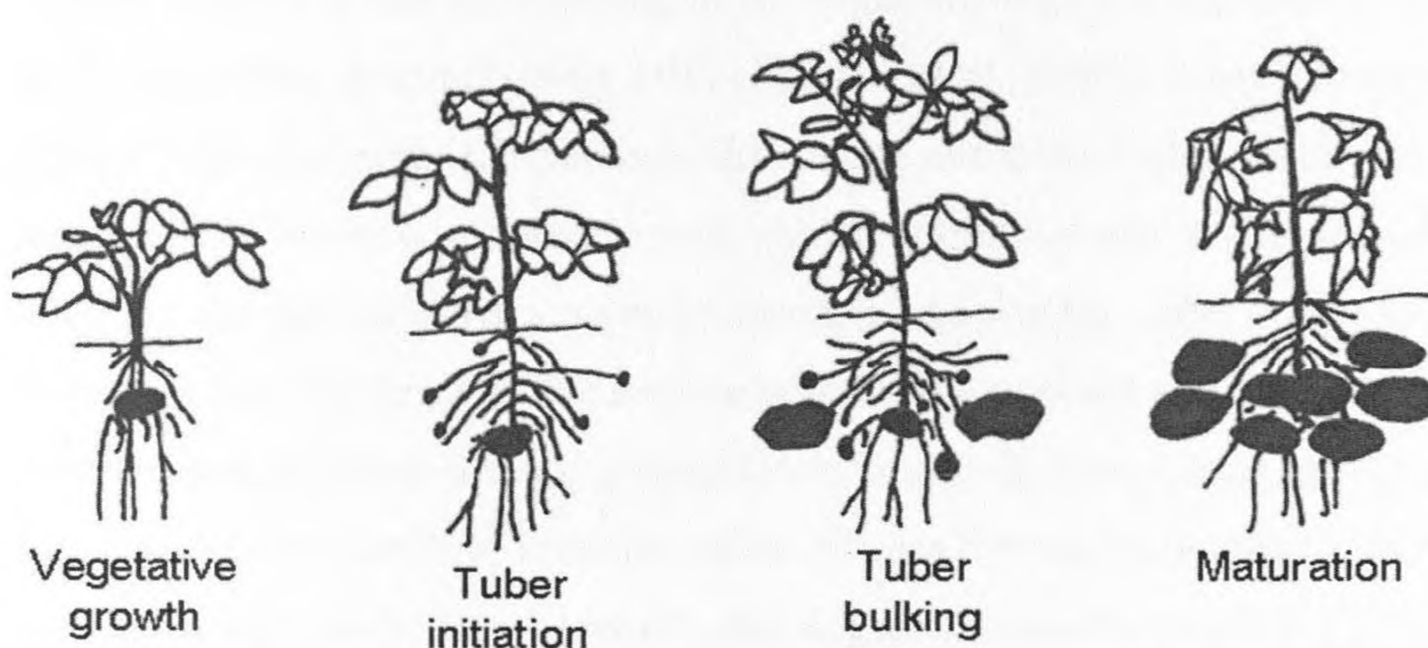



Figure 5.1a- Major stages of growth and development of potatoes (Source: Mikkelsen and Hopkins, 2009).

Tuber initiation occurs by the inhibition of the longitudinal growth of the stolon and is characterized by radical growth of the subapical region of the stolon tip (Appendoorl *et al.,* 1999). Since the developing tubers ultimately become the largest sinks in the potato plant, the transformation of stolons into tubers impacts greatly on the physiology of the entire plant

(Oparka, 1985). An adequate supply of nutrients is important during this stage to get abundant tuber initiation. At the bulking stage, tubers occur very rapidly as they become the major sink for carbohydrates and nutrients (Mikkelsen and Hopkins, 2009). The potato tuber functions as a massive storage reserve for a range of macromolecules. The accumulation of starch and storage proteins, quantity and composition changes of reducing sugars and changes in sucrose at the tuberizing stolons are the major biochemical changes associated with this process (Visser *et al.*, 1994; Minhas, Rai and Saini, 2004).These biochemical alterations at the tuber initiation and bulking phase leading to the tuberization, are regulated by different climatic, physiological and biochemical stimuli and provides signals to the plant to channel excess carbohydrate from vegetative and root growth to begin setting tubers (Mikkelsen and Hopkins, 2009).

Day/night temperature fluctuation (thermo period) and photoperiod are some of the key climatic factors in regulating growth and yield in potatoes (Ewing, 1981; Tibbitts *et al.*, 1989). Lower day/night temperature difference with high night temperatures inhibit tuber initiation whereas higher day night temperature difference with low night temperatures induce both initiation and bulking of the organ through the regulation of starch accumulation at the tips of the stolon (Ewing, 1981; Minhas *et al.*, 2004). It has been observed that, induced plants (high day/night temperature difference and cold nights) accumulate three times more starch in the leaves compared to non-induced potato plants during the day and then transport it out of the leaves during night (Lorenzen and Ewing, 1992). In conditions that are non-inductive for tuberization, the stolons often grow upward and emerge out of the soil to form a new shoot. In tuber-inducing conditions, however, the stolons grow underground until the tip of the stolon swells to form the tuber. Stolon formation occurs in both tuber-inducing and non-inducing conditions; however, the degree of swelling stolon tip has been significantly correlated with the strength of the inductive signal or stimuli (Van den Berg *et al.,* 1996).

It has been found that the external and internal cell signaling ions and molecules with different functional groups alter the biochemical and physiological status of plants directly (Banerjee *et al.*, 2006). Like other biochemical and physiological changes, phloem translocation and the starch/sugar movement through the plant body are induced by different cell signaling molecules (Harmey *et al.*, 1966). According to previous studies, it has been confirmed that potato tuberization is sensitive to different external factors which act as exogenous inductive signals (Okazawa, 1967; Minhas *et al.,* 2004) such as temperature

(Ewing and Struik 1992), light, photoperiod (Jackson, 1999), Calcium ($Ca^{2+}$) level in soil (Balamani, Veluthambi and Poovaiah, 1986) plant growth regulators like IAA and N availability (Krauss, 1978). The tuberization might be influenced either by one or several combinations of such external inductive signals via the alternation of internal biochemical parameters. Therefore, the current study was focused and proposed a possible mechanism of signaling molecules from BFBFs (as illustrated in figure 5.1b) to induce tuberization through the alteration of internal biochemical and physiological status.

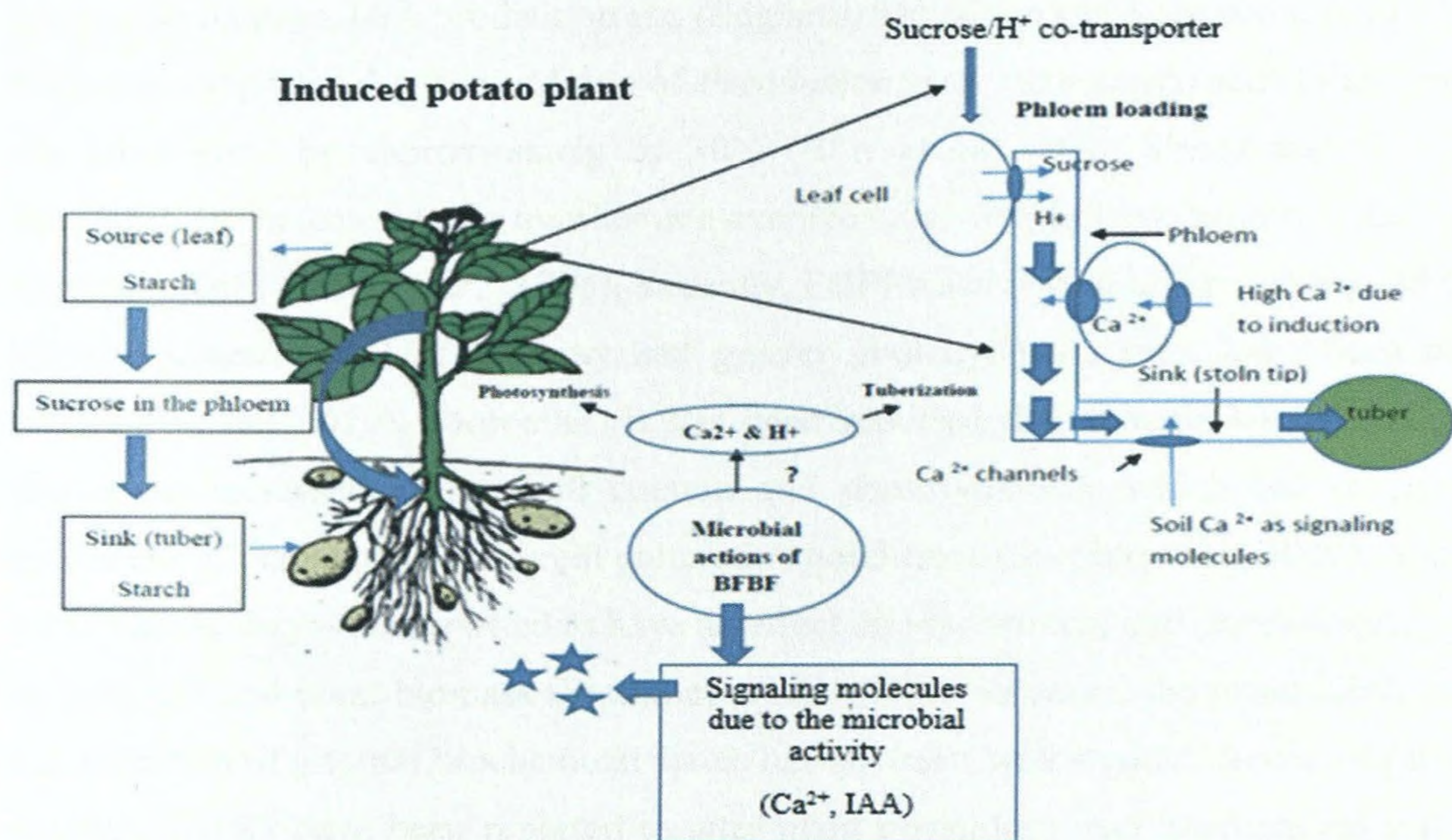



Figure 5.1b- Schematic diagram representing biochemical pathway leading to tuberization of induced plants through signaling molecules

As illustrated in figure 5.1b, soil $Ca^{2+}$ nutrition around the rhizosphere through application of fertilizers plays an important role in potato tuberization. Recent studies have provided evidences for the role of soil $Ca^{2+}$ near the rhizosphere in potato tuberization by improving the tuber number and tuber yield (Nookaraju *et al.*, 2012). Studies further suggested that elevated soil $Ca^{2+}$ facilitates tuberization by altering the internal hormonal and biochemical (starch, sucrose and total sugar) balance (Ozgen *et al.*, 2006). For instance, an external stimuli causes the rapid increment of cytosolic $Ca^{2+}$concentration (Nookaraju *et al.*, 2012) through the massive influx of $Ca^{2+}$ from $Ca^{2+}$ channels (Fig. 5.1b) enhances the tissue sucrose level which ultimately serves as an internal signal for tuberization (Perl *et al.*, 1991; Gibson, 2005). Further, it has been reported that the enhancement of cytosolic sucrose

content is also stimulated by the elevated extracellular proton concentration. Therefore, sucrose concentration increases along with the massive influx of $H^+$ (Boorer *et al.*, 1996) through sucrose/$H^+$ co-transporter protein (Fig. 5.1b).

In addition to these factors, some strains of beneficial rhizobacteria were reported to influence tuber number and tuber yield in potato (Vrany and Fiker 1984; Sturz, 1995; Oswald *et al.*, 2010). The addition of beneficial soil microorganisms are proven to improve fertility and quality of tuber, which is a larger size of potato tuber through their beneficial activities such as $N_2$ fixation, IAA production etc. (Sugiarto, Sulistiono and Soemarno, 2013). Further, it has been reported that the addition of *Pseudomonas* sp. with potato seed tubers enhanced the tuber yield by approximately by 30% (Burr *et al.*, 1978; Vrany and Fiker 1984). Similarly, an increased tuber number and average tuber weight have been reported with the application of PGPRs (Sturz, 1995). Recently, PGPRs activity on higher tuber yield through the enhancement of leaf anatomy and greater photosynthetic rates have been recorded (Oswald *et al.*, 2010). Moreover, it has been reported that inoculation of *Azospirillum brasilense* increased chlorophyll content and photosynthesis, which led to dry matter increment of plants under different cultivation conditions (Swedrzynska, 2000). Apart from tuberization, they were reported to have an effect on biochemical and physiological changes in both soil and plant biomass (Frommel *et al.*, 1991). However, the exact mechanism for the alteration of internal biochemical status has not been well studied. According to several studies, PGPRs have been reported to alter plant physiology and biochemical status by a variety of mechanisms such as fixation of atomospheric $N_2$ that is transferred to the plant (Kennedy *et al.*, 2004), production of siderophore that chelate iron and make it available to the plant root, solubilization of minerals such as P (Banerjee and Yasmin, 2002), Zinc (Zn) (Iqbal *et al.*, 2010), Potassium (K), $Ca^{2+}$ (Han and Lee, 2006) and synthesis of phytohormones such as IAA (Patten and Glick, 2002; Kumar and Dubey 2012).

It has been estimated that up to 80% of the rhizosphere bacteria can synthesize IAA (Khalid *et al.*, 2004; Patten and Glick, 1996). Bacteria which produce IAA can act as a signaling molecule that influences the levels of endogenous biochemical status (Patten and Glick, 1996). It is assumed that plant growth promotion by exogenously applied auxin like IAA acts by increasing root growth, length and surface area, thereby allowing the plant to access more nutrients and water from the soil (Vessey, 2003; Malfanova, 2013). It has been reported that the application of IAA led to earlier tuber initiation and tuber enlargement (Harmey *et*

*al.*, 1966; Sasamoto and Suzuki, 1979). Further the inductive role of IAA on stolon initiation and tuber induction in potato has been documented in earlier studies (Dragicevic *et al.*, 2008). A similar influence of *Bacillus* strains in promoting potato tuberization under *in vitro* and *in vivo* conditions has been observed (Nookaraju, 2011).

Soil fertility is another major factor that affects the yield and quality of the potato. Several studies describe relationships between fertilizer applications, leaf nutrient concentrations, potato (*Solanum tuberosum* L.) tuber yield and tuber quality (Rykbost, Christensen and Maxwell, 1993; Adikari and sharma, 2004). Few bacteria are also able to release significant amounts of useful minerals to the soil, such as P, K, Mg, Mn, Fe, Cu, and Zn and thereby modify plant growth through the enhancement of soil nutrients (Puente *et al.*, 2004a). Another study reported an enhancement of N,P,K level in soil after the inoculation of PGPRs with the combination of CF (Rodriguez and Fraga 1999). A relatively higher uptake of NPK can be predicted in the treatment of externally applied NPK with the co-inoculation of P solubilizing bacteria (Duarah *et al.*, 2011).

However, direct application of fungal-bacterial communities like BFBFs to the soil has been introduced recently, and observed to be multi-functional and more effective than their monocultures. Beneficial microorganisms including biofilm communities have been recorded higher beneficial activities like $N_2$ fixation, IAA production, soil nutrient enhancement and plant growth promotion (Bandara *et al.*, 2006 Seneviratne *et al.*, 2008; Buddhika *et al.*, 2014). Further, it has been confirmed that the inoculation of biofilms can produce and secrete diverse organic compounds and plant growth regulators like IAA (Herath *et al.*, 2013), some of which may be cell signaling molecules that may help to change the physiology of plants (Fig. 5.1b). Further, according to previous studies, the application of fungal-bacterial BFBFs under greenhouse conditions significantly enhanced the tuber fresh weight and the number of tubers (Henagamage *et al.*, 2014). Interestingly, evidence was found for the enhancement of potato tuberization by the BFBFs inoculation along with 50% CF application even under non- induced climatic factors. This can be explained by the alteration of internal biochemical status through the induction activities of either one or several combinations of exogenous signaling molecules by the inoculation of BFBFs.

## 5.2 Objective

- To evaluate the influence of most effective biofilm on tuberization through biochemical, physiological and physicochemical processes of soil-plant systems.

## 5.3 Methodology

### 5.3.1 Soil quality enhancement by the responsive biofilm

The effect of different CF and BFBF treatments on soil nutrient enhancement (soil pH, Total SOC, MBC, Total N content, Available P content, soil K content and Soil $Ca^{2+}$ content) was evaluated using the field data obtained from Bandarawela, Bibile and Padukka field sites (4.3.1.5). After harvesting the potato biomass, soil samples from each treatment plots were collected into black polythene bags separately and were transported to the laboratory at Uva Wellassa University, Badulla to conduct the soil quality analysis.

Soil pH, SOC content and Total soil N were measured according to the procedure as explained in 4.3.1.5.a 4.3.1.5.b and 4.3.1.5.c respectively (Anderson and Ingram, 1998).

#### 5.3.1.1 Soil available N content

Approximately 10 g of fresh soil sample was weighed into an extracting bottles. Subsequently 20 ml of Potassium sulfate solution was added. Bottles were shaken for 30 minutes and filtered through No. 1 Whatman filter paper. Filtrate was used to analyze the available $NO_3^-$ in soil sample. Then 0.5 mL aliquot of soil extract was transferred to the test tube and 1mL of Salisylic acid solution was added and mixed well immediately with vortex mixture (Stuart SA8, United Kingdom) and left for 30 minutes. After 30 minutes 10 mL of Sodium hydroxide solution was added and mixed well. The test tubes were left for colour development for one hour. Finally absorbance of standards and samples were determined at 410 nm using UV visible spectrophotometer (Anderson and Ingram, 1998).

### 5.3.1.2 Soil MBC content

Soil samples from each treatment were sieved to remove the stones and coarse particles. Approximately 10 g of three soil sub sample was measured and two of them were placed in 50 ml beakers. The remaining sample (un-fumigated sample) was placed in a 125 ml water-tight blue cap bottle. Subsequently, 50 ml of 0.5 M $K_2SO_4$ was added to the un-fumigated soil sample followed by vigorous shaking for 30 min after tighten the cap. The filtrate was obtained after filtering the extract through No. 42 Whatman filter paper. Dissolved SOC ($C_1$) in the filtrate was analyzed as explained in 4.3.1.5.b Fumigation was performed for one of the soil samples placed in 50 ml beaker as follows. The beaker with the soil sample was placed in a vacuum desiccator containing 30 ml of alcohol free chloroform in a shallow dish. Vacuum was applied until the chloroform was clearly evaporated. Subsequently, the desiccator was stored in the dark for 5 days at 25 °C followed by transferring the fumigated soil sample into a water tight 125 ml extraction bottle. The extraction was performed as explained above and the dissolved SOC content ($C_2$) was determined as explained in 4.3.1.5.b Dry mass of the soil was determined using the remaining soil sample which was placed in 50 ml beaker according to the oven drying method. Soil MBC was determined using the following equation.

Percentage of MBC = $C_2$-$C_1$ (Vance, Brookes, Jenkinson, 1987: Anderson and Ingram, 1998)

### 5.3.1.3 Available P content

Available P content was measured according to Olsen *et al.,* (1954) method. Approximately 2.5 g of soil (< 2mm size) sample was weighted into a conical flask and added 0.2 g of P free-C into it. Subsequently, 50 ml of 0.5 M $NaHCO_3$ (extracting solution, pH = 8.5) was added and mixed thoroughly for 30 min using a shaker. The solution was then kept standing for a few minutes until the supernatant became clear. The supernatant was filtered through Whatmann No. 5 filter paper and 5 ml of the filtered extract was added to another 50 ml conical flask. Subsequently, 5 ml of colour developing reagent (Solution 'A'- 12 g of $NH_4MoO_4$ in 250 ml distilled water. Solution 'B'- 0.29 g of antimony potassium tartrate in 1L of 5 N $H_2SO_4$. Solution 'A' and 'B' was mixed together and made up to 2 L (solution 'C'). Then, 0.739 g of ascorbic acid was mixed with 140 ml of solution 'C') was added to the extract and mixed gently for few minutes. Approximately 15 ml of distilled water was added into the solution and kept for 15 minutes for the colour development. Colour intensity

was measured using UV spectrophotometer (Spectronic 21, Milton Roy Company at Regional Agriculture Research and Development Center, Bandarawela) at 880 nm with $KH_2PO_4$ solution as a blank. Phosphorous content in the soil sample was calculated as follows.

$$P \text{ in soil (ppm)} = \frac{A \times 25 \times 50}{5 \times 2.5} \quad (A = \text{calibration plot reading})$$

#### 5.3.1.4 Soil available K content

Approximately 5 g of soil (< 2mm size) was weighed into a 100 ml conical flask and added 50 ml of extracting solution (19.27 g of ammonium acetate in 250 ml distilled water (pH=7). pH was adjusted using acetic acid or $NH_4OH$) followed by vigorous shaking for 1 hour. Subsequently, the extraction was filtered in to a flask through a Whatman No.1 filter paper. The extraction was subjected to the flame photometer (Jenway, Model PFP7 at Regional Agriculture Research and Development Center, Bandarawela) to detect the K content (Laboratory handbook on methods of soil analysis, DOA, Sri Lanka) in the soil sample (calibration plot was drawn using standard KCl solution. % transmittance- Y axis, K concentration- X axis).

#### 5.3.1.5 Soil available $Ca^{2+}$ content

Soil available $Ca^{2+}$ content was measured according to Plank (1992) method. Approximately 1 g of dried (80 °C) soil sample (particle size < 1.0 mm) was placed in a muffle furnace and was heated to 500 °C for 4 hours to convert the sample to ash. After cooling, the ash sample was moisten using Conc. $HNO_3$ and repeated the heating step again for 4 hours. Subsequently the resulting product was wetted with a small amount of deionized water and brought into solution using 2 ml Conc. HCl. Dilution was performed using deionized water up to 100 ml final volume. An aliquot of the solution was diluted (10-fold) again with Lanthanum standard by mixing 1 ml of sample with 9 ml of 1000 mg/ L lanthanum chloride. $Ca^{2+}$ concentration of the lanthanum treated solution was determined using AAS (Model No. Varian AA 240) with $CaCO_3$ standard.

#### 5.3.1.6 Microbial diversity

After harvesting, soil samples collected from Bandarawela field site were analyzed to determine the effect of different treatments on microbial diversity. Isolation of microbial strains from soil samples treated with different CF and BFBFs combinations were performed as explain in 2.3.1.3 and the identification of the strains was performed based on morphological and biochemical experiments as explain in 2.3.2. Further, CFU based on different treatments were counted using a colony counter.

### 5.3.2 The effect of different CF and BFBFs treatments on biochemical parameters of potato plant biomass

The effect of different CF and BFBFs treatments on potato plant biochemical properties (Chlorophyll content of leaves, $H^+$ content of different plant tissues, starch content of different plant tissues, sucrose content of different plant tissues, reducing sugar content of plant tissues, tuber nutrient content and tissue $Ca^{2+}$ content) was evaluated using the field data obtained from Bandarawela, Bibile and Padukka field sites. After harvesting, potato plant biomass from each treatment plots were collected in to black polythene bags separately and were transported to the laboratory at Uva Wellassa University, Badulla to conduct further analysis as follows.

#### 5.3.2.1 Photosynthetic efficiency using chlorophyll content of leaves

The effect of different CF and BFBFs treatments on photosynthetic efficiency was determined using the chlorophyll content of leaves. A digital chlorophyll meter was used to measure the chlorophyll content of the leaves in potato plants treated with different fertilizer combinations. The chlorophyll contents of $3^{rd}$ and $5^{th}$ fully expanded leaves from the apical bud were measured after six weeks from seed sowing. Three readings were obtained randomly for each leaf. Chlorophyll content of the leaf tissues of potato plants from different treatments were compared on treatment basis to find the effect of treatments on the efficiency of the photosynthesis.

### 5.3.2.2 The effect on biochemical parameters of plant tissues

The effect of CF and BFBFs treatments on biochemical properties of different tissues (leaf, areal shoot, stolon and tubers) of potato plant was evaluated using different colorimetric analysis. The analysis was performed for plant tissues obtained from all three field sites.

#### 5.3.2.2.a $H^+$ content of different plant tissues

$H^+$ content of different plant tissues were measured based on the pH according to the procedure as explained in 4.3.1.5a (Anderson and Ingram, 1998).

#### 5.3.2.2.b $Ca^{2+}$ content of different plant tissues

Ca2+ of different tissues of the potato plants treated with different CF and BFBF combinations were extracted using the method explained in 5.3.1.5.The concentration of the $Ca^{2+}$ were measured using AAS with $CaCO_3$ standard.

#### 5.3.2.2.c Reducing sugar content

Reducing sugar content was measured according to the method explained by Somogyi, (1952) and Minhas *et al.*, (2004). Approximately 0.1 g of dried tissue sample (ground) was weighed in a 10 ml centrifuge tube, and 5 ml of 80 % ethanol was added. The sample was heated in an 80 °C water bath for 30 min and centrifuged (3000 r/min) for 5 min. The supernatant was collected and the extraction was repeated twice (3000 r/min for 10 min each). Aliquots of 0.2 ml of the supernatant were pipetted into separate test tube and were made up to 2 ml with distilled water. Working standard solution series (0.01 mg/ml- 0.05 mg/ml) was prepared using standard glucose solution. 1 ml of alkaline copper tartrate reagent (Solution A- 2.5g anhydrous $Na_2CO_3$ + 2 g $NaHCO_3$+ 2.5 g potassium sodium tartrate + 20g anhydrous $Na_2SO_4$ and diluted up to 100 ml with water. Solution B- 15 g $CuSO_4$ + one drop of $H_2SO_4$ and diluted up to 100 ml with water. Solution A was mixed with solution B according to 24:1 ratio before use) was added to both working standard solutions and the extracted solution separately and kept for 10 min under boiling water for colour development. After cooling, 1 ml of arsenomolybolic acid reagent (2.5 g $NH_4MoO_4$, 2.5 ml $H_2SO_4$, 0.3 g $Na_2AsO_4$) was added to all the tubes and diluted up to 10 ml with water.

Absorbance values were measured using UV visible spectrophotometer (Model No. Genetics 6) at 620 nm with distilled water as a blank. Amount of reducing sugars in the sample was calculated from the graph drawn.

#### 5.3.2.2.d Starch content

Starch content was measured according to the method explained in Hodge and Hofreiter, (1962) and Luo & Huang, (2011). Approximately 0.5 g of dried tissue sample (ground) was weighed in a 10 ml centrifuge tube, and 10 ml of 80 % ethanol was added. The sample was heated in an 80 °C water bath for 30 min and centrifuged (3000 r/min) for 5 min. The residue was collected and washed repeatedly with hot 80 % ethanol till the washings were not given colour with anthrone reagent. The dried residue was extracted with a solution containing 5 ml of water and 6.5 ml of 52 % percloric acid for 20 min at 0 °C. The mixture was centrifuged (3000 r/min) and the supernatant was collected. Extraction procedure was repeated and was pooled the supernatant followed by dilution up to 100 ml with water. Aliquots of 0.2 ml of the diluted supernatant was pipetted in to separate test tube and was made up to 1 ml with distilled water. Working standard solution series was prepared using standard glucose solution. 4 ml of anthrone reagent was added to both working standard solutions and the extracted solution separately and heated for eight minutes under the boiling water bath for the colour development. Absorbance was measured using UV visible spectrophotometer at 630 nm with distilled water as a blank. Results were extrapolated from a glucose standard curve; amount of starch was calculated by multiplying the equivalent by 0.90 and was expressed as mg of starch $g^{-1}$ fresh weight.

#### 5.3.2.2.e Sucrose content

Sucrose content was measured according to Luo & Huang, (2011) method. 0.1 g of dried tissue sample was weighed in a 10 ml centrifuge tube, and 6-7 ml of 80% ethanol was added. The sample was heated in 80 °C water bath for 30 min and centrifuged (3000 r/min) for 5 min. The supernatant was collected and the extraction was repeated twice (3000 r/min for 10 min each). Supernatant was collected into a flask and 80 % ethanol was added to a final volume of 50 ml. Subsequently 1 ml of the extracted solution was added to a test tube and heated in a boiling water bath until it condensed to 0.05-0.1 ml (not greater than 0.1 mL to avoid longer color developing time). Next, 0.1 ml of 30% KOH was added and the solution

was incubated in boiling water for 10 min. After the solution cooled down to room temperature, 3 ml of anthrone reagent was added, and the solution was incubated at 40 °C for 10-15 min. Absorbance was measured using UV visible spectrophotometer at 630 nm with distilled water as a blank. Sucrose content was calculated from the graph drawn.

#### 5.3.2.3 The effect of different treatments on tuber nutrient content

The effect of CF and BFBFs treatments on nutrient enhancement (starch content, crude protein content, crude fiber content and crude fat content) was evaluated using potato tubers harvested from all three field sites as follows.

#### 5.3.2.3.a Starch content

Analysis of starch content was measured as explained in 5.3.2.2.d

#### 5.3.2.3.b Crude protein content

Crude protein content of tuber tissues was determined according to Gul and Safdar (2009) and Naz *et al.* (2011) methods as follows. Approximately 2 g of powdered tissue sample was obtained and placed in digestion flask. Subsequently, 15 g of $Na_2SO_4$, 1g of $CuSO_4$, 1 g of selenized boiling granules and 25 ml of Conc. $H_2SO_4$ were added to the flask and the mixture was digested for about 2 hours until solution was almost colorless (blue green in color). The digest was cooled and transferred to 100 ml volumetric flask and volume was made up to mark by the addition of distilled water. 10 ml of digest was introduced in the distillation tube then 10 ml of 0.5 N NaOH was gradually added through the same way. Distillation was continued for at least 10 min and $NH_3$ produced was collected as $NH_4OH$ in a conical flask containing 20 ml of 4% boric acid solution with few drops of methyl red indicator. During distillation yellowish color appeared due to $NH_4OH$. The distillate was then titrated against standard 0.1 N HCl solution till the appearance of pink color. A blank was also run through all steps as above. Percent crude protein content of the sample was calculated by using the following formula:

Percentage Crude Protein = 6.25* x %N (* Correction factor)

$$\text{Percentage of N} = \frac{(S-B) \times N \times 0.014 \times D}{\text{Weight of the sample (g)} \times V} \times 100$$

Where

S = Sample titration reading

B = Blank titration reading

N = Normality of HCl

D = Dilution of sample after digestion

V = Volume taken for distillation

0.014 = Milli equivalent weight of N

**5.3.2.3.c Crude fat content**

Crude fat content was determined according to the methods explained in Gul and Safdar, (2009) and Hassan and Umar (2006). Approximately 2 g (W1) of dried (moisture free by heating at 100 °C for 24 hours) powdered tissue sample was weighed and placed into the extraction thimble of soxhelt apparatus (weight of the extraction flask was obtained previously- W2). The sample was extracted with petroleum ether using the soxhelt apparatus for 8 hours. After the extraction, the extraction flask was kept in the oven and heated at 100 °C to remove the trace of solvent remaining in the flask. Subsequently, the flask with the extraction was weighed to a constant weight (W3). Crude fat content was calculated as follows. The fat free sample in the extraction thimble was kept for crude fiber determination.

$$\text{Percentage of crude fat} = \frac{W3-W2}{W1} \times 100$$

**5.3.2.3.d Crude fiber content**

Crude fiber content was determined according to the methods explained in Naz *et al.* (2011) and Gul and Safdar, (2009). The dry weight of the fat free sample in the extraction thimble (remaining fat free sample after the extraction in 5.3.2.3.c) was weighed (W1) and transferred in to a fresh beaker. Subsequently, 200 ml of 1.25 % $H_2SO_4$ was added to the beaker with the sample and digested it by boiling for 30 min on reflux apparatus. After digestion, the contents in the beaker was filtered through a clean muslin cloth using a suction

pump. The residue was washed several times with hot water until it free from acid and transferred the washed solid residue into another clean beaker. The solid sample was digested again with 200 ml of 1.25% NaOH for 30 min using the reflux apparatus. After the second digestion with NaOH, the residue was filtered again and the washing was repeated with hot water. Subsequently, the residue was placed on a pre-weighed crucible and placed it at 100 °C in hot air oven until obtaining a constant weight (W2). The dried sample was then placed in a muffle furnance and ignited at 660 °C for two hours followed by obtaining the final weight of the sample (W3). Fiber content of the residue was calculated as follows.

$$\text{Percentage of the crude fiber} = \frac{W2 - W3}{W1} \times 100$$

W1- weight of the sample

W2- weight of the crucible+ residue left after digestion with acid and alkali solutions

W3- weight of the crucible + ash

W2-W3- weight of the crude fiber

### 5.3.3 Statistical analysis

Normality of the data and constancy of residuals were confirmed. Statistical data analyses were performed on all data collected using the one way Analysis of Variance (ANOVA) Model in MINITAB 16 Statistical Software. The mean values of each soil nutrient parameters were compared on treatment basis using the tukey's simultaneous test at 5% significance level. Correlations between different soil biochemical properties and potato tissue biochemical properties were constructed by using correlation analysis.

### 5.4 Results and Discussion

### 5.4.1 Soil properties

#### 5.4.1.1 Soil pH

In comparison with all other treatments, treatment TR3 significantly reduced ($P < 0.05$) the mean pH ($4.61 \pm 0.02$) of soil at Bandarawela field site (Fig. 5.2). Further, treatment TR3 showed the lowest mean soil pH compared to all other treatments at Bibile ($5.43 \pm 0.058$) and Padukka ($5.28 \pm 0.1$) field sites. The soil pH recorded from all three field sites in the current study were within the range of average pH (Bandarawela- 4.5-5.5, Bibile- 5.5- 6.5 and Padukka-5-6) which have been reported from other studies (Kodikara, Wijesundara and Yatagama, 2008).

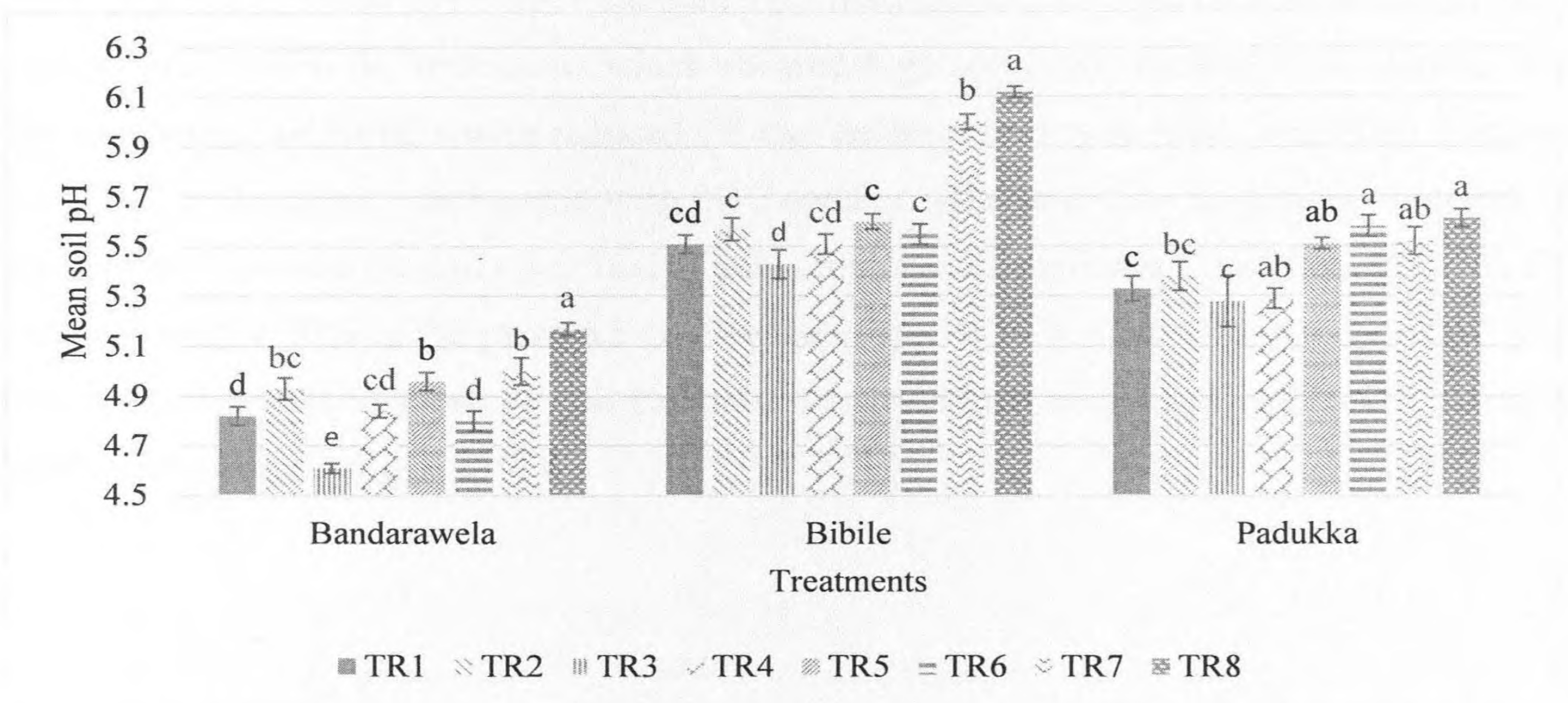
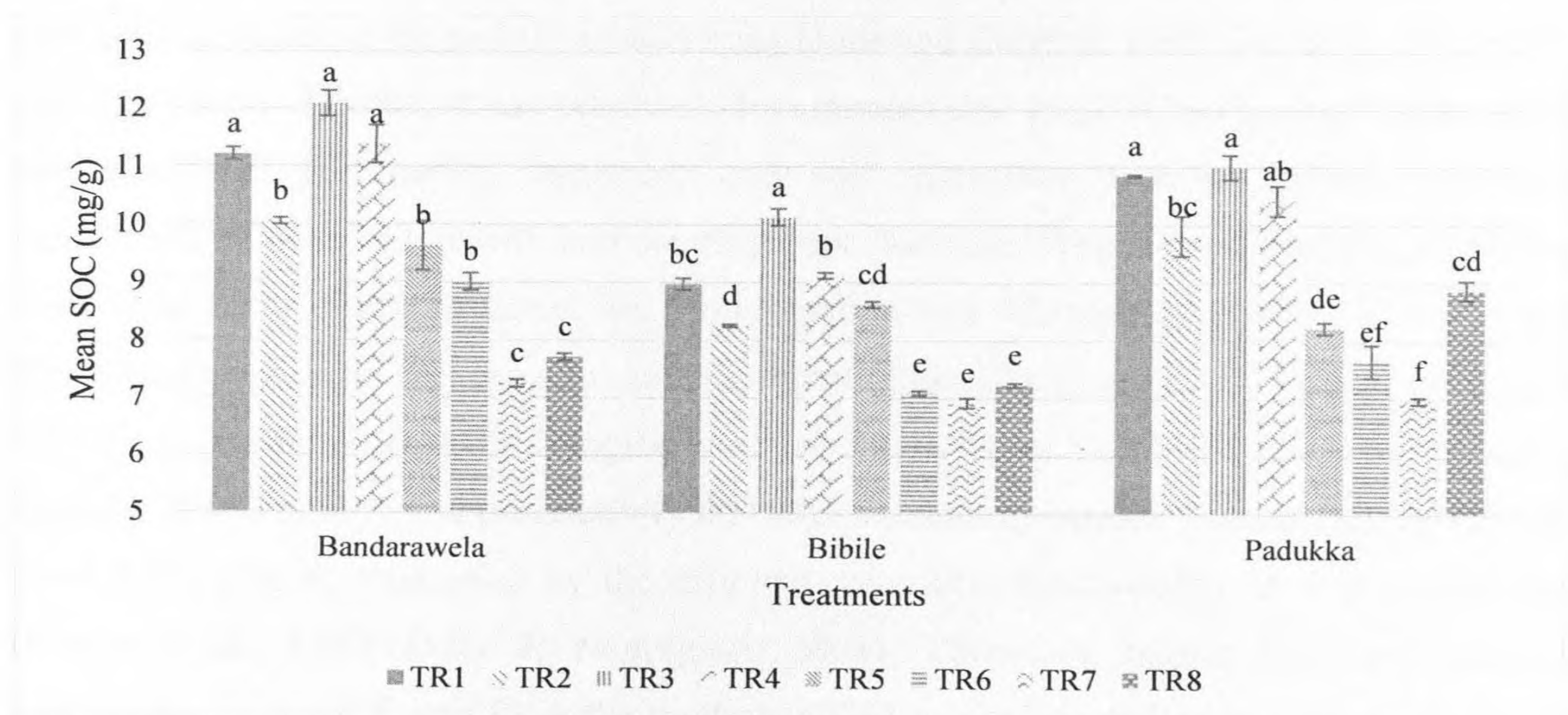



Figure 5.2- The effect of different BFBFs and CF treatments on mean soil pH at different field sites (Bandarawela, Bibile and Padukka). Treatments TR1, TR2, TR3, TR4, TR5, TR6, TR7 and TR8 are 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone No amendments treatments, initial soil without growing plant respectively. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

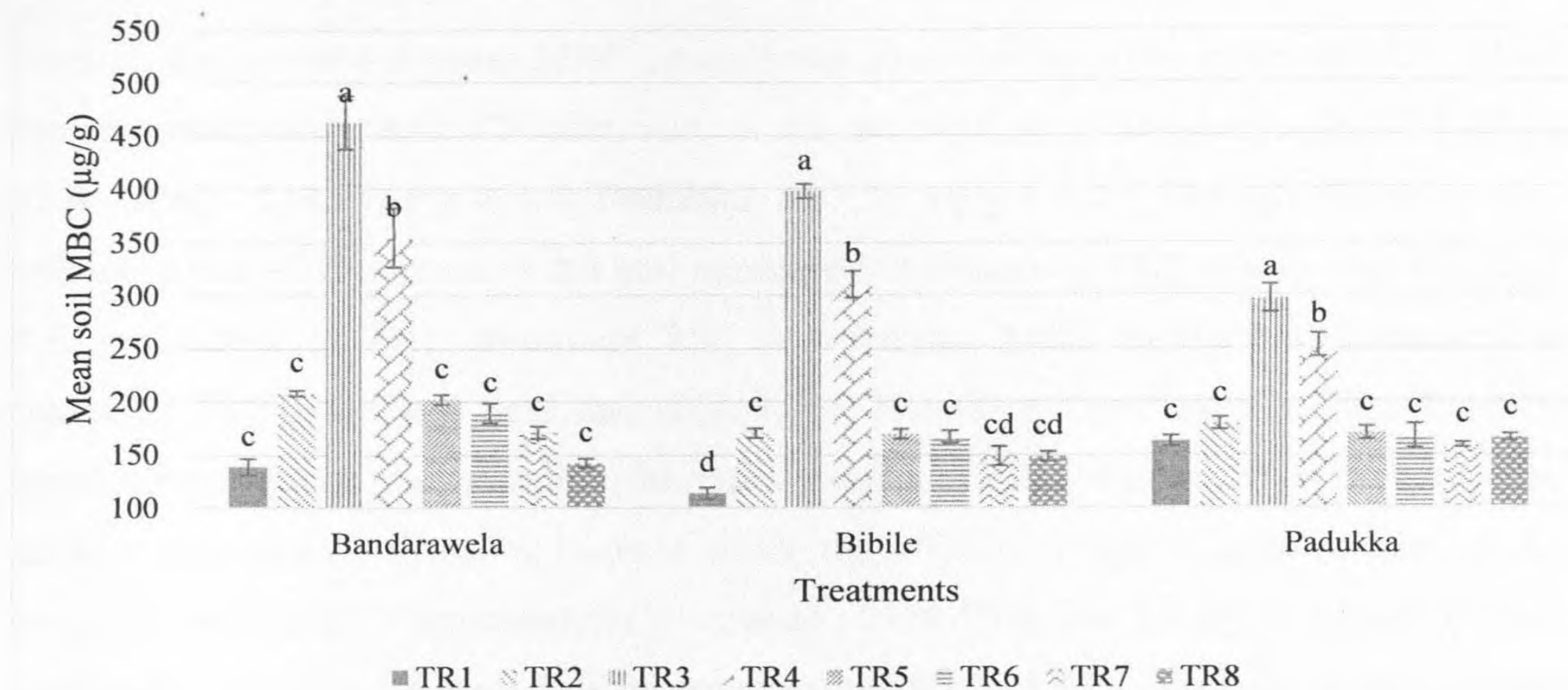
It has been reported that some of the beneficial microbial inoculants produce low molecular weight organic acids mainly gluconic and keto gluconic acids in addition to lowering the pH of rhizosphere (Sharma, Kumar and Tripathi, 2011) and the tuberization is stimulated at low soil pH levels (Wan, Cao and Tibbits, 1994). Moreover, Jeavons, 1974 has been reported that potatoes cultivation can be maximized by reducing soil pH level (pH 4.8).

### 5.4.1.2 Total SOC and MBC contents

According to the results, treatment TR3 showed the highest mean SOC content compared to all other treatments at all three field sites (Bandarawela- 12.1 mg/g ± 0.384, Bibile-10.14 mg/g ± 0.255, Padukka- 11.01mg/g ± 0.363). All treatments enhanced the mean SOC content compared to treatment TR7. Further, all BFBFs treatments with 50% recommended CF combinations (TR3 and TR4) enhanced the mean SOC content compared to treatment TR1 at Bandarawela and Bibile field sites (Fig. 5.3 a). Figure 5.3b shows that the mean MBC content was significantly enhanced ($P < 0.05$) by the treatment TR3 compared to all other treatments at all three field sites (Bandarawela-463.07 μg/g ± 43.8, Bibile- 397.77 μg/g ±11.4, Padukka- 296.52 μg/g ± 22.9). Further, higher MBC was recorded from treatment TR4 (50% CF + BFBF2) except treatment TR3 than that of the other treatments. High SOC was recorded from the treatments which showed high soil MBC content. This implies that the application of BFBFs with reduced CF can enhance both soil MBC and SOC. Further, MBC showed a direct relationship with SOC content. Similar results have been observed by another study where the soil microbial biomass increased significantly by 80%, when the CF rate reduces by 50% in the presence or absence of the BFBFs. Further, the reduced CF rate has been observed to increase soil bacterial density which ultimately reflects in elevated MBC (Seneviratne *et al.*, 2011).




(a)




(b)

Figure 5.3- The effect of different BFBFs and CF treatments on mean soil organic carbon (SOC) content at different field sites (Bandarawela, Bibile and Padukka). (a)- The effect on total mean SOC content. (b)- The effect on mean microbial biomass carbon (MBC) content. Treatments TR1, TR2, TR3, TR4, TR5, TR6, TR7 and TR8 are 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone No amendments and initial soil without plants, respectively. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

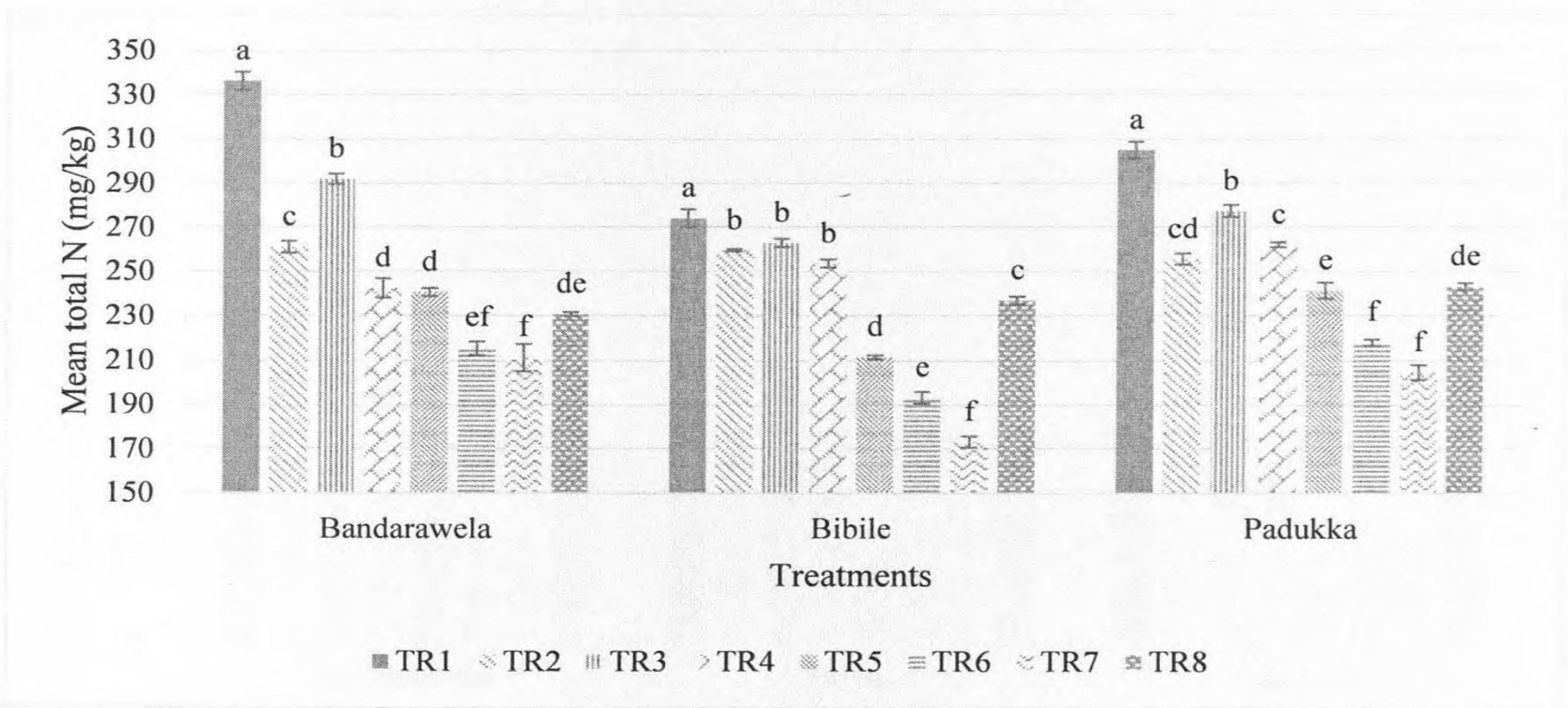
It has been reported that the SOC content can be enhanced either by application of decomposed organic materials externally or by the enhancement of soil decomposing activity (Kara and Bolat, 2007). Although SOC is an important component of soil quality and productivity, its measurement alone does not directly represent changes in the quality and nutrient status of the soil (Franzluebbers, Hons and Zuberer, 1995; Bezdicek, Papendick and Lal, 1996). Further, it has been well documented that the SOC is directly influenced to soil microbial community dynamics and soil microbial diversity which ultimately contributes to the plant growth and development (Kobabe, Wagner and Pfeiffer, 2004; De-Polli *et al.,* 2007). For instance, the growth promotion of sugar beet with PGPRs strains strongly depended on soil organic matter content (Cakmakc *et al.*, 2006; Ekin *et al.*, 2009). Therefore, measurements of biologically active fractions such as MBC could better reflect changes in soil quality and productivity that alter nutrient dynamics. Further, higher nutrient availability can be expressed by the efficient microbial functionality in a particular soil (Goyal *et al*., 1992; Dilly & Nannipieri, 2001). Therefore, higher SOC and nutrient availability (organic C and P) in the treatment TR3 might be resulted in high MBC shown by the treatment TR3. Another reason for higher MBC in treatment TR3 could be the introduction of microorganisms externally in the form of biofilms.

Further, the lowest soil mean MBC content was observed from the treatment TR1 which was the recommended 100% CF treatment, at all the field sites (Bandarawela- 138.67 µg/g ± 13.4, Bibile- 113.77µg/g ± 9.6, Padukka- 162.55 µg/g ± 8.6). Though the treatment TR1 enhanced the SOC content of the soil compared to treatment TR2 which was the exact half CF application of TR1, treatment TR1 reduced the MBC in the soil compared to the treatment TR2 in all three field sites (Fig. 5.3b). Therefore, this clearly implies that more CF applications reduce the microbial biomass in soil. Similar results have been reported by another long term fertilization trials in which the MBC was significantly reduced with eight years of continued CF application (Scott *et al.*, 1998; Fisk and Fahey, 2001). Moreover, the application of 50 % CF with BFBFs treatments (TR3 and TR4) showed an enhancement of mean MBC compared to the treatments with only BFBF applications (TR5 and TR6). Therefore, it was clearly observed that the moderate application of CF helped to enhance the microbial populations in soil. This relationship has been reported by another study that the moderate application of the CF nutrients helps to increase the microbial biomass, which in turn tends to increase the microbial efficiency or the functionality in soil (Seneviratne *et al.*, 2009).

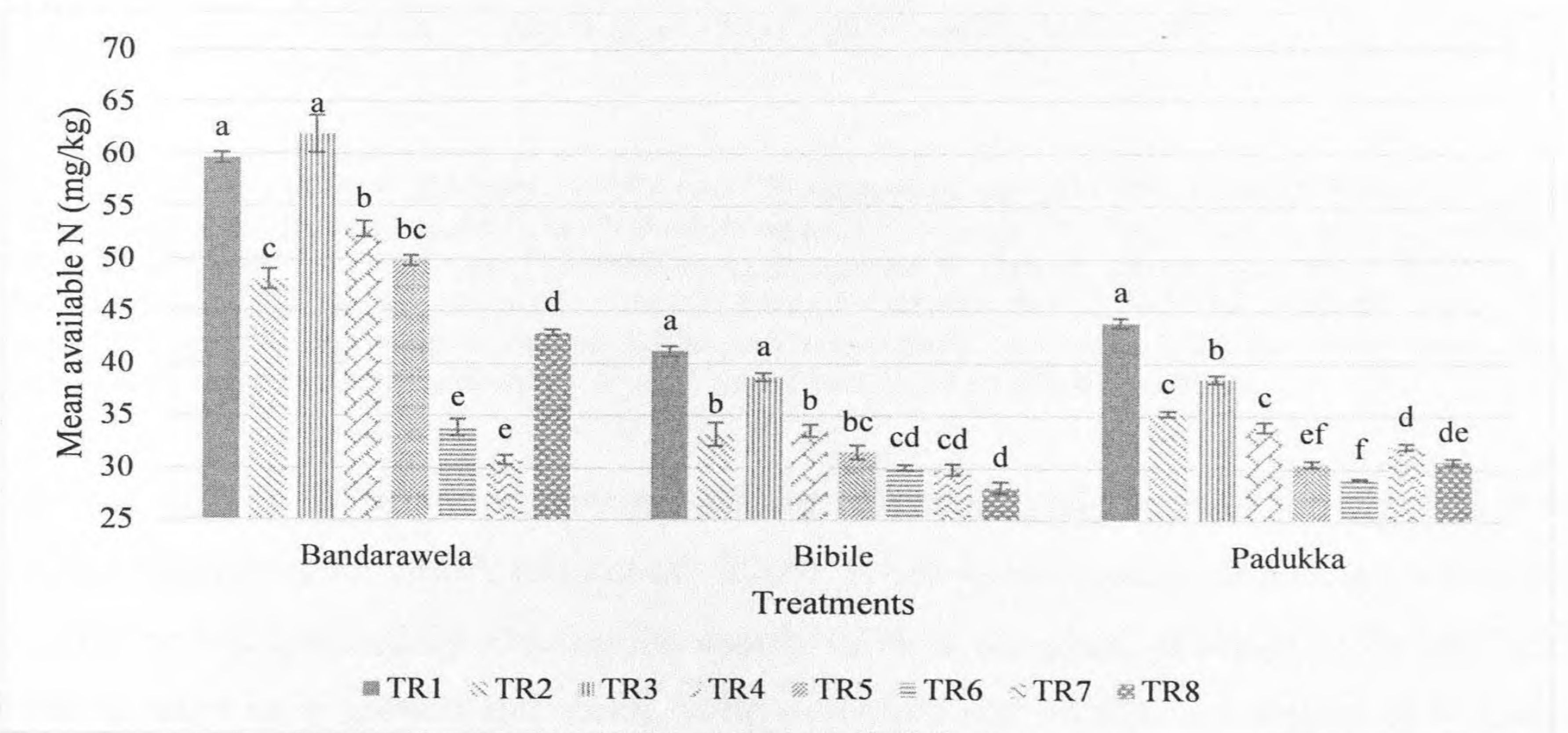
#### 5.4.1.3 Soil NPK level

The current study showed that all treatments enhanced the soil N content compared to treatment TR7 (Fig. 5.4 a). In comparison with all other treatments, treatment TR1 showed the highest soil total N content in all field sites (Bandarawela- 120 mg/kg ± 8, Bibile- 100.8 mg/kg ± 14, Padukka- 118.9 mg/kg ± 12). It has been reported that the CF application is considered as the easiest way of enhancing soil NPK level (Duarah *et al.,* 2011). Therefore, the elevated level of soil N shown by the treatment TR1 might be due to the external CF applications in the form of urea. Further, a significant reduction ($P < 0.05$) of soil N content was recorded by treatment TR3 compared to treatment TR1 at all field sites except Bibile. Moreover, there was no significant difference of mean total N content between treatment TR3 and treatment TR2 at all three field sites. Therefore, it was confirmed that the application of BFBFs with the 50% CF did not contribute to enhance the soil N level significantly. However, treatment TR3 recorded the highest tuber weight and tuber number compared to treatment TR1 (table 4.4, 4.5, 4.6). This implies that N could have been utilized to promote tuberization in TR3. Treatment TR1 showed the highest available N content at

Bibile (41.21mg/kg ± 0.81) and Padukka (43.94 mg/kg ± 0.78) sites compared to other treatments (Fig. 5.4b). However, TR3 (61.93 mg/kg ± 3.07) showed the highest available N content in Bandarawela site compared to the other treatments. Further, available N content was higher in all treatments at Bandarawela site compared to other two sites. Though total N does not express more about the fertility level of the soil, available N is an important measurement which express the usable amount of N for the plant and the microbial biomass (Saeudi *et al.*, 2009). N availability of a particular soil type is affected by different factors. One such factor is having a higher microbial activity. Therefore, the enhanced available N content shown by the treatment TR3 at Bandarawela site might be due to the presence of higher microbial activity.




(a)

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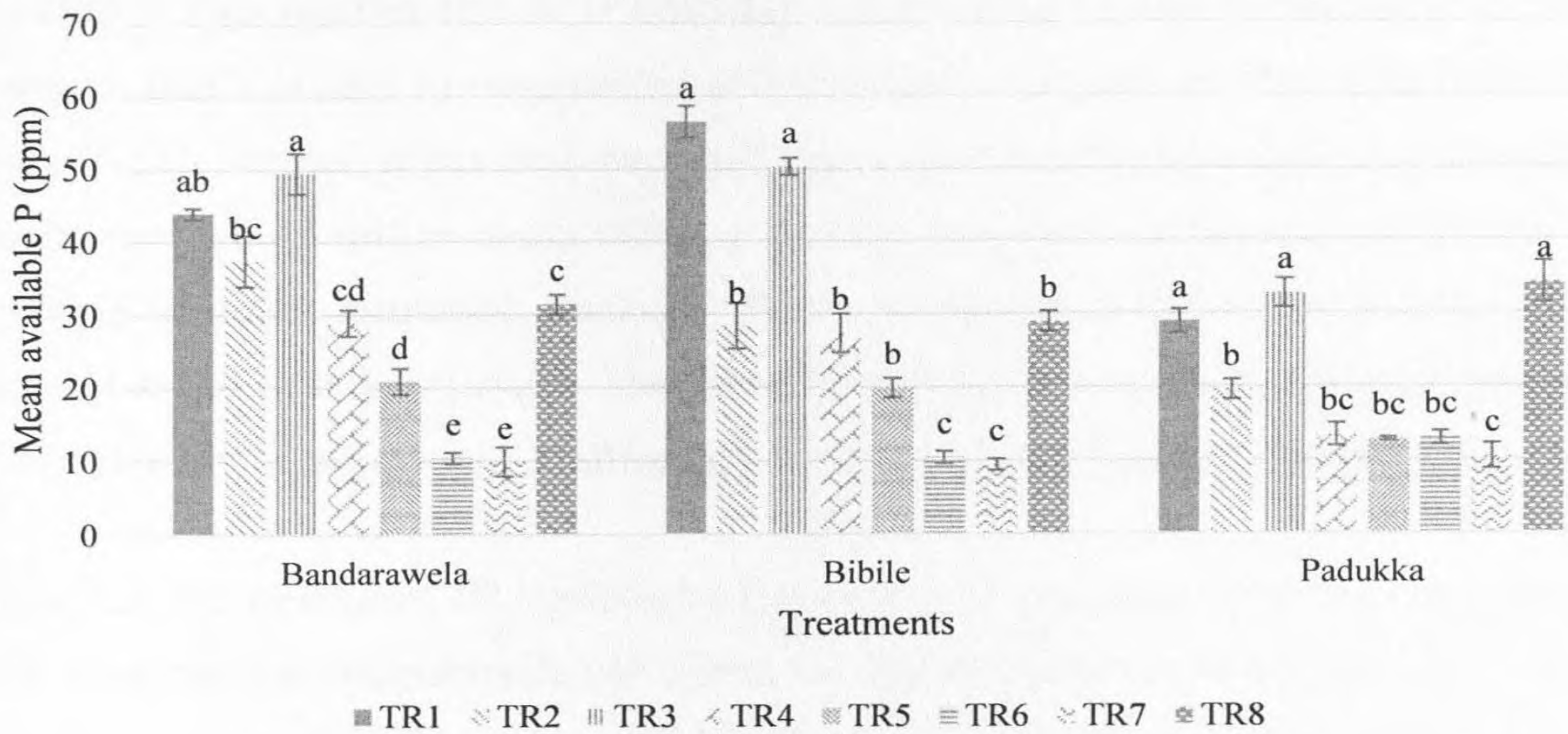


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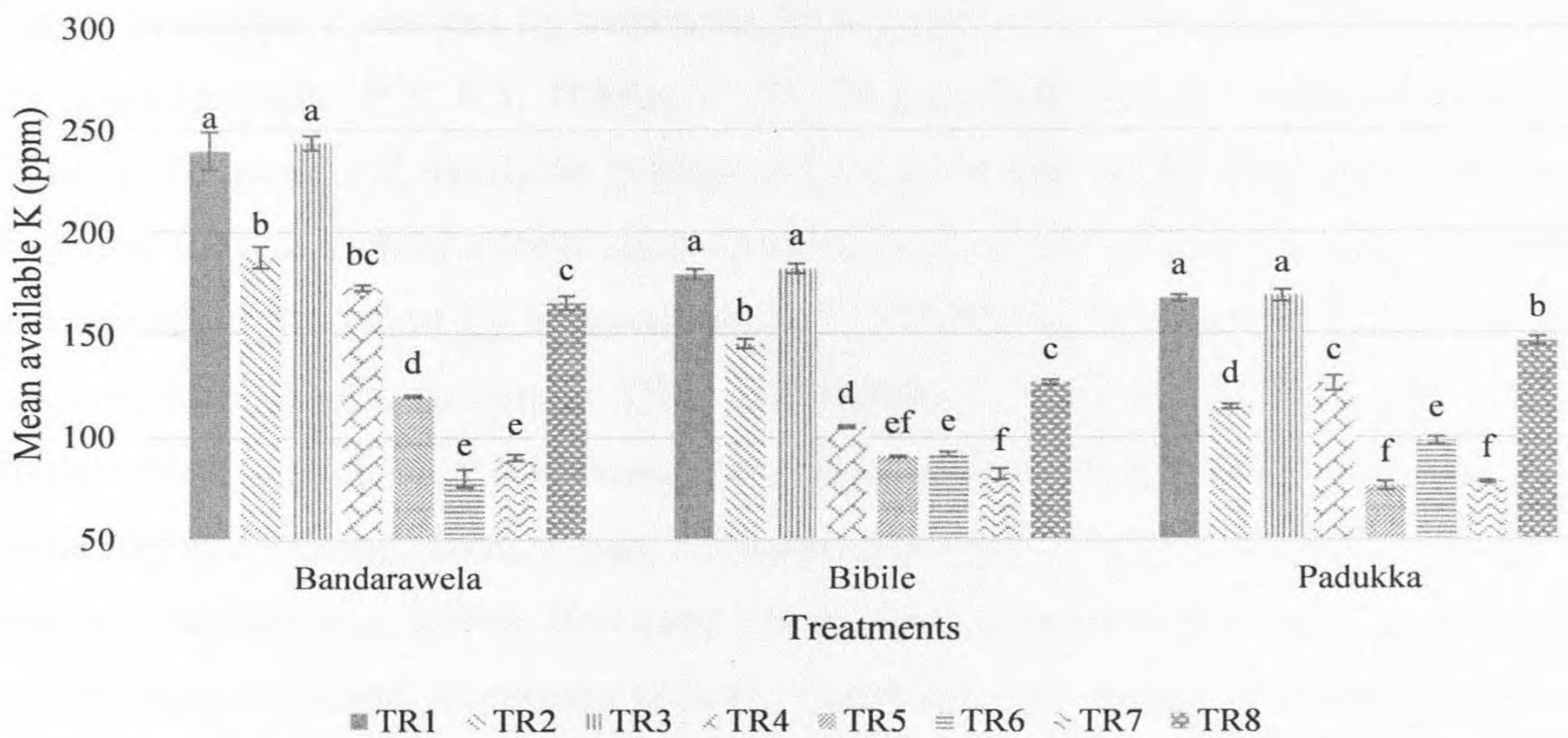



(d)

Figure 5.4- The effect of different BFBFs and CF treatments on soil NPK level at different field sites (Bandarawela, Bibile and Padukka). (a) The effect on total N content. (b)- The effect on available N (c)- The effect on available P content. (d)- The effect on exchangeable K content. Treatments TR1, TR2, TR3, TR4, TR5, TR6, TR7 and TR8 are 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone, No amendments treatments and initial soil respectively. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

The role of N fertilization for tuber growth, development, yield and quality of potato is well known (Kumar *et al.,* 2007; Ekin *et al.,* 2009). It has been reported that tuberization could readily be manipulated by altering the supply of N to the plant. Inadequate N fertilization leads to poor crop growth and yield, while excessive and continuous supply of N leads to delayed maturity, poor tuber quality, severely delayed tuberization (Krauss, 1985; Ekin *et*

*al.*, 2009). This implies that N is probably not involved in the induction of tuberization; however, that it is able to suppress tuber formation once induction has taken place (Davod *et al.*, 2011). Further, it has been recorded that high N levels in the soil promote vegetative growth that would utilize much of the available nutrients and thereby reduce the amount available for tuber formation (Jackson, 1999). Moreover, potato is considered as a heavy accumulator of N in their tissues. Therefore, inappropriate rates of N causes to accumulation of this element as nitrate which ultimately leads ti increase toxicity (Saeudi *et al.*, 2009).

Moreover, the treatment TR3 enhanced the mean soil available P content compared to all other treatments at Bandarawela (49.5 ppm ± 4.8) and Padukka (32.83 ppm ± 3.4) field sites (Fig. 5.4 c). An elevated mean available P content (56.5 ppm ± 3.7) was recorded from treatment TR1 compared to all other treatments at Bibile field site. This might be due to the application of TSP fertilizers. Further, two sample t-test showed a significant enhancement of mean available P content by treatment TR3 compared to treatment TR2 at all three field sites (Bandarawela- $P < 0.1$, Bibile- $P < 0.05$ and Padukka- $P < 0.05$). Treatment TR3 enhanced the mean soil available P content by approximately 32% at Bandarawela, 76% at Bibile and 67% at Padukka field sites compared to treatment TR2 whereas it enhanced the mean available P content by approximately 12.5% at Bandarawela and 13.2% at Padukka field sites compared to treatment TR1. Availability of P of a particular soil is affected by different factors such as P mineralization from organic matter decomposition, degree of weathering and erosion, fertilization, mineralization of P by plant secretions, uptake of P by plants etc (Richardson, 1994). However, solubilization using beneficial microbial species is one such important and prominent factors. Therefore, the enhanced availability of P by the treatment TR3 might be due to the beneficial microbial activity of BFBFs. It has been reported that P is an essential nutrient which stimulates growth of young plants while giving them a vigorous start. In potato, adequate P nutrition is critical for tuber development and high photosynthetic rate maintenance during tuber bulking. P nutrition is also linked to the assimilation of other mineral nutrients and improvement of protein contents (Mishra, Mohan and Mishra, 2007). Since potato has an inherently low root density and restricted ability to uptake P fertilizer, P deficiency is usually a limiting factor to yield in commercial potato production (MacKay, Carefoot and Entz, 1988). Therefore, large amounts of chemical P fertilizers have to be added annually to enhance the yield (Richardson, 1994). However, a large portion of soluble inorganic phosphate applied to soil as CF is rapidly immobilized soon after application and becomes unavailable to plants (Rodriguez and Fraga, 1999). There

is strong evidence that rhizosphere associated soil bacteria are capable of transforming soil P to the forms available to plant (Rodriguez and Fraga, 1999). These microorganisms have the ability to solubilize and mineralize P from inorganic sources and can be used as biofertilizer to reduce CF inputs (Gyaneshwar *et al.,* 2002; Asok and Jisha, 2006; Shah *et al.,* 2007). For instance, higher order biofilm including *Trichoderma harzianum* and several species of *Bacillus* are known to solubilize unavailable forms of P and convert them to available forms, thereby enabling better P uptake (Srinath, Bagyaraj and Satyanarayana, 2003). Moreover, it was clearly observed that the mean P content was reduced in all BFBFs treated soil except TR3 compared to initial P level in the soil (TR8). This might be due to the heavy uptake of the available P by the plants and the introduced microbial biomass for their growth and development.

In comparison with all other treatments, the mean exchangeable K content in soil (241.9 ppm ± 6.0) was enhanced by treatment TR3 only at Bandarawela field site (Fig. 5.4 d). However, two sample t-test showed a significant enhancement of mean exchangeable K content in soil by the treatment TR3 compared to treatment TR2 at all three field sites ($P < 0.05$). Treatment TR3 enhanced the mean soil exchangeable K content by approximately 29% at Bandarawela, 21% at Bibile and 42% at Padukka field sites compared to treatment TR2. However, all BFBF treated soil except treatments TR3 and TR4 reduced the mean K content compared to the initial P level in the soil (TR8). It has been reported that potato plants require much more K than any other vegetable crop to maintain the vigor of the plants. Further, potato crop sometimes is regarded as an indicator crop for K availability because of its high K requirement. (Ulrich and Ohki, 1996; Khan *et al.,* 2010).

Soil micro-organisms play a significant role in regulating the dynamics of organic matter decomposition and the availability of plant nutrients such as NPK and other nutrients (Duarah *et al.,* 2011). The current study confirmed that the inoculation of beneficial microorganisms in the form of BFBFs with only 50% CF enhanced P, K and other nutrient level in soil. Further, it was confirmed that the application of BFBFs along with CF created favorable soil N level for potato tuberization. Considerable evidence exists that soil NPK level influence potato (*Solanum tuberosum* L.) yields and quality (Dobránszki *et al.*, 2008).

#### 5.4.1.4 Soil $Ca^{2+}$ content

In comparison with all treatments, the highest $Ca^{2+}$ concentration was observed from the treatment TR3 (Bandarawela- 0.394 mg/g ± 0.006, Bibile-0.362 mg/g ± 0.001, Padukka-0.37 mg/g ± 0.003) at all field sites (Fig. 5.5). A significant enhancement of soil $Ca^{2+}$ level was recorded in treatment TR3 compared to other treatments at Bandarawela and Bibile field sites ($P < 0.05$). Further, treatment TR3 enhanced the soil $Ca^{2+}$ level by approximately 4 % at Bandarawela field site and by approximately 9.5% at Bibile field site even compared to treatment TR1. Moreover, it was clearly observed that the treatments with biofilm combination BFBF1 significantly enhanced the mean soil $Ca^{2+}$ level compared to the treatments with biofilm combination BFBF2 (TR4) at Bandarawela ($P < 0.05$) and Bibile ($P < 0.05$) field sites.

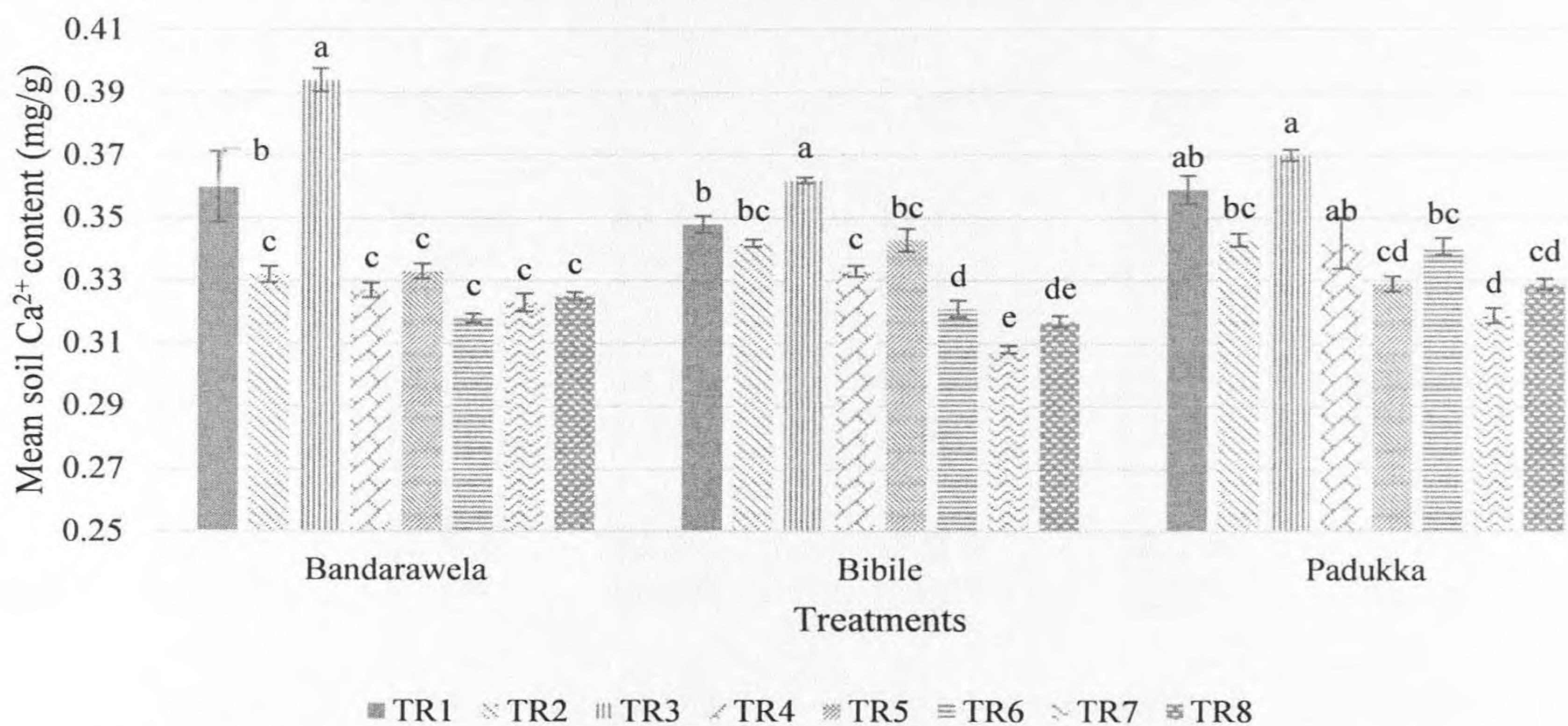



Figure 5.5- The effect of different BFBFs and CF treatments on soil $Ca^{2+}$ level at different field sites (Bandarawela, Bibile and Padukka). Treatments TR1, TR2, TR3, TR4, TR5, TR6, TR7 and TR8 are 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone, No amendments treatments and initial soil respectively. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

### 5.4.2 Potato plant biochemical parameters

#### 5.4.2.1 Chlorophyll content in leaves

Chlorophyll intensity of potato leaves were estimated using a digital chlorophyll meter. The chlorophyll meter is for simple, rapid, and nondestructive estimation of chlorophyll contents in different plant leaves. In comparison with all other treatments, the highest mean

chlorophyll contents of both 3rd ( Bandarawela- 24.2 ± 1.04, Bibile- 48.7 ± 1.26, Padukka- 15.5 ± 1.05) and 5th ( Bandarawela- 29.0 ± 0.83, Bibile- 55.6 ± 1.71, Padukka- 18.5 ± 1.09) potato leaves were observed from the treatment TR3 at all three field sites (table 5.1). All treatments enhanced the mean chlorophyll content in both leaves compared to treatment TR7 at all field sites. It was observed that the mean chlorophyll content of 5th leaf was higher than that of the 3rd leaf at all field locations.

Table 5.1 - The effect of different BFBFs and CF treatments on chlorophyll content of 3rd and 5th leaves of potato at different field sites.

| | Bandarawela Chlorophyll intensity Mean ± SD | | Bibile Chlorophyll intensity Mean ± SD | | Padukka Chlorophyll intensity Mean ± SD | |
|---|---|---|---|---|---|---|
| | 5th Leaf | 3rd Leaf | 5th Leaf | 3rd Leaf | 5th Leaf | 3rd Leaf |
| TR1 | 24.5 ± 0.99$^{ab}$ | 21.7 ± 0.86$^{ab}$ | 47.5 ± 1.13$^{bc}$ | 45.6 ± 1.49$^{ab}$ | 18.1 ± 1.03$^{ab}$ | 14.5 ± 0.60$^{ab}$ |
| TR2 | 21.8 ± 0.82$^{bc}$ | 19.2 ± 0.72$^{bc}$ | 46.6 ± 1.72$^{bc}$ | 43.8 ± 1.50$^{ab}$ | 15.5 ± 1.05$^{abc}$ | 13.2 ± 0.55$^{ab}$ |
| TR3 | 29.0 ± 0.83$^{a}$ | 24.2 ± 1.04$^{a}$ | 55.6 ± 1.71$^{a}$ | 48.7 ± 1.26$^{a}$ | 18.5 ± 1.09$^{a}$ | 15.4 ± 0.65$^{a}$ |
| TR4 | 26.0 ± 1.51$^{ab}$ | 20.9 ± 1.16$^{ab}$ | 52.7 ± 1.67$^{ab}$ | 42.8 ± 1.35$^{ab}$ | 18.1 ± 1.07$^{ab}$ | 14.4 ± 0.84$^{ab}$ |
| TR5 | 18.4 ± 0.97$^{c}$ | 15.6 ± 0.68$^{c}$ | 40.9 ± 1.61$^{cd}$ | 38.3 ± 1.96$^{b}$ | 14.2 ± 0.54$^{bc}$ | 11.8 ± 0.48$^{ab}$ |
| TR6 | 21.7 ± 1.08$^{bc}$ | 18.7 ± 0.72$^{bc}$ | 42.1 ± 1.69$^{cd}$ | 39.0 ± 2.40$^{b}$ | 13.6 ± 0.58$^{c}$ | 13.2 ± 0.57$^{ab}$ |
| TR7 | 19.8 ± 0.81$^{c}$ | 16.8 ± 0.74$^{c}$ | 35.3 ± 1.88$^{d}$ | 27.6 ± 0.87$^{c}$ | 8.3 ± 0.91$^{d}$ | 6.1 ± 0.95$^{c}$ |

Treatments TR1, TR2, TR3, TR4, TR5, TR6 and TR7 are 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone and No amendments treatments respectively. Same letter are not significantly different at 5% probability level.

It has been reported that the young leaves of potato plants have rather less photosynthetic activity than the old matured leaves (Okazama, 1967). Average chlorophyll contents of leaves for all treatments were higher at Bibile field location compared to the other two field locations. This might be due to the presence of extreme climatic conditions like high sunlight intensity at Bibile field site. It has been reported that most plants have the ability to acclimate to a specific light environment and the chlorophyll concentration is considered as an indicator of plant responsiveness to light intensity (Vieira, 1996). Another study has reported that the chlorophyll concentration, chloroplast density and carotenoid composition can fluctuate according to the light intensity (Dem-mig-Adams *et al.*, 1997). It has been reported that there is a relationship between chlorophyll content and rate of photosynthesis, it seems likely that equal amounts of chlorophyll should cause equal rates of photosynthesis (Vieira, 1996). Moreover, PGPRs inoculation has been reported to enhance the physiological properties of the host plants namely, photosynthetic rate (Mia *et al.*, 2005). Leaf area, leaf chlorophyll content and total dry matter have been increased significantly due to PGPRs inoculation. This might be due to the balanced N accumulation by bacterial $N_2$ fixation and better root growth, which promoted the greater uptake of water and nutrients. Favorable soil N level has been reported to be contributed for the formation of chlorophyll, which consequently, increased the photosynthetic activity (Mia *et al.*, 2005; Mia *et al.*, 2012).

### 5.4.3 The effect of different CF and BFBF treatments on tuber nutrient content

In comparison with all other treatments, the highest significant tuber starch content was recorded from treatment TR3 at Bandarawela ( $P < 0.05$, 24.1 % ± 0.53) and Bibile ($P < 0.05$, 14.24% ± 0.16) field sites (table 5.2). Treatment TR3 enhanced the starch content of the tubers by approximately 33% at Bandarawela field site and by approximately 18% at Bibile field site even compared to treatment TR1. The highest tuber mean crude protein content (table 5.2) was observed from the treatment TR3 compared to the other treatments at all field sites (Bandarawela- 2.04% ± 0.03, Bibile- 1.88% ± 0.02). It was clearly observed that the BFBF1 treatment combinations enhanced the tuber crude protein content compared to the BFBF2 treatment combinations at all field sites. Further, all treatments enhanced the tuber crude protein content compared to control (TR7). Moreover, in comparison with all other treatments, treatment TR3 significantly enhanced the tuber mean crude fiber content at Bandarawela ($P < 0.05$, 2.69% ± 0.01) and Bibile ($P < 0.05$, 2.29% ± 0.02) field sites ( table 5.2). BFBF1 treatment combinations enhanced the mean tuber fiber content compared

to BFBF2 treatment combinations. However, it was clearly observed that the treatment TR3 reduced the mean crude fat content of tubers compared to the treatment TR1 and TR2 at both field sites (table 5.2).

Table 5.2- The effect of different BFBFs and CF treatments on nutrient content of tuber at different field sites.

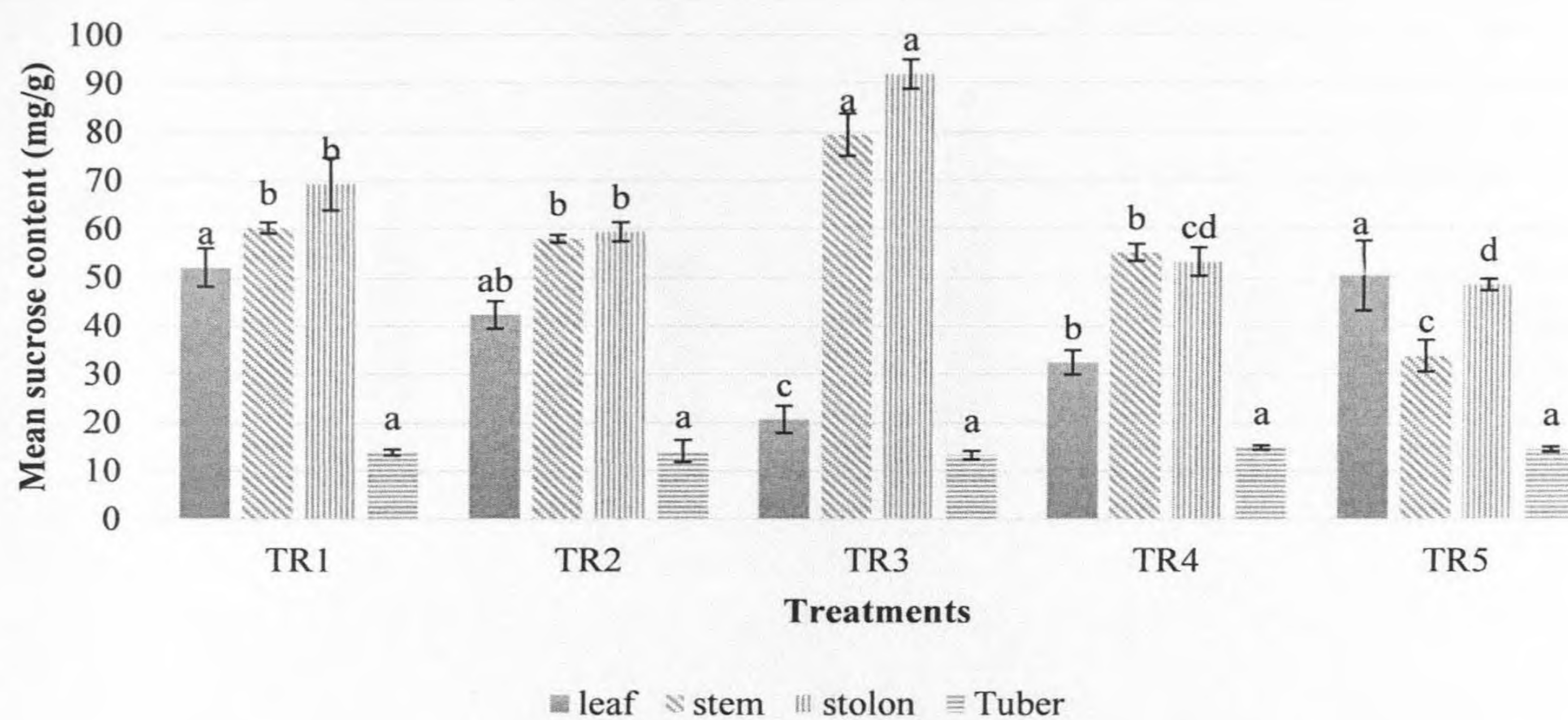
| | Starch (%) | | Crude protein (%) | | Crude fiber (%) | | Crude fat (%) | |
|---|---|---|---|---|---|---|---|---|
| | Mean ± SD | | Mean ± SD | | Mean ± SD | | Mean ± SD | |
| | Ban | Bi | Ban | Bi | Ban | Bi | Ban | Bi |
| TR1 | 18.09 ±0.22$^{b}$ | 12.05± 0.59$^{b}$ | 2.02± 0.03$^{a}$ | 1.86± 0.02$^{a}$ | 2.53± 0.03$^{b}$ | 2.1±0.02$^{b}$ | 0.11±0.01$^{a}$ | 0.0973 ±0.01$^{a}$ |
| TR2 | 16.47± 0.69$^{b}$ | 11.64± 0.20$^{bc}$ | 2.02± 0.05$^{ab}$ | 1.84± 0.02$^{ab}$ | 2.5±0.02$^{b}$ | 2.12±0.01$^{b}$ | 0.103±0.01$^{ab}$ | 0.09±0.01$^{ab}$ |
| TR3 | 24.1± 0.53$^{a}$ | 14.24± 0.16$^{a}$ | 2.04± 0.03$^{a}$ | 1.88± 0.02$^{a}$ | 2.69± 0.01$^{a}$ | 2.29±0.02$^{a}$ | 0.103±0.01$^{ab}$ | 0.089± 0.01$^{ab}$ |
| TR4 | 13.85± 0.31$^{c}$ | 12.12± 0.50$^{b}$ | 1.89± 0.02b$^{c}$ | 1.72± 0.02$^{c}$ | 2.53± 0.02$^{b}$ | 1.99±0.01$^{c}$ | 0.079±0.01$^{ab}$ | 0.0793 ±0.01$^{cd}$ |
| TR5 | 14.25± 0.35$^{c}$ | 10.95± 0.05$^{bc}$ | 1.92± 0.03$^{abc}$ | 1.76± 0.01$^{bc}$ | 2.56± 0.01$^{b}$ | 2.14±0.01$^{b}$ | 0.078±0.01$^{ab}$ | 0.079± 0.01$^{cd}$ |
| TR6 | 13.45± 0.12$^{c}$ | 10.16± 0.20$^{c}$ | 1.78± 0.01$^{cd}$ | 1.67± 0.01$^{c}$ | 2.4±0.02$^{c}$ | 1.91±0.02$^{c}$ | 0.085±0.02$^{ab}$ | 0.0837 ±0.01$^{bc}$ |
| TR7 | 13.64± 0.36$^{c}$ | 10.7± 0.31$^{bc}$ | 1.73± 0.01$^{d}$ | 1.56± 0.02$^{d}$ | 2.24± 0.02$^{d}$ | 1.68±0.01$^{d}$ | 0.068±0.01$^{b}$ | 0.0713 ±0.01$^{d}$ |

Treatments TR1, TR2, TR3, TR4, TR5, TR6 and TR7 are 100% CF, 50% CF, 50% CF+ BFBF1, 50% CF+ BFBF2, BFBF1 alone, BFBF2 alone and No amendments treatments respectively. Same letter are not significantly different at 5% probability level.

The results clearly imply that the biofilm combination BFBF1 along with 50% CF enhanced tuber nutrient content (starch, crude protein and crude fiber) compared to the treatment TR1 which was DOA recommended CF treatment. Further, it was noted that BFBF2 treatments (TR4 and TR6) reduced the tuber nutrient level compared to treatment TR2. This implies that BFBF2 treatment has no effect on the enhancement of tuber nutrient level.

### 5.4.4 The effect of CF and BFBFs combinations on biochemical properties of different potato tissues

Treatment TR3 showed the highest significant mean sucrose content in stolon tissues ($P < 0.05$, 91.86 mg/g ± 3.01) and stem tissues ($P < 0.05$, 79.33 mg/g ± 4.3) compared to all other treatments at Bandarawela field site (Fig. 5.6a). Further, TR3 enhanced the sucrose content in stolon tissues by approximately 31% and the sucrose content in stem tissues by 32% compared to the treatment TR1. However, the mean sucrose content of the leaf tissues for the treatment TR3 was significantly low ($P < 0.05$, 20.65 mg/g ± 2.8) compared to other treatments. Further, significant difference in sucrose content was not observed by the treatment TR3 compared to the other treatments in tuber tissues at Bandarawela field site.
In comparison with all other treatments, treatment TR3 showed the highest significant reducing sugar content ($P < 0.05$, 68.78 mg/g ± 2.9) in leaf tissues at Bandarawela field site (Fig. 5.6b). Further, TR3 enhanced the reducing sugar content in leaf tissues by approximately 46% compared to the treatment TR2 and by approximately 26% even compared to treatment TR1. However, treatment TR3 which showed the highest tuberization effect (table 4.4), resulted the lowest mean reducing sugar contents in stem (15.99 mg/g ± 1.1) and stolon tissues (15.34 mg/g ± 0.8) compared to the other treatments at Bandarawela field site. Another study has been reported that the reducing sugar content of the stem and stolon tissues dropped sharply upon the formation of potato tubers (Minhas *et al.*, 2004). Treatment TR5 which showed the lowest tuberization effect (table 4.4), resulted the highest reducing sugar content in stem (33.68 mg/g ± 3.5) and in stolon (34.91 mg/g ± 0.944) tissues at Bandarawela field site. The highest significant tuber starch content ($P < 0.05$, 241.5mg/g ± 3.2) and the lowest leaf starch content (38.22 mg/g ± 0.6) were recorded by the treatment TR3 compared to the all other treatments (Fig. 5.6c) at Bandarawela field site. Another study has reported significantly low amounts of starch content in potato leaf tissues (Minhas *et al.*, 2004). According to literature, this is due to the speed translocation of majority of the leaf photosynthate in to the tubers (Moorby, 1978; Wolf, Marani and Rudich, 1990; Frommer and Sonnewald, 1995).

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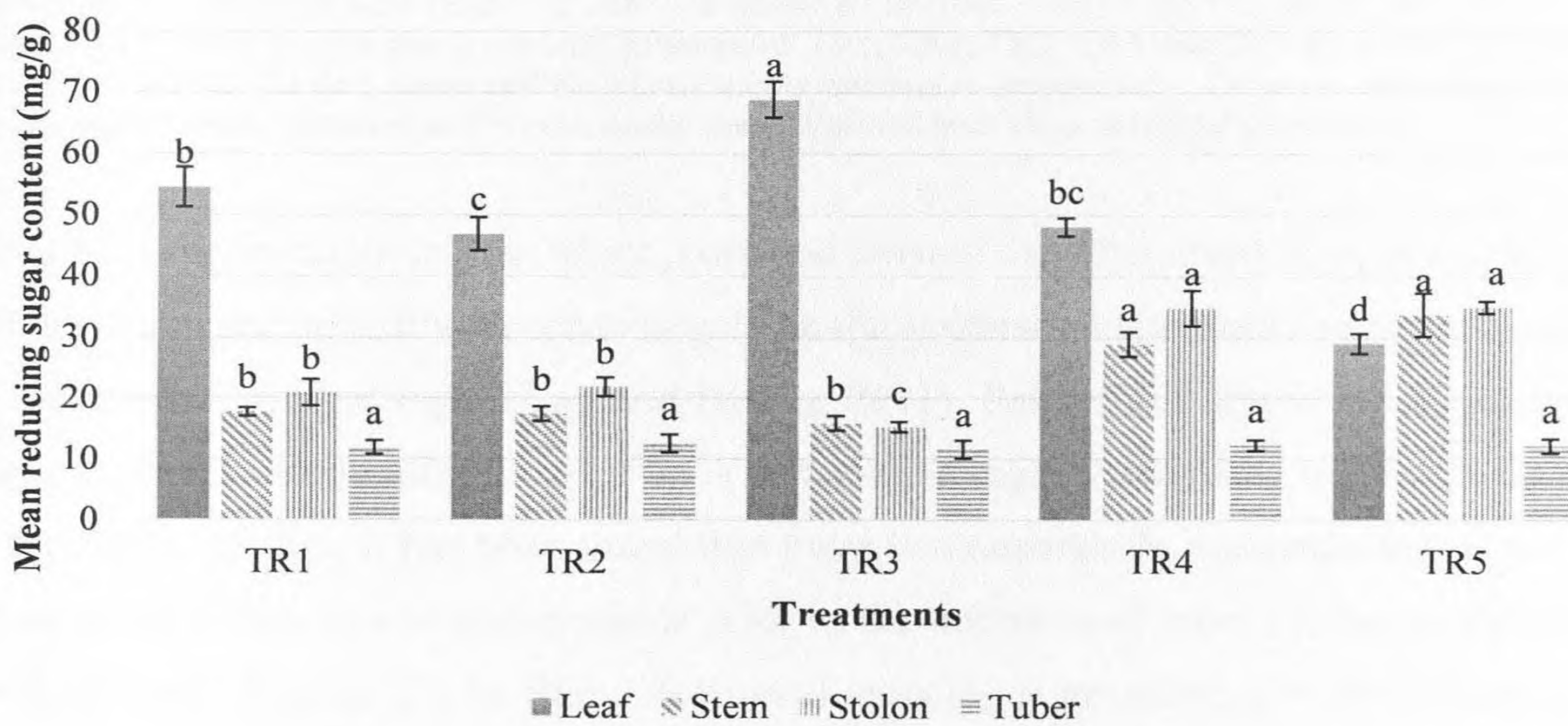


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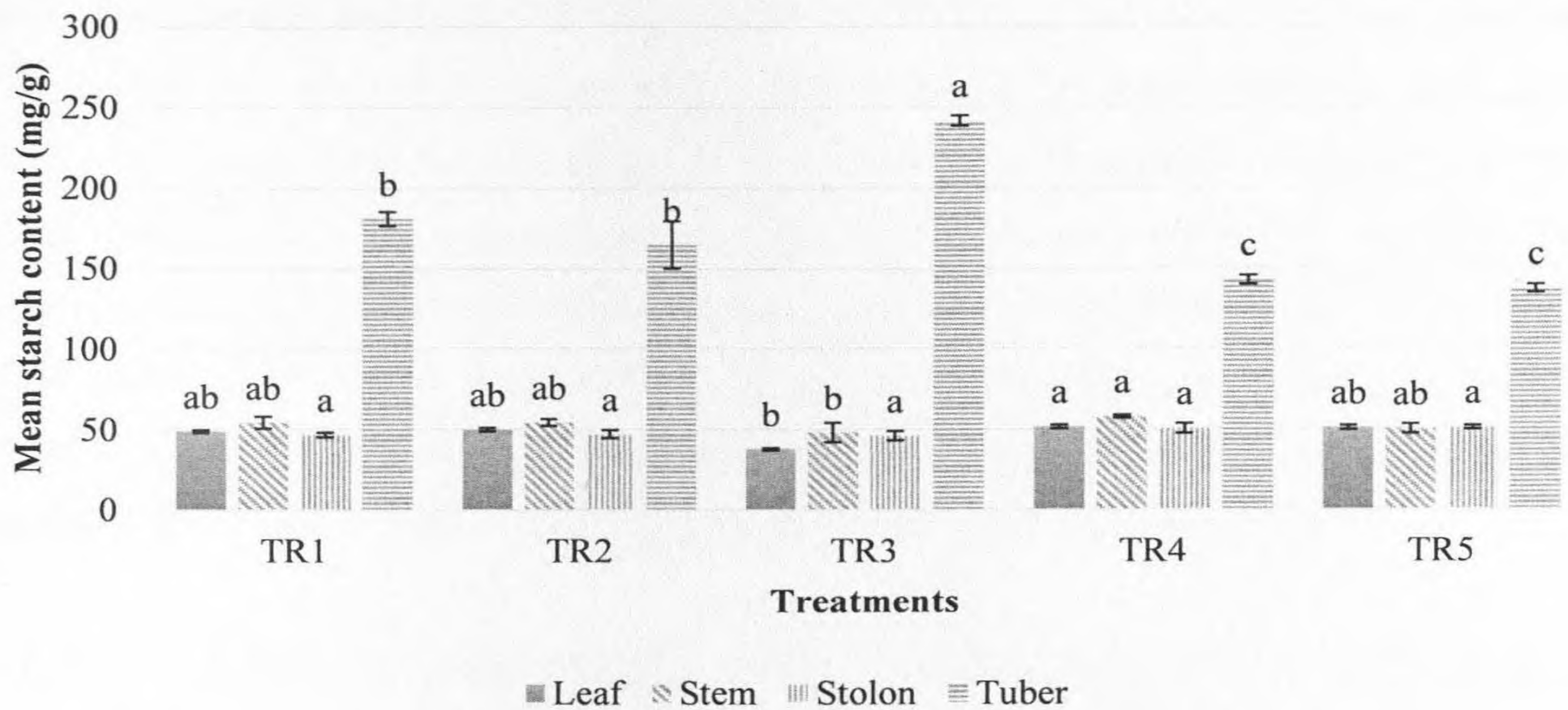



(c)

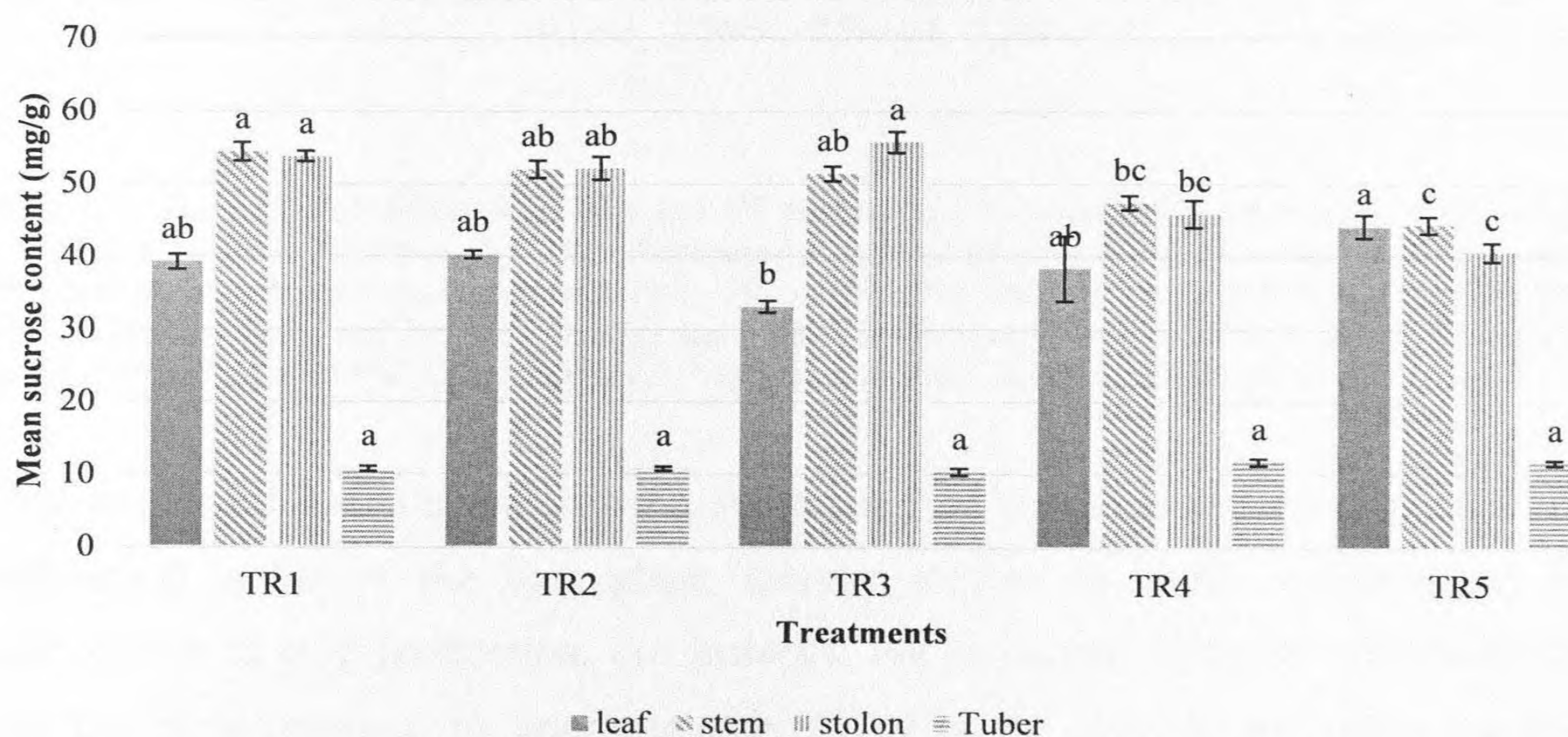
Figure 5.6- The effect of different BFBF1 and CF treatments on biochemical properties of different potato plant tissues at Bandarawela field site. (a)- The effect on sucrose content (b)-The effect on reducing sugar content (c) - The effect on starch content. Treatments TR1, TR2, TR3, TR4 and TR5 are 100% CF, 50% CF, 50% CF+ BFBF1, BFBF1 alone and No amendments treatments respectively. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

It has been reported that in rice, wheat, corn and most of the tuber crops like potato and sweet potato, starch accumulation depends largely on the synthesis, translocation and accumulation of the internal soluble sugar (Luo and Huang, 2011). Reducing sugar is the major soluble sugar in leaves, accounting for over 74% of the total sugar. According to the results stated in the earlier studies, it has been found that there is a remarkable accumulation of reducing sugar in the stolon tips of potato plants prior to the initiation of tuber formation (Okazawa, 1967; Luo and Huang, 2011). Rapid decline of reducing sugar content in the stolon tips has been recorded while the gradual increment of starch accumulation immediately after the onset of tuberization (Okazawa, 1967).

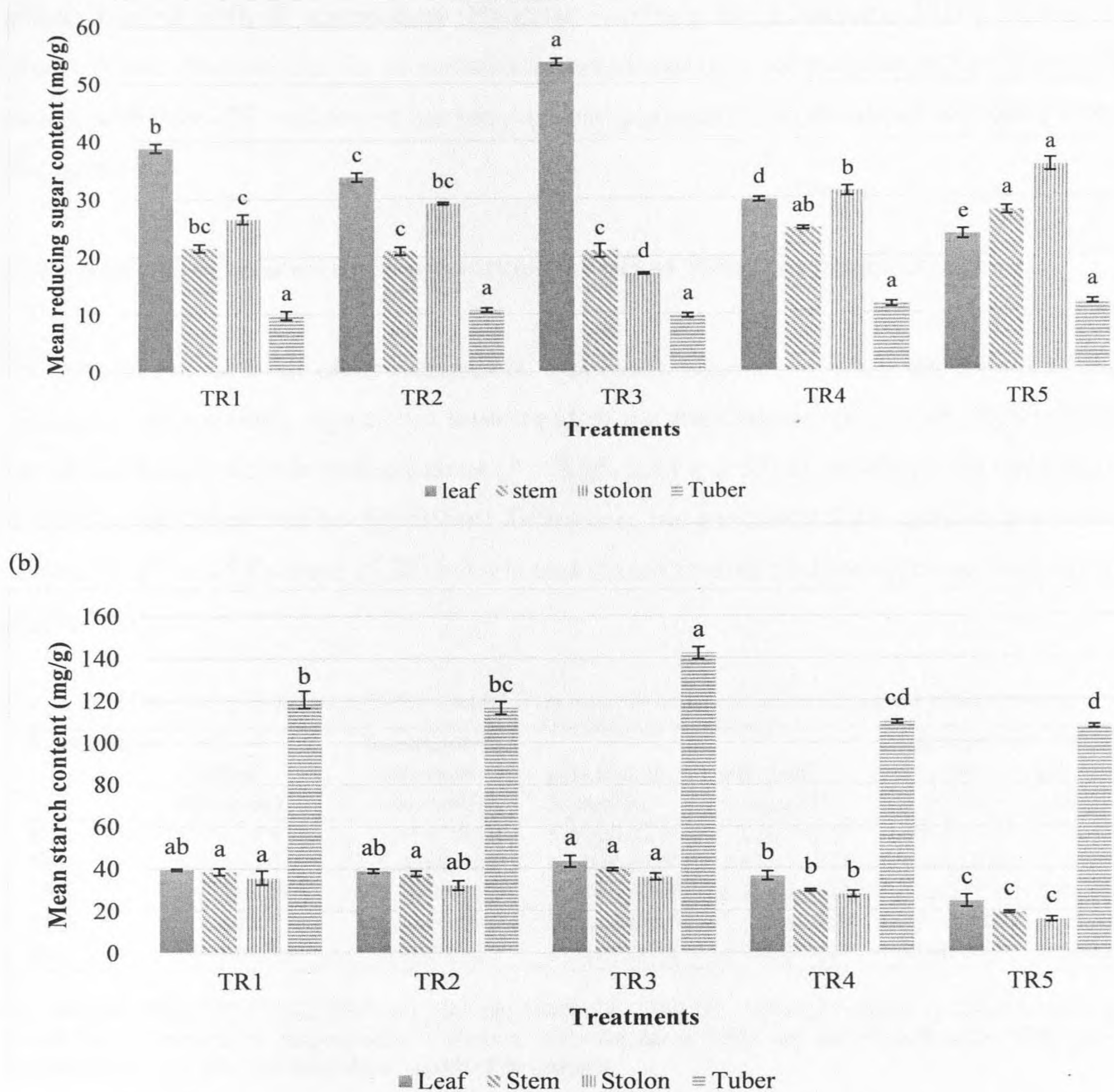
Sucrose is the major transport form of fixed photo assimilates into developing potato tubers and its concentration gradually declined along the axis (Viola *et al.,* 2001). According to the mechanisms, sucrose synthase enzyme cleaves the sucrose into the reducing sugars like glucose and fructose after unloading the sucrose from the phloem tissues at the sink organs (stolon tip). When the tuber enlargement is proceeding, the increasing reducing sugar content at the sink (stolon tip) tissues stimulates the enzymes which promotes starch synthesis from the reducing sugars, thereby decreasing the reducing sugar content at the stolon tip (Hawker, Marschner and Krauss, 1979; Sasamoto and Suzuki, 1979). Further, it has been reported that

the sucrose content is very low in stolon tips and newly formed tubers and increases later to a constant level, whereas reducing sugar content is high in stolon tips but decreases to a lower level during tuberization (Hawker *et al.,* 1979). Low sucrose content in the source leaves could boost the photosynthesis rate by less sugar inhibition (Youjun, 2011). Moreover, the starch content of the tubers increases rapidly after tuberization and then remain constant at the later stages (Hawker *et al.,* 1979; Sasamoto and Suzuki, 1979). Similar results were observed in the current study in terms of the quantity of reducing sugar, starch and the sucrose in the stolon tissues and tuber tissues (sink) at tuberization.

Further, the current study showed a high sucrose content in stolon tissues (55.76 mg/g ± 2.5) and low sucrose content in leaf tissues (33.13 mg/g ± 1.4) by the treatment TR3 at Bibile field site (Fig. 5.7a). Significant differences were not observed in tuber sucrose content by the treatments. The highest significant reducing sugar content in leaf tissues ($P < 0.05$, 53.74 mg/g ± 1.1) and the highest significant starch content in tuber tissues ($P < 0.05$, 142.37 mg/g ± 2.8) were observed by the treatment TR3 at Bibile field site (Fig. 5.7b). Treatment TR3 enhanced the leaf reducing sugar content by approximately 61% compared to treatment TR2 and by 39% even compared to treatment TR1. Further, it enhanced the tuber starch content by approximately 22% compared to treatment TR2 and by approximately 18% even compared to treatment TR1 (Fig. 5.7c).




(a)




(c)

Figure 5.7- The effect of different BFBF1 and CF treatments on biochemical properties of different potato plant tissues at Bibile field site. (a)- The effect sucrose content (b)-The effect on reducing sugar content (c) - The effect on starch content. Treatments TR1, TR2, TR3, TR4 and TR5 are 100% CF, 50% CF, 50% CF+ BFBF1, BFBF1 alone and No amendments treatments respectively. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

It has been well documented that the inoculation of beneficial microorganisms alter the biochemical status of the host plant; thereby creates favorable environment for the enhancement of crop production. For instance, the increment of lignin content of the rice plant has been recorded by approximately 300% in the plant treated with *Trichoderma asperellum* and by 200% in the plants treated with *Pseudomonas fluorescens*. Further, total phenol content has been enhanced by 42% and the flavonoids by approximately 26% in the

plants treated with *T. asperellum* (Shoresh, Harman and Mastouri, 2010). In the current study, it was obvious that the inoculation of beneficial microorganisms in the form of BFBFs along with 50% CF, enhanced the biochemical parameters in the tuber and other tissues of the host plant.

### 5.4.5 The effect treatment combinations on pH of different plant tissues

In comparison with all other treatments, treatment TR3 showed the lowest pH of the leaf tissues (5.44 ± 0.006), significant lowest pH of the stem tissues ($P < 0.05$, 5.24 ± 0.03) and the significant lowest in stolon tissues ($P < 0.05$, 5.24 ± 0.02) at Bandarawela field site (table 5.3). Though there was no significant difference, the treatment TR3 showed the lowest pH in leaf (5.57 ± 0.04), stem (5.36 ± 0.03) and stolon tissues (5.31 ± 0.05) at Bibile field site (table. 5.3).

Table 5.3- The effect of different BFBF1 and CF treatments on pH of different potato plant tissues.

| Treatments | Bandarawela | | | Bibile | | |
|---|---|---|---|---|---|---|
| | pH-leaf Mean±SD | pH- stem Mean±SD | pH- stolon Mean±SD | pH- leaf Mean±SD | pH- stem Mean±SD | pH- stolon Mean±SD |
| TR1 | $5.45\pm0.031^{b}$ | $5.35\pm0.021^{c}$ | $5.32\pm0.021^{c}$ | $5.61\pm0.025^{b}$ | $5.40\pm0.025^{c}$ | $5.36\pm0.025^{b}$ |
| TR2 | $5.50\pm0.015^{b}$ | $5.37\pm0.026^{c}$ | $5.36\pm0.026^{c}$ | $5.61\pm0.017^{b}$ | $5.40\pm0.016^{c}$ | $5.37\pm0.020^{b}$ |
| TR3 | $5.44\pm0.006^{b}$ | $5.24\pm0.032^{d}$ | $5.24\pm0.020^{d}$ | $5.57\pm0.038^{b}$ | $5.36\pm0.025^{c}$ | $5.31\pm0.050^{b}$ |
| TR4 | $5.72\pm0.057^{a}$ | $5.52\pm0.032^{b}$ | $5.51\pm0.031^{b}$ | $5.74\pm0.015^{a}$ | $5.48\pm0.020^{b}$ | $5.49\pm0.023^{a}$ |
| TR5 | $5.77\pm0.025^{a}$ | $5.68\pm0.025^{a}$ | $5.71\pm0.015^{a}$ | $5.74\pm0.025^{a}$ | $5.55\pm0.032^{a}$ | $5.51\pm0.015^{a}$ |

Treatments TR1, TR2, TR3, TR4 and TR5 are 100% CF, 50% CF, 50% CF+ BFBF1, BFBF1 alone and No amendments treatments respectively. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

It has been reported that $H^+$ concentration of potato tissues is regulated accompany with the sucrose content due to the presence of sucrose- $H^+$ co transporter protein in the cell membrane. Therefore, $H^+$ concentration increases along with the influx of sucrose in potato tissues (Boorer *et al.,* 1996). It has been proven that the sucrose content of the stolon tissues is remarkably increased along with the tuberization (Hawker *et al.*, 1979). Therefore, in the current study, low pH shown by the stolon and stem tissues might be due to the massive influx of $H^+$ along with the influx of sucrose.

### 5.4.6 The effect of different treatments on $Ca^{2+}$ content of different plant tissues

In comparison with other treatments, significant enhancement of $Ca^{2+}$ content was observed from the treatment TR3 in leaf ($P < 0.05$, 0.54 ppm ± 0.07) and stolon ($P < 0.05$, 0.42 ppm ± 0.006) tissues compared to the other treatments at Bandarawela field site (table 5.4). Though there was no significant difference, treatment TR3 showed the highest $Ca^{2+}$ content in stem tissues (0.422 ppm ± 0.006). Further, the highest significant $Ca^{2+}$ content was observed from the treatment TR3 in all tissues (leaf- $P < 0.05$, 0.56 ppm ± 0.02. stem- $P < 0.05$, 0.52 ppm ± 0.03. stolon- $P < 0.05$, 0.58 ppm ± 0.004) compared to the other treatments at Bibile field site (table 5.4).

Table 5.4- The effect of different BFBF1 and CF treatments on $Ca^{2+}$ content of different potato plant tissues.

| Treatments | Bandarawela | | | Bibile | | |
|---|---|---|---|---|---|---|
| | $Ca^{2+}$-leaf Mean±SD | $Ca^{2+}$- stem Mean±SD | $Ca^{2+}$- stolon Mean±SD | $Ca^{2+}$- leaf Mean±SD | $Ca^{2+}$- stem Mean±SD | $Ca^{2+}$- stolon Mean±SD |
| TR1 | $0.37 \pm 0.013^{b}$ | $0.33 \pm 0.008^{bc}$ | $0.32 \pm 0.001^{bc}$ | $0.38 \pm 0.025^{c}$ | $0.40 \pm 0.023^{b}$ | $0.33 \pm 0.014^{c}$ |
| TR2 | $0.29 \pm 0.041^{bc}$ | $0.30 \pm 0.012^{c}$ | $0.24 \pm 0.037^{cd}$ | $0.30 \pm 0.013^{d}$ | $0.27 \pm 0.044^{c}$ | $0.25 \pm 0.027^{d}$ |
| TR3 | $0.54 \pm 0.071^{a}$ | $0.42 \pm 0.006^{a}$ | $0.60 \pm 0.090^{a}$ | $0.56 \pm 0.018^{a}$ | $0.52 \pm 0.033^{a}$ | $0.58 \pm 0.004^{a}$ |
| TR4 | $0.49 \pm 0.007^{a}$ | $0.38 \pm 0.002^{ab}$ | $0.43 \pm 0.012^{b}$ | $0.47 \pm 0.038^{b}$ | $0.42 \pm 0.014^{b}$ | $0.45 \pm 0.032^{b}$ |
| TR5 | $0.19 \pm 0.037^{c}$ | $0.17 \pm 0.040^{d}$ | $0.16 \pm 0.006^{d}$ | $0.11 \pm 0.018^{e}$ | $0.09 \pm 0.025^{d}$ | $0.11 \pm 0.023^{e}$ |

$Ca^{2+}$ concentrations – ppm values. Treatments TR1, TR2, TR3, TR4 and TR5 are 100% CF, 50% CF, 50% CF+ BFBF1, BFBF1 alone and No amendments treatments respectively. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

Though Bandarawela has potential to cultivate potato, favorable climatic conditions (thermoperiod) for commercial potato cultivation has not been recorded at Bibile and Padukka regions. Therefore, those regions are considered as potato non- inducing regions. However, in the current study high potato tuberization was observed by the application of beneficial microorganisms in the form of BFBFs treatments irrespective of the climatic conditions even at the Bibile field site. Further, it was noted that all biofilm treatments along with 50% CF (TR3 and TR4) enhanced soil and tissue $Ca^{2+}$ content compared to the other treatments at both Bandarawela and Bibile field sites. Therefore, there might be a relationship between the enhancement of tuberization and $Ca^{2+}$ content of the soil and tissues. Similarly reports have suggested the possible involvement of $Ca^{2+}$-sensor proteins such as calmodulin (CaM) (Jena, Reddy and Poovaiah, 1989) and potato $Ca^{2+}$ dependent protein kinase (StCDPK1) in tuberization of potato (MacIntosh *et al.*, 1996; Jackson, 1999). In spite of evidences for the positive role of $Ca^{2+}$ and various $Ca^{2+}$- regulated proteins in potato tuberization, the exact molecular mechanism of $Ca^{2+}$ and $Ca^{2+}$- induced signal

pathways controlling tuberization is not studied well. However, it has been reported that cytosolic $Ca^{2+}$ in plant cells is maintained at a low concentration in the absence of a stimulus but in response to an external stimuli including light, touch, wind, gravity, hormones, abiotic and biotic stresses, the concentration is rapidly elevated via an increased $Ca^{2+}$ influx due to the release of $Ca^{2+}$ by $Ca^{2+}$ channels (Poovaiah and Reddy, 1993; Nookaraju *et al.*, 2012) Therefore, in the current study, the elevation of tissue $Ca^{2+}$ content might be due to the induction activity of introduced biofilm in the form of BFBFs. This implies that the induced $Ca^{2+}$ ions have a particular mechanism to regulate tuberization of potato.

### 5.4.7 Correlations between different biochemical and physiological properties of potato tissues and soil

Significant negative correlation was observed between mean tuber weight and mean soil pH at both Bandarawela ($r = 0.890$, $P = 0.043$) and Bibile ($r = 0.809$, $P = 0.047$) field sites (table 5.5). Further, another significant negative correlation was observed between mean tuber starch content and mean soil pH at both field sites (Bandarawela- $r = 0.991$, $P = 0.001$ and Bibile- $r =0.812$, $P = 0.043$). This implies that potato tuberization is greatly affected by the soil pH and this might be due to the introduction of beneficial microorganisms in the form of BFBFs which creates low pH around the rhizosphere. In the current study, the lowest soil pH (Fig. 5.2) and the highest potato tuberization (table 4.4, 4.5, 4.6) were recorded from BFBF1 treatment along with 50% CF at all three field sites. Further, higher IAA production was observed by the biofilm combination BFBF1 (Fig. 3.7b).

Table 5.5- Correlation coefficients (r) between different potato plant and soil properties at Bandarawela (Ba) and Bibile (Bi) field sites

| | | Soil pH | TW | TS | LRS | Ch.5th | Ch.3rd |
|---|---|---|---|---|---|---|---|
| TW | Ba | -0.890 (0.043) | - | - | - | - | - |
| | Bi | -0.809 (0.047) | - | - | - | - | - |
| TS | Ba | -0.991 (0.001) | 0.891 (0.043) | - | - | - | - |
| | Bi | -0.812 (0.043) | 0.948 (0.014) | - | - | - | - |
| LRS | Ba | -0.911 (0.031) | 0.865 (0.058) | 0.900 (0.038) | - | - | - |
| | Bi | -0.808 (0.098) | 0.969 (0.007) | 0.987 (0.002) | - | - | - |
| Ch.5th | Ba | -0.963 (0.008) | 0.926 (0.024) | 0.974 (0.005) | 0.819 (0.090) | - | - |
| | Bi | -0.887 (0.045) | 0.974 (0.005) | 0.942 (0.017) | 0.972 (0.006) | - | - |
| Ch.3rd | Ba | -0.929 (0.022) | 0.961 (0.009) | 0.945 (0.015) | 0.807 (0.098) | 0.989 (0.001) | - |
| | Bi | -0.974 (0.005) | 0.901 (0.037) | 0.788 (0.113) | 0.865 (0.059) | 0.946 (0.015) | - |
| SCa | Ba | -0.994 (0.000) | 0.893 (0.041) | 0.972 (0.006) | 0.914 (0.030) | 0.947 (0.014) | 0.914 (0.030) |
| | Bi | -0.988 (0.002) | 0.836 (0.077) | 0.884 (0.037) | 0.866 (0.058) | 0.914 (0.030) | 0.959 (0.010) |

(TW- tuber weight, TS- tuber starch content, LRS- leaf reducing sugar content, Ch. $5^{th}$- Chlorophyll content of $5^{th}$ leaf, Ch. $3^{rd}$- Chlorophyll content of $3^{rd}$ leaf, Sca- soil $Ca^{2+}$ content)
Values within parentheses are probability levels.

It has been reported that beneficial microbial communities reduce soil pH through secretion of different types of organic acids e.g. carboxylic acid (Deubel and Merbach, 2005) and plant growth promoting hormones like IAA. It is well-established that the rhizosphere is generally possess a low pH, and many microbial strains interact efficiently with plants at this low pH (Ahamad *et al.*, 2014). Further, a significant enhancement of mean tuber weight, plant height and the tuber number has been recorded by the inoculation of *Bacillus* sp. (Ekin *et al.*, 2009). Another study has shown that the inoculation of *Azotobactor* sp. and *Glumus* sp. significantly enhanced the tuber number and the plant height of potato (Yao *et al.*, 2002; Douds *et al.*, 2007). It has been recorded that most of the *Bacillus* sp and *Azotobactor* sp possess the ability to produce elevated amount of IAA and thereby enhanced the plant growth and development (Saharan and Nehra, 2011). Therefore, the relationship between soil pH and the tuber weight

might be due the production of plant growth hormones like IAA by the biofilm combination BFBF1.

Strong significant positive correlation was observed between mean tuber starch content and mean leaf reducing sugar content at both Bandarȧwela ($r = 0.9$, $P = 0.038$) and Bibile ($r = 0.987$, $P = 0.002$) field sites (table 5.5). It has been well documented that reducing sugar is the substrate for starch synthesis and there is a close relationship between the reducing sugar content and starch synthesis in plants (Xing-lu, Zhong-yong and Ying-hua, 2006; Luo and Huang, 2011). This study has further shown that the starch accumulation in the tuberous roots had a positive correlation with the soluble sugar content in cassava leaves and a negative correlation with the leaf reducing sugar content as well as the sucrose contents in leaves (Luo and Huang, 2011). However, in the current study, it was clearly observed that the reducing sugar content had a strong positive correlation with tuber starch accumulation of potato plant. Similar relationship has been observed by another study that the fluctuation of sugar content in wheat leaves and stems has correlated with the starch accumulation in the seeds and the trend is that increasing of sugar content in leaves and stems is good for starch accumulation in the seeds. (Luo and Huang, 2011).

Significant positive correlations were observed between mean tuber starch content and mean chlorophyll contents $5^{th}$ leaf of potato at Bandawela ($r = 0.974$, $P = 0.005$) and Bibile ($r = 0.942$, $P = 0.017$). Further, though a significant positive correlation was observed between tuber starch and $3^{rd}$ leaf of potato at Bandarawela field site ($r = 0.945$, $P= 0.015$), significant correlation was not observed at Bibile site ($r = 0.788$, $P= 0.133$). Similar positive relationships between chlorophyll content and the carbohydrate content of the plants have been reported by several other studies (Kapulnik *et al.*, 1981; Chavan *et al.*, 1997). It has been reported that the chlorophyll content of potato leaves is directly enhanced by the activity of $N_2$ fixing microorganisms through the enhancement of plant N and other nutrient content. Further, this study has shown that the carbohydrate assimilates of potato plant has direct relationship with the chlorophyll content of the leaf (Kapulnik *et al.*, 1981). Moreover, another study has reported that the inoculation of beneficial microorganisms enhanced the metal elements like zinc, manganese, iron and copper which help to synthesize chlorophyll of tissues in wheat, sorghum and panicum plants (Chavan *et al.*, 1997).

Significant positive correlations were observed between mean soil $Ca^{2+}$ content and mean tuber starch content at both Bandarawela (r = 0.972, P = 0.006) and Bibile (r = 0.884, P = 0.037) field sites. Further, a significant positive correlations were observed between tuber starch content and tuber weight of potato at both field sites (Bandarawela- r = 0.891, P = 0.043 and Bibile- r = 0.948, P = 0.014). It has been reported by another study that the starch content is positively correlated with dry matter percentage of stolon tips, and is a good indication of transition of stolon tips to tubers (Helder and Vreugdenhil 1999; Minahas *et al.*, 2004). This implies that the starch deposition process is greatly regulated by the soil $Ca^{2+}$ content and the tuber weight is mostly due to the starch accumulation. Similar relationship has been observed by another study that the increment of starch and protein content in the tuber is proportional to the fresh weight during the whole period from tuber initiation to tuber enlargement (Sasamoto and Suzuki, 1979). Though a significant correlation was observed between soil $Ca^{2+}$ content and tuber starch (r = 0.893, P = 0.041) at Bandarawela site, a significant correlation was not observed between those parameters (r = 0.836, P= 0.077) at Bibile site.

The potato tuber is a massive storage of macromolecules, most notably starch and protein. Therefore, the tuberization is mostly regulated by the movements of macromolecules from leaf tissues to sink organs such as stolon tips where the tuber is formed (Okazava, 1967). It has been reported that a wide variety of soil, environmental and hormonal stimuli are known to be involved either directly or indirectly in the induction of potato tuberization through starch deposition (Sasamoto and Suzuki, 1979; Nookaraju *et al.*, 2012). Further it has been noted that increased sucrose transport would greatly affect carbon allocation and sugar signal generation (Youjun, 2011). Therefore, it is important to study the effect of an exogenous stimuli on the endogenous levels of different biochemical processes which induce tuberization.

Among the soil factors, $Ca^{2+}$ nutrition plays an important role in potato tuberization. Experiments with single-node leaf cuttings from induced potato (*Solanum tuberosum* L.) plants proved the possible role of $Ca^{2+}$ as a mediator of tuberization stimulus (Balamani *et al.,* 1986). Several other studies reported the effect of $Ca^{2+}$ on tuberization of potato under field conditions (Chang *et al.*, 2007; Nookaraju *et al.,* 2012). It has been reported that $Ca^{2+}$ acts as intracellular messenger in the cytosol (Marschner, 1995). Further, it acts as a mediator of stimulus-response and provide signals in the regulation of diverse biochemical and

morphological responses (Nookaraju *et al.,* 2012). It has been shown that a given signal may induce a different $Ca^{2+}$ levels in different cell types (Kiegle *et al.,* 2000). Most of the $Ca^{2+}$-dependent cellular processes is facilitated through intracellular $Ca^{2+}$ - binding or $Ca^{2+}$ modulator proteins, also known as $Ca^{2+}$-sensors. The increase in the cytosolic $Ca^{2+}$ concentration stimulates various $Ca^{2+}$-sensor proteins that convert the external signals into a wide variety of biochemical changes. It has been reported that soil $Ca^{2+}$ influences tuberization by altering the hormonal balance at the stolon tip (Nookaraju *et al.*, 2012). In a field study, the influence of $Ca^{2+}$ application on potato tuber yield has been investigated (Simmons and Kelling, 1987). It has been reported that application of exogeneous $Ca^{2+}$ resulted high quality tubers with increased tuber size. This might be due to the induction activity of exogenous $Ca^{2+}$ on starch accumulation process, thereby enhances tuber weight (Sasamotto and Suzuki, 1979). This indicates the induction activity of $Ca^{2+}$ on tuberization signal. According to a previous study, compared to non-induced plants, induced plants accumulate three times more starch in the leaves during the day and subsequently transport it out of the leaves towards tubers during the dark period (Lorenzen & Ewing, 1992; Minhas *et al.*, 2004). Application of P solubilizing microorganisms has been reported to enhance soil $Ca^{2+}$ content by releasing the $Ca^{2+}$ from Ca- Phosphates due to the acid production (Khan *et al.,* 2009). This clearly implies that the tuberization induction activity is regulated by exogenous signals like $Ca^{2+}$, other biochemical signals or physiological factor through sugar-starch conversion process.

Plant hormones are also considered as chemical messengers that affect a plant's ability to respond to its environment. Further, it has been well documented that exogenous plant growth regulators play a prominent role in the induction of potato tuberization (Ewing, 1987; Ghavidel *et al.,* 2012). The possible role of GA and Auxin in this process was extensively studied, mainly in experiments in which this compound was applied exogenously. These experiments showed that application of GA promotes stolon elongation and inhibits tuber formation (Smith and Rappaport, 1969; Xu *et al.,* 1998). It was also reported that a decline of GA activity in potato (*Solanum tuberosum* L.) plants is associated with tuberization (Okazawa, 1959; Xu *et al.,* 1998). It has been observed that IAA treatment induces larger tubers at an earlier stage and auxin content is high in the stage before tuber initiation and decreases during tuber development (Harmey *et al.,* 1966; Sasamoto and Suzuki, 1979). Many plant-associated microorganisms have the capability of producing plant hormones such as IAA, GA and Cytokinin which act as signaling molecules to alter the biochemical

and physiological status of the host plant (Sreenivasa, Naik and Bhat, 2009; Saharan and Nehra, 2011). Plant-associated IAA producers include many species of bacteria, as well as fungi and yeasts (Costacurta and Vanderleyden, 1995; Kim *et al.*, 2011).

In the current study, it was recorded that the application of BFBF1 along with 50% CF enhanced soil and tissue $Ca^{2+}$ content (Fig. 5.5 and table 5.4), tuber starch content (Fig. 5.6) and ultimately tuber fresh weight at Bandarawela field site (table 4.4) which possess a tuber inducing climatic condition. It is noteworthy that similar responses were recorded from Bibile field site though it possesses non-induce climatic conditions for potato cultivation. Further, the biofilm combination in the treatment BFBF1 did not produced tuber inhibiting hormone, GA (Smith and Rappaport, 1969) and produced elevated amount of tuber inducing IAA hormone. Therefore, this implies that the elevated IAA production and soil $Ca^{2+}$ content by the treatment TR3 (BFBF1 along with 50% CF) might act as potential stimulators for the tuberization enhancement by altering the essential biochemical processes in potato tissues irrespective of the climatic conditions.

### 5.4.8 The effect of biofilm treatment combinations on microbial diversity of soil

The highest significant mean CFU value was observed from treatment TR3 ($P < 0.05$, 23.67 $g^{-1} \pm 2.5$) compared to all other treatments except treatment TR4 (Fig. 5.8). It is noteworthy that treatment TR3 enhanced the CFU by approximately 39% compared to treatment TR2 (17 $g^{-1} \pm 1$) and by approximately 69% compared to treatment TR1 (14 $g^{-1} \pm 1.7$). Another study has reported the enhancement of CFU in soil microorganisms by the application of higher order biofilm including three different bacteria with *Trichoderma harzianum* (Srinath *et al.,* 2003). Further, it was noticed that the treatment TR1 reduced the CFU by approximately 21.5% compared to treatment TR2 which was half CF application of TR1. It has been reported that the N fertilizers suppress the action of microbes (Kolb and Martin, 1988; Cruz *et al.,* 2009), thus, reduced fertilizer rates should also enhance growth of the microbes and consequently their beneficial effects on the plant growth and the soil (Seneviratne *et al.*, 2011). Moreover, all the BFBFs treatments (TR3 and TR4) enhanced the CFU compared to the treatments with only CF applications. CFU represents the species richness and the species abundance of soil (Bloem *et al.*, 2006). Therefore, this clearly implies that higher rate of CF applications reduces the species richness and abundance.

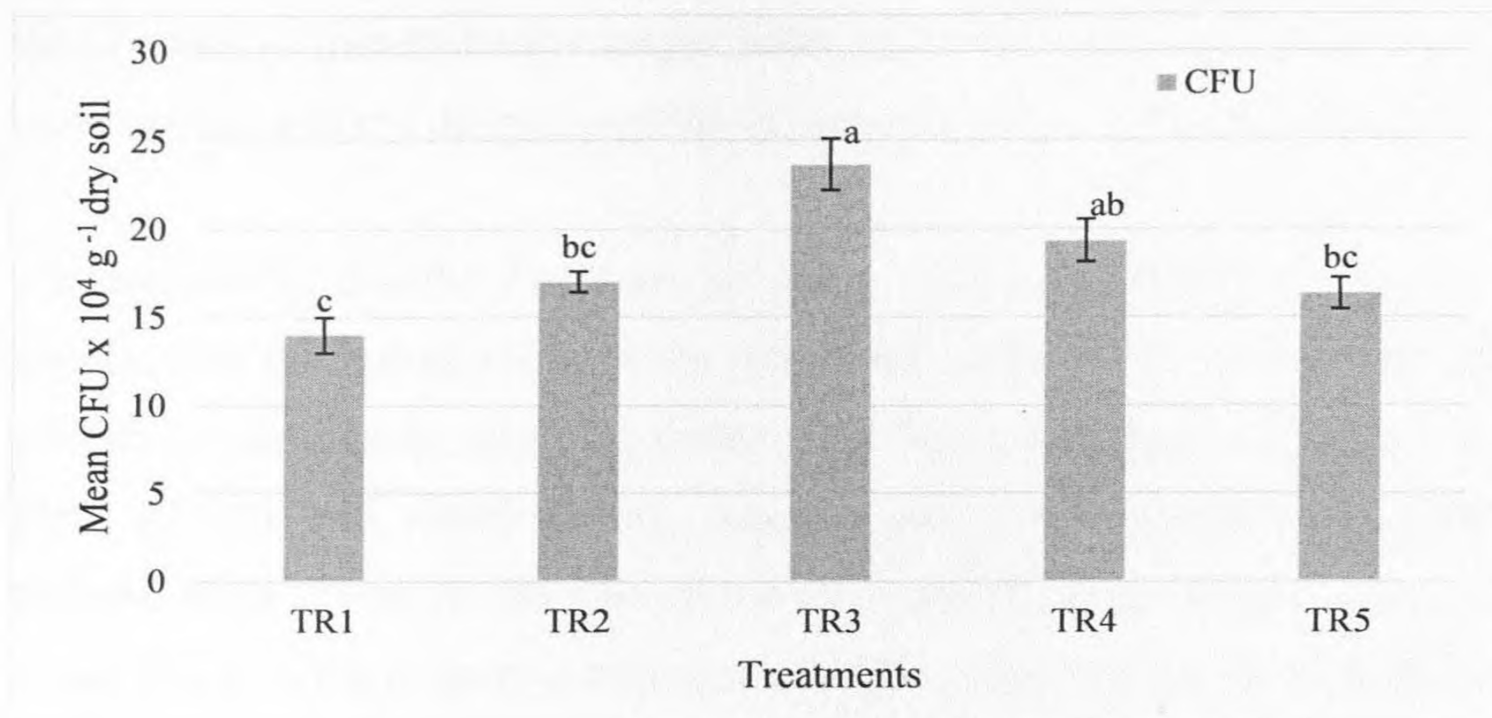



Figure 5.8- Mean CFU values of soil treated with different BFBFs and CF combinations at Bandarawela field site. Treatments TR1, TR2, TR3, TR4 and TR5 are 100% CF, 50% CF, 50% CF+ BFBF1, BFBF1 alone and No amendments treatments respectively. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

It was noticed that the species richness of both bacteria and fungi was high in treatment TR3 compared to the other treatments (Table 5.6). Originally, the BFBF1 contained two bacteria and one fungi, of which the bacterial (*Bacillus* sp.) and the fungal species (*Trichoderma* sp.) were isolated from BFBFs treated soil. Major bacterial species observed from the soil treated with treatment TR3 were three different *Bacillus* sp. and one Gram's negative rod shape bacterial strain whereas major fungal species were *Trichoderma* sp., *Colletrotrichum* sp. and two *Aspergillus* sp. Further, *Pseudomonas* sp. and one Gram's positive bacterial sp. were the common species found in all treatments whereas *Phytopthora* sp. was identified as the common fungal species in all treatments. *Phytopthora* sp. is a common plant pathogen found in most of the potato fields which causes severe disease known as 'potato late blight' (Wu *et al.,* 2013).

It was noted that the application of CF (treatments TR1 and TR2) reduced the species richness and the CFU compared to the treatments with BFBFs (Fig.5.8 and table 5.6). Similar reduction of species richness has been observed by another study (Buddhika *et al.,* 2013). *Fusarium* sp which is a common plant pathogen (Daboussi and Langin, 1994), and *Phytopthora* sp were prominent in the soil treated with only CF (TR1 and TR2). This may be due to the destruction of natural balance of soil microbial diversities and the lack of natural control of pathogens due to the application of CF (Seneviratne, 2009; Buddhika *et al.,* 2013). It has been reported that the adverse soil conditions generated by the 100% CF application collapse beneficial microbial communities (Seneviratne, 2009), leading the

inactivation of metabolically active microbial cells (Buddhika *et al.,* 2013). Therefore, the composition and the diversity of the microbial communities in such soil become altered.

It is noteworthy that the *Fusarium* sp was not identified in the soil treated with BFBFs. This implies that the introduction of the beneficial microorganisms through BFBF suppress the growth of pathogenic microorganisms and thereby creates favorable soil environment for plant growth and development. Another study has reported the ability of producing antimicrobial agents by *Bacillus* sp which suppress the pathogenic microorganisms (Kumar *et al.,* 2012). This is an indication of possible effects of the BFBFs along with 50% CF in maintaining microbial balance for natural biocontrol of pathogens. This was confirmed by another study expressing that many wild strains of *Bacillus subtilis* are capable of forming biofilms on plant root surfaces and that this capability is critical during the suppression of phytopathogens through the production of antimicrobial agents (Chen *et al.,* 2012). It has been reported that the beneficial biofilm inoculum suppress the pathogenic microbial growth through the production of diverse biochemical compounds (Seneviratne, 2003a; Herath *et al.*, 2013). These active compounds including enzymes, antibiotics, siderophores, and the plant hormone IAA, produced by potato-associated endophytes, have been reported to antagonize fungal or bacterial pathogens in the rhizosphere (Sessitsch *et al.,* 2004; Wu *et al.,* 2013). Further, it has been reported that the biocontrol amendments *Trichoderma virens*, has increased microbial activity and bacterial populations in organic and conventional potato production systems (Bernard *et al.,* 2012). Moreover, fungus *Glomus intraradices* has been reported to suppress the development of potato dry rot, a post-harvest disease caused by the fungus *Fusarium sambucinum* (Niemira, Hammerschmidt and Safir, 1996; Wu *et al.,* 2013).

Table 5.6-Microbial isolates found in potato rhizosphere under different treatments

| Treatments | Microbial diversity | |
|---|---|---|
| | Bacteria | Fungi |
| TR1 | *Pseudomonas* sp.<br>Un-identified Gram's positive rod | *Phytopthora sp.*<br>*Fusarium* sp. |
| TR2 | *Pseudomonas* sp.<br>*Bacillus* sp. 1<br>Un-identified Gram's positive rod | *Phytopthora* sp.<br>*Fusarium* sp |
| TR3 | *Bacillus* sp. 1<br>*Bacillus* sp. 2<br>*Bacillus* sp. 3<br>*Pseudomonas* sp.<br>Un-identified Gram's negative rod<br>Un-identified Gram's positive rod | *Trichoderma* sp.<br>*Phytopthora* sp.<br>*Aspergillus* sp. 1<br>*Aspergillus* sp. 2<br><br>*Colletotrichum* sp. |
| TR4 | *Bacillus* sp. 2<br>*Bacillus* sp. 3<br>Un-identified Gram's negative rod<br>Un-identified Gram's positive rod | *Trichoderma* sp.<br>*Phytopthora* sp. |
| TR5 | *Pseudomonas* sp.<br>*Bacillus* sp. 1<br>Un-identified Gram's positive rod | *Phytopthora* sp<br>*Fusarium* sp. |

Further, results indicated that the application of BFBF1 together with 50% CF (TR3) supported emergence of new fungal species (*Aspergillus* 1, *Aspergillus* 2 and *Colletotrichums* sp) which were not identified in other treatments. This might be due to the induction of dormant microbial seed bank which already exists in the soil. It has been reported that the application of the BFBFs along with 50% CF tends to break dormancy of microbial seeds bank in the soil, which causes emergence of diverse microbes (Buddhika *et al.*, 2013). Further, it has also been reported that FBBs produce diverse and unique set of biochemical compounds (*e.g.* low molecular weight sugars and amino acids) which have the ability to break dormancy of microbial spores and conidia (Saini *et al.*, 1986; De Boer, Folman and Summerbell, 2005; Herath *et al.*, 2013).

### 5.5 Conclusions

It is noteworthy that the treatment TR3 (50% CF + BFBF1) responded positively to create favorable soil biochemical (SOC, MBC, N, P, K and $Ca^{2+}$), physiological (pH) and biological conditions (microbial diversity) while improving the internal biochemical (tissue $Ca^{2+}$, tuber nutrients, leaf reducing sugar, stolon starch) and physiological (pH, chlorophyll content) status of the potato plant, leading to the enhanced tuberization. It was confirmed that BFBF1 treatment along with 50% CF increased tuberization at all tested field sites, irrespective of the climatic conditions. Thus, it can be concluded that the exogenous favorable biochemical physiological and physiochochemical factors created by the biofilm BFBF1 along with the 50% CF was capable of inducing the internal biochemical and physiological status, leading to the improved tuberization. The biofilm action has compensated for the climatic conditions required for increased tuber production.

# CHAPTER 6

# THE EFFECT OF DEVELOPED FUNGAL-BACTERIAL BIOFILMS ON RHIZO-REMEDIATION OF POTATO

## 6.1 Introduction

In the last five decades, the rate of NPK fertilizer and agrochemical application has increased tremendously. Although mineral fertilization has been shown to have beneficial effect on crop yield, incorrect agro technical measures and improper fertilization practices can lead to seriously disturbed functions of the entire agro ecosystems and contribute to the formation of different contaminants in soil (e.g. nitrosamines and heavy metals) which are harmful for soil microorganisms and cultivated plants, and in turn for animals and humans. This detrimental phenomenon, a side-effect of the use of chemicals in agriculture, definitely influences the activity of soil microorganisms, and consequently it indirectly affects fertility of arable soils (Barabasz *et al.*, 2002).

The application of N fertilizers in large amounts is associated with risks such as increased nitrate concentration in the edible parts (Schenk, 2004). The nitrate ion has a low level of acute toxicity, however it can be transformed into nitrite, which has much higher acute toxicity (Fishbein, 1979). In addition, mineral N, introduced into soil environments, undergoes various biochemical transformations and produce numerous toxic chemicals like nitroso compounds (Barabasz *et al.*, 2002). The most recent studies have indicated that out of numerous contaminants in the soil, nitrosamines deserve special attention as they are included among the most dangerous ecological poisons (Barabasz *et al.*, 2002). The studies have demonstrated that nitrosamines endanger also all organisms inhabiting ecosystems and biological productivity of agro ecosystems, field crops, food products, and health of animals and humans.

Nitrosamines are candidates for serious consideration as human carcinogens because they are such potent producers of cancer in a variety of animals (Barabasz, *et al.*, 2002). To date numerous nitrosamines have been tested and approximately 80% have proved to be carcinogenic, primarily in the liver, esophagus, respiratory system and kidney (Fishbein, 1979). Dimethyl nitrosamine is an extremely toxic compound belongs to the nitroso group

commonly found in soil in low concentrations (Pancholy 1977; Mhlongo, Mamba, Krause, 2009). They can generate in agricultural soils treated with pesticides, fungicides like thiram and receiving heavy applications of N fertilizer like urea (Ayanaba *et al.*, 1973a). Elevated levels of nitrosamines in soils and numerous agricultural products like potato have been previously reported (Anon, 1976; Raymond and Alexander, 1976). Nitroso compounds have been found in amounts ranging from $10^{-3}$ ppm to as high as about 48 ppm (Fishbein, 1979). Another study has reported that level exceeded even 25 ppm in highly contaminated soil (Barabasz *et al.*, 2002). Further, it has been reported that the precursor generation and formation of different nitrosamines in soil environments depends on many ecological and physicochemical factors such as pH, type of soil, vegetation cover, agrotechnical measures, applied pesticides and N fertilization rate (Brewer, Draper and Wey, 1980; Barabasz *et al.*, 2002). The yield of nitrosamines has been reported to increase with decreasing soil pH (Sander and Schwinsberg, 1973; Mirvish, 1972). The acidic sandy loam contained twice as much nitrosamines as the neutral sandy loam and clay loam (Pancholy, 1977).

It has been reported that the natural degradation of nitrosamine contaminants in soil is very slow (Tate and Alexander, 1975). A greater persistence of dimethyl nitrosamine in soil with low organic matter and low microbial activity has been reported by several other studies (Yang *et al.*, 2005; Gan *et al.*, 2006). Studies conducted with undefined aerobic microbial consortia have demonstrated that dimethyl nitrosamine is biodegradable in laboratory incubations (Gunnison *et al.*, 2000). In addition, biological activity has been implicated for the disappearance of dimethyl nitrosamine from groundwater at a field site in Colorado (Gunnison *et al.*, 2000). Another study has reported that a variety of monooxygenase expressing bacterial strains are capable of degrading most of the nitrso compound available in soil (Sharp, Wood and Alvarez-Cohen, 2005).

Moreover, heavy metals are another important group of environmental pollutants threatening the health of human population and natural ecosystems. Heavy metals can affect the quality of agricultural soils, including phytotoxicity and transfer of heavy metals to the human diet from crop uptake (Nicholson *et al.* 2003; Premarathna, Hettiarachchi and Indraratne, 2011). Elevated concentrations of heavy metals in harvested plant tissue could expose consumers to excessive levels of potentially hazardous chemicals (George *et al.*, 2011).Metals such as lead (Pb), mercury (Hg), cadmium (Cd), and copper (Cu) are cumulative poisons, which cause environmental hazards and are reported to be exceptionally toxic (Ellen, Loon and

Tolsma, 1990). It has been well documented that synthetic fertilizers and pesticides contain trace metals as impurities or active ingredients (McLaughlin *et al.*, 2000; Pierzynski, Sims and Vance, 2000). For example, chemical phosphatic fertilizers such as TSP can contain appreciable amounts of Cd (Pierzynski *et al.*, 2000) and Cd contamination in soils of many countries is mainly a result of the use of P fertilizers (McLaughlin *et al.*, 1996). These P fertilizers also unintentionally introduce other contaminants, such as Hg, As, and Pb, into agricultural soils (Premaratne *et al.*, 2005). Further, those contaminants in plants are directly associated with their concentrations in soils (Ozturk *et al.*, 2011). For instance, high concentrations of elemental arsenic (As), Cd, Pb, and Zn have been found in potatoes samples from overused phosphate-fertilized soils, which increased the daily intake of metals in food (Cheraghi *et al.*, 2013).

Generally, heavy metals cannot be degraded easily and persist in the environment indefinitely (Khan *et al.*, 2009). Basically, the removal of those metals is often performed by oxidation– reduction processes, filtration, evaporation, ion exchange or reverse osmosis (Quintelas *et al.*, 2009). However, these methods present several disadvantages like high energy and chemical requirements. In this context, rhizo-remediation through soil beneficial microorganisms has been considered as one of the promising methods to reduce soil toxicities. Numerous strains of PGPRs possessing metal reducing ability have been identified (Faisal and Hasnain 2005; Khan *et al.*, 2009). The removal of metals from contaminated soils by beneficial microbial communities can be carried out by artificial introduction of viable population to contaminated sites (bio-augmentation), or stimulation of viable native microbial population (bio-stimulation). Once accumulated in the soils, the toxic metals inversely affect the microbial communities in the rhizosphere including biofilms. Biofilms have been found to be suitable for the remediation of pollutants because of their high microbial biomass and ability to immobilize pollutants (Quintelas *et al.*, 2009). There are reports on the application of biofilms for the removal of heavy metals. Recently, bioremoval of Cr (III) using bacterial biofilm in a continuous flow reactor has been reported. The ability of a biofilm of *Escherichia coli* supported on Na zeolite for the removal of Cr (VI), Cd (II), Fe (III), Ni (II) from wastewater was also reported (Das *et al.*, 2012). For survival under metal-stressed environment, microbial biofilm communities in the soil have evolved several mechanisms including (1) the pumping of metal ions exterior to the cell (2) accumulation and sequestration of the metal ions inside the cell (3) transformation of toxic

metal to less toxic forms (Wani, Khan and Zaidi, 2008) and adsorption/desorption of metals (Mamaril, Paner and Alpante, 1997; Khan *et al.*, 2009).

## 6.2 Objective

- To investigate the rhizo-remediation ability of nitrosamine and heavy metal contaminations by the effective BFBF1.

## 6.3 Methodology

### 6.3.1 Rhizo-remediation abilities of different treatments on nitrosamine

A pot experiment was conducted (as a simulation experiment) under greenhouse conditions to evaluate the rhizo-remediation abilities of different treatments on nitrosamine. The experiment was conducted from May 2014 to June 2014 at the Regional Agriculture Research and Development Center, Bandarawela, Sri Lanka.

#### 6.3.1.1 Media preparation and planting seed potato

River sand was sieved (particle size ≤ 1mm) and washed three times with tap water before sterilization. Then the sand medium was heat sterilized using a dry oven at 160 °C for 2 hours after adjusting the pH to 5-7. Six inch diameter black plastic pots were used for the experiment and were filled with sterilized sand medium. Disease free seed tubers ('Granola' variety) were obtained from government certified Regional Agriculture Research and Development Center, Bandarawela. Two seeds were grown in each pot with sand medium separately.

#### 6.3.1.2 Application of biofilm cultures

Responsive biofilm culture (BFBF1) was maintained in a biofilm forming medium under continuous mixing. Low cost medium was used as the carrier material to inoculate the biofilm culture to the plants. Approximately 5 ml of broth culture was inoculated initially with 100 ml of sterilized low cost medium and incubated for 5 days with continuous mixing. In the meantime, sustaining ability of the microorganisms in the biofilm with the low cost medium was confirmed through the microscopic observations. At the time of seed sowing,

diluted biofilm mixture (250 times with clean water) was sprayed directly around the root zone of the potato plants grown in sand pots. Biofilm inoculation was repeated again after five weeks from seed sowing.

**6.3.1.3 Fertilizer application**

Albert solution (1%) was used as the basal CF and applied at the time of the seeds were sown. After 8- 10 days, the concentration of the Albert solution medium was increased to 10% and applied once in two days until the harvesting. Rates of the CF application was calculated per plant basis according to the DOA recommendations. Water level of the medium was maintained constantly by applying 250 ml of water for each pot every day. Plants were grown with a daily minimum-maximum temperature range of 20 °C – 30 °C.

**6.3.1.4 Application of nitrosamine**

Approximately 3 ppm nitrosamine stock solution was prepared using N-butyl-N- (4-hydroxybutyl) nitrosamine original sample. The sand medium in each pot was amended with the diluted nitrosamine solution after 8- 10 days from seed sowing simultaneously with BFBFs application. The levels of nitrosamines present in soil are much lower than those used in this investigation. Subsequently all the pots were arranged according to the CRD inside the greenhouse and five replicates were maintained for each treatment. Treatments are as follows.

TR1- 100% CF
TR2- 50% CF
TR3- 50% CF + BFBF1
TR4- BFBF1 alone
TR5- No amendments (including nitrosamine)
TR6- No amendments (excluding nitrosamine)

#### 6.3.1.5 Harvesting

After 90 days from seed sowing, plants were uprooted without damaging the tubers and were washed carefully with water to remove unwanted materials. Subsequently, the tubers were transferred into black polythene bags accordingly with the different treatments and were brought in to the laboratory for further analysis. Sand samples were also collected into black polythene bags separately to analyze the nitrosamine contamination.

#### 6.3.1.6 Analysis of nitrosamine contaminations

Rhizo-remediation abilities of different treatments on nitrosamine was evaluated by analyzing the tuber and sand samples separately. Tuber samples obtained from each treatment were washed thoroughly with clean water to remove the direct nitrosamine contaminations at the tuber surface. Subsequently, the peel of the tubers was removed carefully using a sharp knife and tuber mass were obtained up to 5 mm depth from the peripheral surface of the peeled off tuber (as shown in the diagram 1).

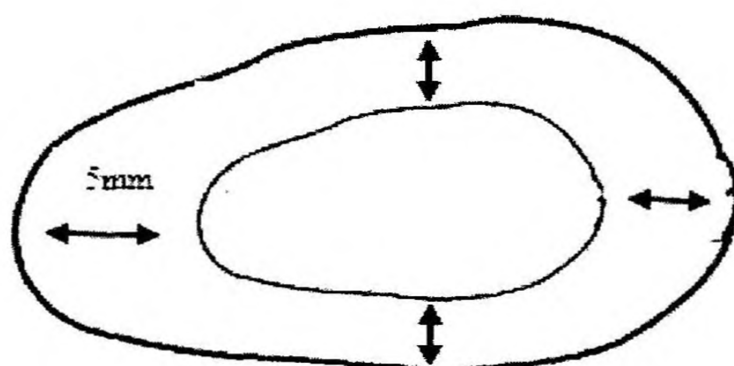



Diagram 1

Twenty grams of tuber mass obtained from the peripheral region of the tubers was used to extract nitrosamine with dichloromethane at 55 °C using Soxhlet apparatus. Subsequently, the extractions were evaporated using rotary evaporator to concentrate the available nitrosamines. The resulting extracts were analyzed by fourier transform infrared (FTIR) spectroscopy for the availability of nitrosamine. Peak resembles to 1440 $cm^{-1}$- 1460 $cm^{-1}$ (NNO bond) wavelengths was considered as the responsible peak for nitrosamine detection (Mistry, 2009). Nitrosamine availability of the sand medium was evaluated using the same method as explained above. Statistical data analyses was performed on all data collected using the one way Analysis of Variance (ANOVA) Model in MINITAB 16 Statistical

Software. The mean values of the absorbance obtained from the FTIR spectroscopy (Nicolet 6700 FTIR, available at Institute of Fundamental Studies, Kandy, Sri Lanka) for nitrosamine availability (at 1440 $cm^{-1}$- 1460 $cm^{-1}$wavelengths) in sand and tuber biomass were compared separately on treatment basis using the Tukey's simultaneous test at 5% significance level.

### 6.3.2 Rhizo-remediation abilities of different treatments on heavy metals

A pot experiment was conducted to evaluate the rhizo-remediation abilities for heavy metals by different treatments including the responsive biofilm under greenhouse conditions. The experiment was conducted from May 2014 to June 2014 at Regional Agriculture Research and Development Center, Bandarawela, Sri Lanka.

#### 6.3.2.1 Media preparation and planting seed potato

Soil collected from 0 - 10 cm depth of a loamy soil in Regional Agriculture Research and Development Center, Bandarawela was used as the medium for the pot experiment. The soil sample was sieved to obtain uniform soil particles (particle size $\leq$ 2 mm). The initial concentrations of Cd and Pb were determined (Cd-0.3 ppm and Pb- 3.5 ppm). Then the soil medium was artificially spiked with a solution containing known concentrations of Cd and Pb compounds (Cd- 5 ppm and Pb- 25 ppm). The heavy metal containing solution was mixed thoroughly with the soil medium to distribute the heavy metal compounds evenly throughout the soil. The levels of heavy metals that may be formed in soil are much lower than those used in this investigation. Subsequently, disease free potato tubers (two seed tubers in each pot) were sown in each pot with the contaminated soil mixture and kept inside the greenhouse at Regional Agriculture Research and Development Center, Bandarawela, Sri Lanka.

#### 6.3.2.2 Application of cultures

Responsive biofilm culture (BFBF1) was prepared with the carrier material as explained in 6.3.1.2. At the time of seed sowing, diluted biofilm mixture (250 times with clean water) was sprayed directly around the root zone of the potato plants grown in contaminated soil medium. Biofilm inoculation was repeated again after five weeks from seed sowing.

#### 6.3.2.3 Fertilizer application

A mixture of urea (2.0 g/kg), TSP (3.33 g/kg), MOP (1.33 g/kg) was mixed with the soil as a basal fertilizer mixture at the time of the seeds were sown. Fertilizer application (2.0 g/kg of urea and 1.33 g/kg of MOP) was repeated after five weeks from the seed sowing simultaneously with the biofertilizer application. Rates of the chemical fertilizer application was calculated per plant basis according to the DOA recommendations. Moisture level of the medium was maintained constantly by applying 250 ml of water for each pot every day. Plants were grown with a daily minimum-maximum temperature range of 20 °C – 30 °C. All the pots were arranged according to the CRD inside the greenhouse and five replicates were maintained for each treatment. Treatments are as follows.

TR1- 100% CF
TR2- 50% CF
TR3- 50% CF + BFBF1
TR4- BFBF1 alone
TR5- No amendments

#### 6.3.2.4 Harvesting

After 90 days from seed sowing, plants were uprooted without damaging the tubers and were washed carefully with water to remove attached soil particles. Subsequently, the tubers were transferred into black polythene bags accordingly with the different treatments and were brought in to the laboratory for further analysis. Soil samples were also collected into black polythene bags separately to analyze the heavy metal contamination.

#### 6.3.2.5 Determination of the availability of heavy metals

Rhizo-remediation abilities of different treatments on heavy metals was evaluated by analyzing the availability of heavy metals in the soil samples and by analyzing the heavy metal uptake in the tuber biomass.

Tuber samples obtained from each treatment were washed thoroughly using clean water to remove airborne pollutants at the tuber surface. The peel of the tubers was removed carefully

using a sharp knife and middle tuber mass was obtained for further analysis. Subsequently, 10 g of chopped tuber samples were measured and air-dried for a day separately, to reduce the water content, followed by oven-drying at 70 °C for 48 hours to constant weight. Preparation of soil samples was performed as explained above.

The dried samples (both tuber and soil samples separately) were ground manually with ceramic mortar and pestle to pass through a 2 mm non- metal sieve to ensure uniform particle size. To 1 g of each dry sample, 10 ml of concentrated acid solution (1 perchloric acid: 4 nitric acid) was added and the mixture was allowed to stand overnight, and then heated for 4 hours at 125 °C on a hot plate. The hydrolyzed samples were transferred to a centrifuge tube for centrifugation at the rate of 3000 rpm to remove solid particles. The presence of lead (Pb detection limit- 0.5 ppm) and cadmium (Cd detection limit- 0.1 ppm) were analyzed using the AAS (Model No. GBC 933AA, Australia) at 217.0 nm and 228.8 nm wavelengths, respectively (standard samples for Pb- $Pb(NO_3)_2$ and Cd-$Cd(NO_3)_2$ ) Samples were analyzed in triplicates. Based on the absorbance data, heavy metal concentrations in the tuber and soil samples were determined. Mean concentration values of the soil for treatments were compared with the concentration obtained from initial soil sample (Kisku *et al.*, 2011).

#### 6.3.2.6 Statistical analysis for heavy metal content

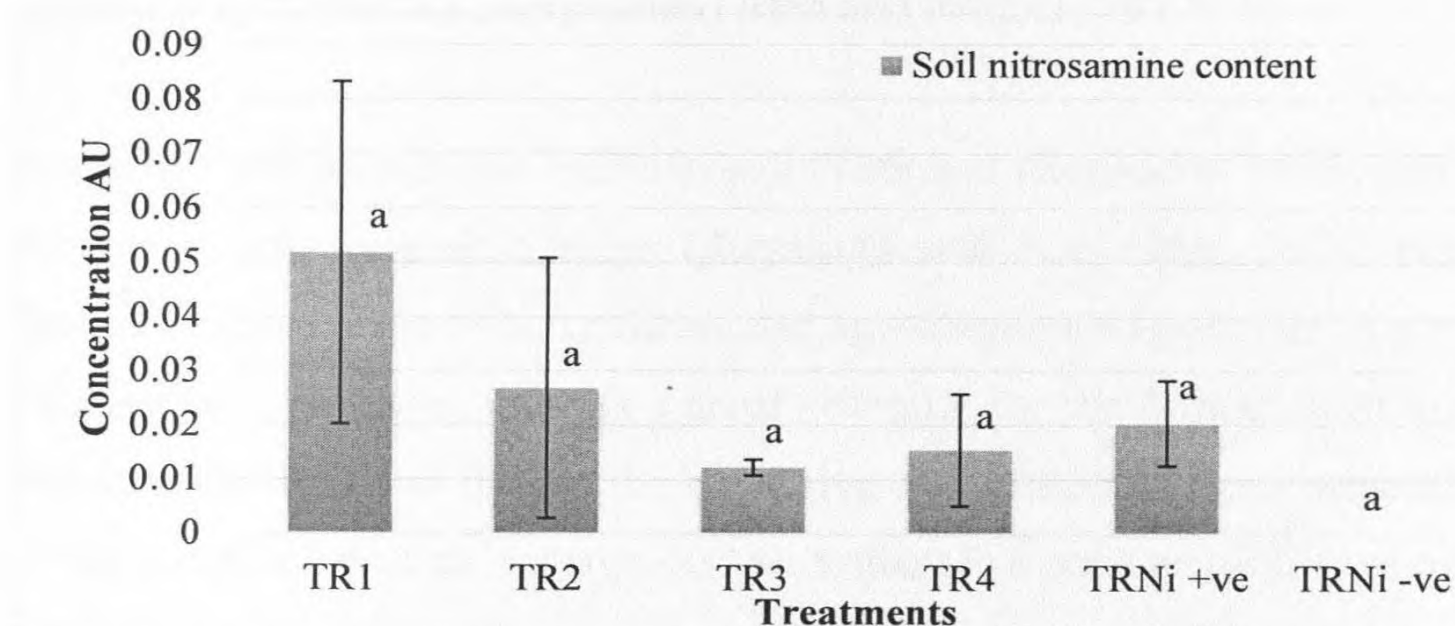
Statistical data analyses was performed on all data collected using the one way Analysis of Variance (ANOVA) Model in MINITAB 16 Statistical Software. The mean values of the heavy metal content of soil and tuber biomass were compared separately on treatment basis using the Tukey's simultaneous test at 5% significance level.

## 6.4 Results and Discussion

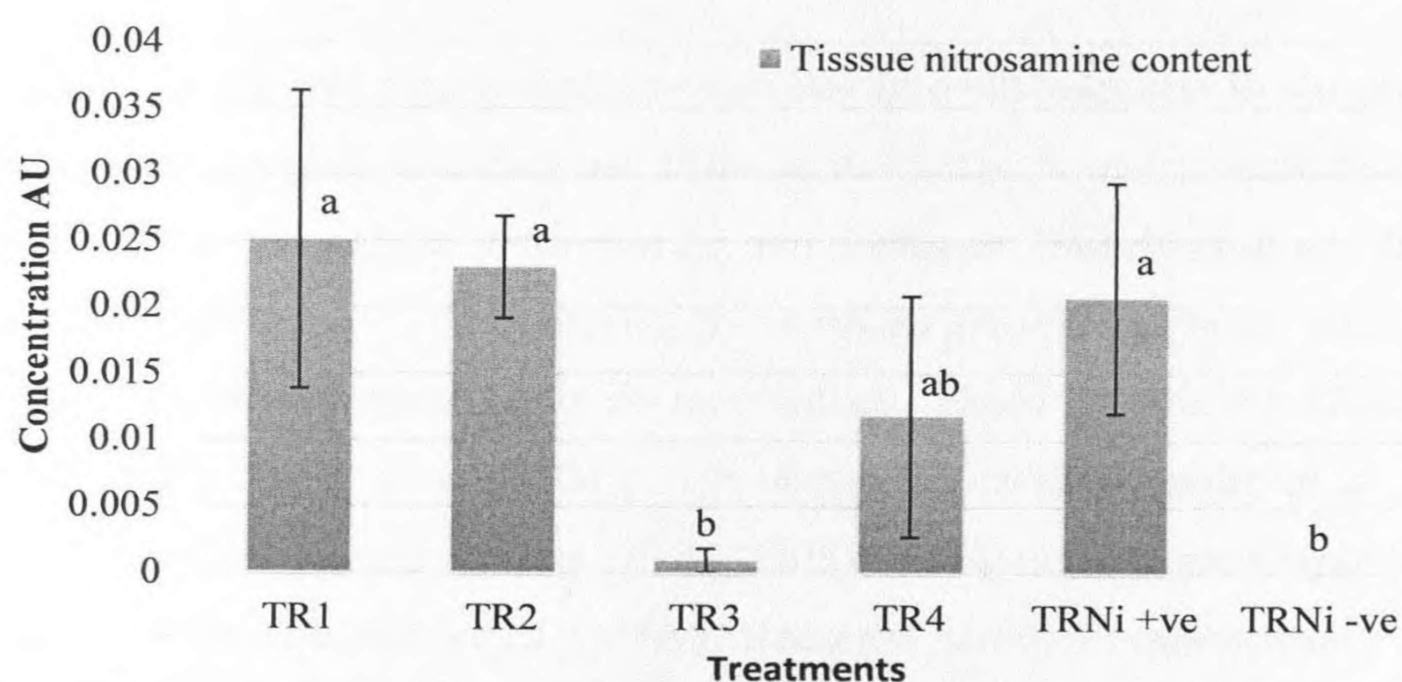
### 6.4.1 The effect of different treatments on nitrosamine content

Due to the unavailability of dimethyl nitrosamine which is the most commonly found nitroso compound in the soil, N-butyl-N- (4- hydroxybutyl) nitrosamine, a hydroxylated derivative of nitrosamne was used in the current study. In comparison with all other treatments, the lowest soil nitrosamine content (0.00123 AU ± 0.001) was observed in the treatment TR3 except treatment TRNi-ve (Fig. 6.1a). It is noteworthy that the treatment TR3 reduced the nitrosamine content of the sand medium by approximately 76% compared to treatment TR1.

Furthermore, the significant lowest ($p < 0.05$) nitrosamine content was detected from the treatment TR3 ($0.000963 \pm 0.0002$) except treatment TR4 and TRNi -ve in tuber biomass (Fig. 6.1b). This implies that the beneficial microbial community in the biofilm combination BFBF1 along with 50% CF effectively remediate nitrosamine in the sand medium. Further, the microbial biomass of the biofilm combination BFBF1 reduces the penetration of nitrosamine into the tuber mass. It was noted that the treatment TR3 reduced the tuber nitrosamine content by approximately 96% compared to treatment TR1 and by approximately by 95% compared to the treatment TR2. Further, treatment TR4 ($0.0118 \pm 0.003$) reduced the tuber nitrosamine content compared to the treatment TR1 and TR2.




(a)




(b)

Figure 6.1- The effect of different CF and BFBFs treatments on nitrosamine content in sand and tuber tissues analyzed by FTIR spectroscopy. (a)- Nitrosamine content in sand medium. (b)- Nitrosamine content in tuber tissue. Treatments TR1, TR2, TR3, TR4, TRNi+ve and TRNi-ve are 100% CF, 50% CF, 50% CF+ BFBF1, BFBF1 alone, No amendments (including nitrosamine) and No amendments (excluding nitrosamine)treatments respectively. Columns with the same letter are not significantly different at 5% probability level. Vertical bars show standard deviations.

Studies have reported that soil nitrate, generated from heavy CF applications is correlated with the formation of *N*-nitroso compounds (Hotchkiss *et al.* 1992; Zhong, Hu, Wang, 2002). Another study has also reported that the occurrence of the nitrosamines in the soil, due to the presence of precursor compounds (e.g., secondary amines, nitrogen oxides, nitrate, nitrites), by the pesticides, herbicides, fungicides like thiram (Tesfai, Mallik and Pancholy, 1977) heavy applications N fertilizers or nitrogenous organic materials (Ayanaba *et al.*, 1973a; Oliver and Kontson, 1978; Fishbein, 1979). In certain soils under fairly high ammonia resulting from heavy fertilization with urea, nitrite and N have been reported to persist for several months (Chapman and Liebig, 1952) and thereby creates favorable conditions for the synthesis of N-nitroso compounds (Aten and Bourke, 1977).

N-nitroso compounds are stable in soil (Tate and Alexander, 1976) and can be translocated from soil into vegetable crops (Raymond and Alexander, 1976; Brewer *et al.*, 1980). Intensive application of N fertilizers and agrochemicals is a common practice in cultivations like potato. Therefore, there is a great potential for the formation of nitroso compounds in potato cultivated soil due to the excessive application of agrochemicals and N fertilizers. Since potato tuber is an underground part, there is a great possibility to contaminate the tuber with the soil toxicities like nitrosamine. It has been reported that nitrosamines can be remain in soil for 90-150 days, and their biodegradation is very slow (Barabasz *et al.*, 2002).

However, some soil microorganisms can degrade nitrosamines to simple compounds in co-metabolic processes, and then use them as nutrients. Some of those bacteria are belonging principally to the genera *Arthrobacter* and *Eubacter* (Barabasz *et al.*, 2002). Several other bacteria expressing broad-specificity monooxygenase enzymes have been reported to degrade nitroso compounds via co-metabolism. These include the *Rhodococcus* sp. (Sharp *et al.*, 2005; Sharp *et al.*, 2007), *Rhodococcus ruber* (Streger *et al.*, 2003) as well as *Mycobacterium vaccae* (Sharp *et al.*, 2005), *Methylosinus trichosporium* (Yoshinari and Shafer, 1990; Fournier *et al.*, 2009). Bacterial strain *Pseudomonas mendocina* has also recently been reported to be capable of metabolizing dimethyl nitrosamine (Sharp *et al.*, 2005), but the degradation pathway is unknown. Interestingly, bioremediation abilities of *Bacillus* sp. for different nitroso compounds have been reported recently (Oliveira el al., 2010). The data presented herein suggest that the bacterium oxidizes the nitrosamine primarily to *N*-nitro dimethyl amine (NTDMA), which is then metabolized further to

produce less toxic compounds like *N*-nitro methyl amine (NTMA) and formaldehyde (Fournier, *et al.,* 2006). Nitrosamine availability in the natural soil is very low and the detection and the quantification are highly impossible using FTIR spectroscopy. Further, Soil is a complex medium contains different N containing compounds which can be interfere with FTIR spectroscopy. The main intention of the current study was to detect and analyze the Nitrosamine availability of the medium and the tissues after the respective treatments. Therefore, purified sand was used as the medium to avoid the major interferences from other N containing compounds and to obtain clear FTIR spectrum.

### 6.4.2 The effect of different treatments on heavy metal contamination

In comparison with all other treatments, BFBF1 treated soil showed lower Pb availability and the lowest (17.07 μg/g ± 0.48) was recorded (per one gram of soil) from the treatment TR4 (table 6.1). The mean Pb content was significantly reduced by the treatment TR4 ($P < 0.05$) compared to treatment TR1 whereas a significant difference of soil Pb content was not observed between the treatment TR2 and TR3 at 5% significance level. Further, it was observed that significance difference of the tissue Pb availability was not observed by any treatment. However, the lowest tissue Pb availability was observed in the treatment TR4 (2.94 μg/g ± 0.30).

Moreover, the lowest significant soil Cd content was observed in the treatment TR3 ($P < 0.05$, 1.64 μg/g ± 0.34) compared to all other treatment except treatment TR4 (table 6.1). All BFBF1 treated soil (TR3 and TR4) reduced the Cd availability compared to all other treatments. Treatment TR3 reduced the soil Cd level by approximately 59.5% compared to treatment TR2 and by approximately 67% compared to treatment TR1. It is noteworthy that the Cd availability after the treatment TR1 (5.58 μg/g ± 0.3) was higher than the initial soil Cd level (5.26 μg/g ± 0.21). This might be due to the effect of CF application, especially due to the Cd contaminations in TSP fertilizers. It has been reported that phosphate fertilizers are the most important source of Cd contamination of agricultural soils among mineral fertilizers. These fertilizers are manufactured from phosphorites (phosphate rocks), which can contain relatively high concentration of Cd (Casova *et al.*, 2009). For instance, TSP has been recorded the highest Cd concentration (23.5 mg/kg) among the phosphate fertilizers used in potato cultivation (Premarathne *et al.*, 2011). Further, the lowest significant tissue

Cd level was recorded in the treatment TR3 ($P < 0.05$, 0.10 μg/g ± 0.02) compared to all other treatments except treatment TR4 (0.12 μg/g ± 0.02). All the BFBF1 treatments significantly reduced ($P < 0.05$) the Cd level in tissues compared to all other treatments. Treatment TR3 reduced the tissue Cd level by approximately 86.6% compared to treatment TR2 and by approximately 87.6% compared to treatment TR1.

Table 6.1- The effect of different CF and BFBFs treatments on heavy metal contaminants in soil and tuber tissues analyzed by AAS.

| Treatments | Soil Cd (μg/g) Mean±SD | Tissue Cd (μg/g) Mean±SD | Soil Pb (μg/g) Mean±SD | Tissue Pb (μg/g) Mean±SD |
|---|---|---|---|---|
| TR1 | 5.58±0.30[a] | 0.90±0.07[ab] | 21.90±1.31[b] | 3.90±0.60[a] |
| TR2 | 4.40±0.21[b] | 0.85±0.06[b] | 18.38±0.74[cd] | 3.54±0.81[a] |
| TR3 | 1.64±0.34[c] | 0.10±0.02[c] | 17.10±0.67[d] | 2.98±0.69[a] |
| TR4 | 2.21±0.73[c] | 0.12±0.02[c] | 17.07±0.48[d] | 2.94±0.30[a] |
| TR5 | 4.25±0.09[b] | 0.98±0.06[a] | 20.81±1.42[bc] | 4.00±0.81[a] |
| Initial | 5.26±0.21[a] | | 28.39±2.31[a] | |

Treatments TR1, TR2, TR3, TR4 andTR5are 100% CF, 50% CF, 50% CF+ BFBF1, BFBF1 alone and No amendments treatments respectively. Initial- soil just after artificial spiking of heavy metals. Columns with the same letter are not significantly different at 5% probability level.

It has been reported that intensification of agricultural practices such as excessive use of synthetic agrochemicals, CF, organic manures result in accumulation of heavy metals like Cd and Pb in cultivated lands (Lambert and Indraratne, 2014).The average soil Cd level in up country soil has been recorded as approximately 4.0 ppm (Premaratne *et al.*, 2005) and the average Pb level as approximately 20 ppm (Premaratne *et al.*, 2011). Therefore in the current study, the initial levels of the Cd and Pb were adjusted to the available standards (Cd- 5 ppm, Pb- 25 ppm).

Generally, heavy metals are impossible to be degraded biologically and persist in the environment for extended periods of (Khan *et al.*, 2009). However, soil beneficial microorganisms have been proved to be effective in detoxifying pollutants in the soil that include heavy metals such as Pb and Cd (Harrison, 1997;Casova *et al.*, 2009).For instance, a strain of *Pseudomonas maltophilia* has been shown to minimize environmental mobility of toxic ions such as $Hg^{2+}$, $Pb^{2+}$, and $Cd^{2+}$ (Blake *et al.*, 1993; Park, Keyhan and Matin, 1999; Khan *et al.*, 2009). In a study, Cd, Cu, Se and Zn were reported to be biosorbed by *Streptococcus aureus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Pseudomonas aeruginosa*

and *Serratia marscecens*, in the mixtures of Gram positive and Gram negative bacteria (Khan *et al*., 2009). Recently, the inoculation effects of plant growth promoting rhizobacteria *Methylobacterium oryzae* and *Burkholderia* sp. isolated from rice (*Oryza sativa*) tissues, on potato, grown in Ni and Cd treated soil has been studied (Madhaiyan, Poonguzhali and Sa, 2007). These bacterial strains have significantly reduced the toxicity of both metals under pot culture conditions (Khan *et al.*, 2009).

Further, toxicity increment has been reported with CF application, due to phytotoxins accumulation in rhizosphere (Monnier *et al.*, 2011). However, reduced rates of CF, when coupled with BFBF allow detoxification of allelochemicals (i.e phenolic compounds). Biofilms have been reported as good candidates for bioaccumulation studies (Doering and Uehlinger, 2006; Ogbuagu *et al.*, 2011). Rhizo-remediation of certain heavy metals has been studied with the use of *E. coli* biofilms. (Quienteles *et al.*, 2009). Therefore, managing the microbial populations in the rhizosphere by using microbial inoculum consisting of a consortium of plant growth promoting rhizobacteria could provide plants with benefits in terms of rhizo-remediation of most of soil toxicities (Khan, 2004). In the current study, it was obvious that the biofilm combination BFBF1 reduced the level of nitrosamine and heavy metals (Cd and Pb) in soil and thereby reduced the possibilities of those soil toxicities accumulating in tuber tissues. Effect of BFBFs on rhizoremediation in this manner aids for sustainability of maize agriculture in Sri Lanka with increased biological functioning which cannot be observed in the CF application.

## 6.5 Conclusions

All BFBF1 treatments (TR3 and TR4) reduced the level of nitrosamine and targeted heavy metal (Cd and Pb) availability in the soil and the tuber tissues. BFBF1 treatment along with 50% CF was the best treatment in reducing nitrosamine and Cd contaminations in tuber tissues. Therefore, it can be concluded that the biofilm treatment BFBF1 has the ability to remediate nitrosamine, Cd and Pb contaminants in soil and thereby to reduce the possibilities of those soil toxicities accumulating in tuber tissues.

# CHAPTER 7

# DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

## 7.1 Discussion

The results of the current study show that the effective establishment and application of BFBF under greenhouse conditions and different field conditions reduced the CF use by 50% with several other beneficial functions needed for sustainability of potato cultivation. Similar results have been reported by several other studies on reducing CF application by 50% for maize (Buddhika *et al.*, 2012a), tea (Seneviratne *et al.*, 2011) and rice (Weerarathne *et al.*, 2012) cultivations with the inoculation of BFBFs. Further, the proposed mechanism (Fig. 5.1b) for inducing potato tuberization could be clearly explained, based on the obtained results. It was clearly observed that soil and tissue $Ca^{2+}$ contents (Figs. 5.5 and table 5.4), soil nutrient levels (Figs. 5.3 and 5.4) and soil pH (Fig. 5.2) were enhanced by the biofilm BFBF1 while inducing tuberization, irrespective of the climatic factors at all tested field sites. Moreover, the biofilm BFBF1 (*Trichoderma harzianum* + *Bacillus pumilus* + *Bradyrhizobium japonicum* + *Bacillus subtilis*) showed higher beneficial activities, *viz*; production of tuber inducing hormone IAA, higher nitrogenase activity etc. (Fig. 3.7), which induce plant growth promotion. Tuber induction in potato (*Solanum tuberosum* L.) is a complex, multilevel process, which integrates environmental and internal signals to ensure optimal life strategy during the growing season. Generally, photoperiodic signal is integrated with other environmental factors, such as nutrient availability, temperature and light intensity, as well as with the overall metabolic status of the plant (Jackson, 1999; Fischer *et al.*, 2008). Tuber inducing potential of exogenous $Ca^{2+}$ (Nookaraju *et al.*, 2012) and IAA (Harmey *et al.*, 1966; Sasamoto and Suzuki, 1979) has been well documented in several other studies and the production of those signaling compounds and ions by the rhizosphere associated beneficial microorganisms has also been recorded (Spaepen *et al.*, 2007). Further, it was clearly observed that the biofilm BFBF1 inoculation altered the internal biochemical status (sugar and starch compositions) of different tissues (Fig. 5.6, Fig. 5.7, table 5.3 and 5.4), leading to potato tuberization at all field sites (table 4.4, 4.5, 4.6) as proposed in figure 5.1b. Therefore, this implies that the enhancement of exogenous IAA and $Ca^{2+}$ availabilities by the biofilm BFBF1 may have acted as potential stimulators for enhancing tuberization of potato, irrespective of the climatic conditions.

Further, application of BFBF1 along with only 50% recommended CF enhanced the potato yield up to 21 MT/ha and it is noteworthy that this yield limit is beyond the average potato yield (15-18 MT/ha), generally recorded at Bandarawela. Enhanced tuberization was recorded from the same BFBF1application at Bibile and Padukka field sites where potato cultivation is not practiced commercially. Previous climatic data confirmed that Bibile field location has some potential climatic conditions for potato cultivation only during Maha season. However, the current study showed that the average tuber yield obtained from Bibile field site (2.67 MT/ha) and Padukka field site (0.18 MT/ha) were much lower than that of the yield at Bandarawela field site and was not sufficient to establish large scale profitable commercial cultivation.

It has been reported that the annual potato production (approximately 70,000 MT) is not sufficient to cater to demand (approximately 140,000 MT) and the needs of consumers in Sri Lanka. Quality and the size of the potato tubers are the main factors that the consumers in especially urbanized areas of Sri Lanka are greatly concerned. However, it is noticed that the size and the quality of the tubers available in the local market is greatly variable since proper grading or branding is not practiced. Therefore, it is hard for household consumers to choose the best potatoes. Market data indicates that a kilo of potatoes have a high retail price ranging from Rs. 70/- to Rs. 120/- compared to the other vegetable commodities. Therefore, consumers prefer to buy high quality small size potato tubers rather than buying large tubers. This is mainly because, consumers have more chances to buy greater number of tubers per kilo if they buy small tubers. Further, the main part of the potato production in Sri Lanka is sold for fresh consumption without any processing. However, there is a growing demand and market trend for processed food products among the middle and upper middle class society in Sri Lanka. Potatoes possess a great potential to be marketed as processed potatoes like potato chips, frozen potatoes, and minimally processed potatoes and are currently emerging in the supermarket sector in Sri Lanka. Apart from the household consumers, restaurants, hotels and canteens are the major end points of potato market chain, using fresh potato mainly for food preparations. They obtain potato directly from the large scale or small scale growers without much concern about grading or branding. In the current study, it was obvious that there is a potential to cultivate potato in Bibile area though the yield is lower than Bandarawela. However, the size and the quality (nutrient content) of the tubers obtained by the application of BFBF1 along with only 50% CF at Bibile field site was considerably

higher than that of the tubers obtained only by applying the recommended CF. Further, it was noticed that the size of the tubers obtained from Bibile site was higher than some of the potato tubers available in the local market in Sri Lanka. Therefore, if cultivated, there would be a great market potential and demand for the tubers obtained by applying BFBF1 along with 50% CF at Bibile area. As such, it is hoped that it would be possible to popularize potato cultivation among the farmers though potato cultivation is not currently practiced in Bibile area.

Further, global agriculture which had greatly depended on chemical inputs is now being changed into a more ecofriendly sustainable agriculture. Sustainable agriculture is an important element of the overall effort to make human activities compatible with the demands of the earth's eco-system. Low-input agriculture is the base of sustainable agricultural systems which reduces (but not necessarily elimination) CF and agrochemicals while reducing production cost and minimizing the impact on the environment. In the case of Sri Lanka, most of the N fertilizers are not produced within the country itself and has to be imported from other countries. It has been reported that the CF requirement for potato cultivation is around 1000 kg $ha^{-1}$and from that the N requirement is as high as 336 kg $ha^{-1}$ for an expected yield of 5000 kg $ha^{-1}$ (Davies *et al.,* 2005b; Lang *et al.*, 1999). Therefore, the government has to invest huge capital (approximately Rs. 68,000/= per ha, considering 1 kg of fertilizer at Rs. 80/=) to fulfill the CF requirement for potato. However, according to the policy of the Sri Lankan government, imported CF are being given to farmers under a subsidized price (Rs. 24/kg) and thereby the government of Sri Lanka has to bare an extra burden in terms of CF and agrochemicals. Further, the country is experiencing adverse effects of the unnecessary use of CF like some chronic kidney diseases and cancer issues. The government has therefore, initiated measures to control the excessive use of agrochemicals and fertilizer consumption and promote the use of organic fertilizer in farm lands. The findings of the current study imply that the input of CF can be reduced by 50% along with the developed BFBFs during the entire vegetative growth phase of potato. Therefore, halving CF use in the BFBFs application could save half of the expense for CF import, thus contributing to the country‘s economy in terms of fertilizer saving. Moreover, biological functions of microbes in the biofilm BFBF1 seemingly helped the detoxification of toxins like nitrosamine (Fig. 6.1) and heavy metals (table 6.1) accumulated in the soil-plant system during potato cropping with the CF inputs. Further, the yield enhancement of potato by the application of BFBFs + with only 50% CF, cannot be achieved by the

application of 100% recommended CF. Thus, the biofilm BFBF1 assisted to build up a robust soil in agroecosystems to produce high quality tubers while remediating toxicities. This could protect agricultural soils, steering to sustainable agroecosystems. BFBFs which turned a new page in biofertilizer research opened avenues for microbiologist to handle microbes in microbial communities for sustainable agriculture. Therefore, the BFBFs can be recommended strongly as soil microbial ameliorator in the development of sustainable agroecosystems that could gain both economic and ecological benefits. However, the yield enhancement and the soil quality amelioration after the application of the developed BFBF1 to the real potato farmer's fields in the relevant climatic areas of Sri Lanka should be further evaluated since most of the farmer's fields may be highly contaminated or degraded.

## 7.2 Conclusions

Exogenous microbial signals created by the application of the microbial biofilm along with 50% CF induced the required internal biochemical and physiological conditions for increased tuberization of potato while improving rhizo-remediation of soil toxicities like nitrosamine and heavy metals. Thus, it seems that the plant biochemical and physiological cycles required for tuberization, generally induced by the specific climatic factors like day/night temperature difference have been stimulated by the microbial signals of the biofilm. As such, the climatic requirements for potato tuberization have been compensated by the biofilm microbial actions. This is the first report of such a mechanism in potato tuberization. This finding opens a new avenue for potato cultivation in regions where there are no suitable climatic requirements.

## 7.3 Future perspectives

October to April is considered as the best period for potato cultivation in Bandarawela area of Sri Lanka, since the required day/night temperature difference for potato tuberization is reached during this period. The current study was undertaken to evaluate crop responses and soil nutrient improvement by the application of microbial biofilms, using small scale field experiments from November to March during one season. Therefore, it is suggested that further studies are needed to test the biofilm action in large scale cultivations in year-round field experiments. Further, changes of existing soil microbial communities by the application of developed BFBFs should be evaluated using molecular approaches.

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# APPENDICES

## Appendix 1-

### Extraction of genomic DNA from bacteria and fungi using CTAB (Cetyl trimethyl ammonium bromide) method

Approximately 1.5 ml of each broth culture (bacteria and fungi) was added to clean sterile 1.5 ml eppendorf tubes separately. Tubes were centrifuged at maximum rpm (12,000 g) for 10 min using a bench top eppendorf centrifuge (BIORAD). The process was repeated again until obtaining considerable amount of pellet. The pellet was obtained by discarding the supernatant and was re-suspended with 100 µl of lysozyme (lysozyme +TE buffer, 10mg/ml) followed by 20 min incubation at 33°C. After incubation, 700 µl of CTAB buffer (2% hexadecyl trimethyl ammonium bromide-CTAB, 1.4 M NaCl, 100 mM TrisHCl (pH=8.0), 20 mM EDTA) and 10 µl of proteinase K enzyme were added simultaneously to the lysozyme treated samples. The mixture was incubated at 60°C for one hour and 700µl of mixture of chloroform and isoamyl alcohol (chloroform 24: isoamyl 1) were added to the incubated eppendorf tubes. Tubes were shaken thoroughly to mix the two layers (two layers appeared after adding chloroform+ isoamyl alcohol) and centrifuged at 12000 g for 8 min. The contents of each tube appeared as three layers just after centrifugation. The upper transparent aqueous layer of the tube was transferred carefully to another clean eppendorf tube without disturbing the other layers. DNA was precipitated out by adding 560 µl of isopropanol to the transparent upper layer. DNA was visualized as small white cotton strand after gentle mixing. Samples were centrifuged at 12000 g for 10 min and subsequently the pellet was obtained by discarding the supernatant carefully without disturbing the pellet. About 1.5 ml of 70% ethanol was added to each pellet separately and centrifuged again at 12000 g for 5 min. Bulk of the supernatant was removed carefully and centrifuged again at 12000 g for 5 min in order to adhere the DNA to bottom of the tube as a pellet. After the centrifugation, the ethanol remaining in the tubes, was removed as much as possible using a micropipette without disturbing the pellet. Tubes were kept open for 10 min to evaporate the trace amount of ethanol remaining in the tube. Subsequently, 50µl of TE buffer was added to each tube separately and the extracted DNA samples (high molecular weight) were stored in low temperature until further processing.

**Appendix 2-**

PCR master mix reagents

| Reagent | Quantity (from 25 µl reaction mixture) |
| --- | --- |
| dd H2O | 16.8 µl |
| 5x My Taq reaction buffer | 5 µl |
| Forward primer (10µM) | 1 µl |
| Reverse primer (10µM) | 1 µl |
| My Taq polymerase (5U/µl) | 0.2 µl |

RFLP master mix (12 µl reaction mixture)

| Reagent | Quantity |
| --- | --- |
| dd $H_2O$ | 6.3 µl |
| 10X NEB cut smart buffer | 1.2 µl |
| Taq α I/ HhaI/ MSPI 10 µM | 0.5 µl |
| PCR product | 4 µl (depending on the concentration) |
| | 12 µl (Total) |

**Appendix 3-**

**Extraction of DNA from a fungal-bacterial biofilm community**

DNA extraction from the purified biofilm community using MO BIO Power Soil DNA isolation kit (Cat No- 12888-100) with the bead beating method

Approximately 0.25 g of purified biofilm mass was transferred into the Power Bead tubes provided with the isolation kit followed by mixing gently using vortex mixer to mix the biofilm mass with the digestion solution inside the tube. Then, 60 μl of solution C1 (contains SDS and other cell disrupting agents) was added to the power bead tubes with the biofilm mass and was mixed gently using vortex mixer. Tubes were subsequently placed on MO BIO vortex adapter horizontally (tubes were tightly attached to the flat- bed vortex pad with clips) and the content of the tubes was mixed at maximum speed for 10 min. After, mixing with MO BIO vortex, the tubes were centrifuged at 10,000 g for 30 s at room temperature. Supernatant was transferred to a clean 2 ml collection tube and added 250 μl of solution C2 (a reagent to precipitate non- DNA organic and inorganic material)was added followed by incubation at 4 °C for 5 min, followed by centrifugation at 10,000 g for 1 min. The supernatant (maximum 600 μl) was carefully transferred to another clean 2 ml collection tube and 200 μl of solution C3 (a reagent to precipitate additional non- DNA organic and inorganic material and cell debris) was added followed by incubation at 4 °C for 5 min. Again the mixture was centrifuged at 10,000 g for 1 min and 750 μl of the supernatant was transferred to another clean 2 ml collection tube. Then 1.2 ml of solution C4 (high concentrated salt solution) was added to the supernatant and was mixed using vortex mixer for 5 s. Approximately 675 μl of the mixture was loaded onto a spin filter and was centrifuged at 10,000 g for 1 min at room temperature. The flow through was discarded and the process was repeated another two times. Then 500 μl of solution C5 (ethanol based wash solution to clean the DNA that was bound to the silica membrane in the spin filter) was added to the spin filter and centrifuged at 10,000 g for 30 s at room temperature. The spin filter was centrifuged again at 10,000 g for 1 min after discarding the flow through. The spin filter was carefully transferred to a clean 2 ml collection tube without splashing any remaining solution. Finally 100 μl of solution C6 (sterile elution buffer to complete release the DNA attached to the silica membrane) was added to the center of the white spin filter membrane and centrifuged at 10,000 g for 30 s at room temperature. The spin filter was discarded and the DNA in the tube was stored in low temperature (-20 °C)

**Appendix 4-**

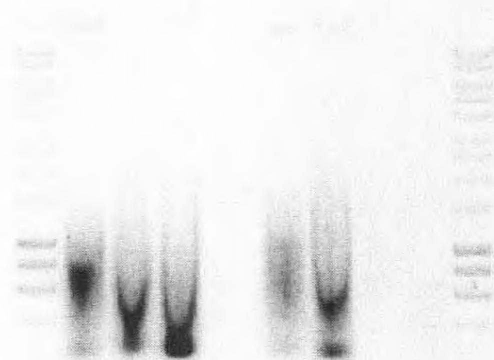
RFLP mastermix (30 µl reaction mixture)

| Reagent | Quantity (taken from 30 µl reaction mixture) |
|---|---|
| dd $H_2O$ | 5.5 µl |
| 10X NEB cut smart buffer | 3 µl |
| Taq α I/ HhaI 10 µM | 0.5 µl |
| PCR product | 21 µl (depending on the concentration) |
| | 30 µl (Total) |

## Extraction of genomic DNA from microorganism

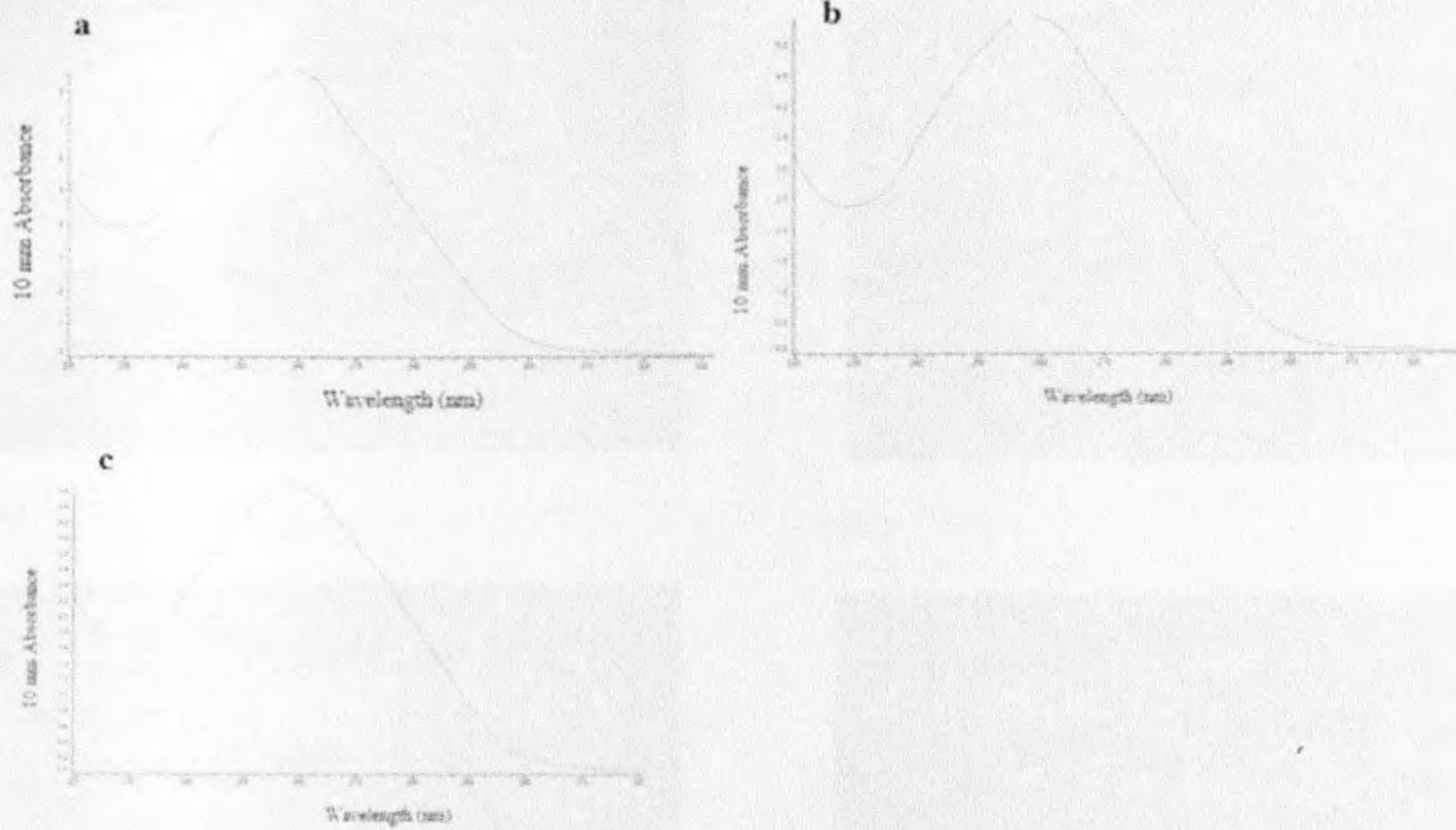
The CTAB method that was followed in the current study resulted in a high molecular weight DNA from all biofilm forming microbial strains. Proteinase K and the heat treatment of the CTAB method for all microbial strains including fungi was very effective to extract microbial genomic DNA. Further, lysozyme treatment of the CTAB method was useful to extract genomic DNA with greater purity. All the bands were visualized as smears rather than having clear sharp bands. The dark black smear was due to the RNA contamination and the faint smear at the top of the gel was due to the genomic DNA. However, the presence of RNA contamination was not considered as a major effect on further DNA manipulations.

M 1 2 3 4 5 6 M



Agarose gel electrophoresis of the extracted genomic DNA from biofilm forming bacterial and fungal strains. Lanes M, banding pattern of 1Kb (Hyperladder 1Kb, BioLine). Lanes 1-2, DNA extracted from *Bacillus* sp. Lane 3, DNA extracted from *Rhizobium* sp. Lane 4-5, negative and positive control. Lane 6, DNA extracted from *Trichoderma* sp.

## Appendix 5-




UV spectra for different PCR products obtained from Nano Drop 2000 spectrophotometer. (a) – (b), spectra obtained for PCR products of *Bacillus* sp. and *Rhizobium* sp. respectively. (c)- Spectrum obtained for PCR products of *Trichoderma* sp.

Concentration of different PCR products obtained from Nano Drop 2000 spectrophotometer

| Sample | Concentration (ng/µl) | A260/280 |
|---|---|---|
| B1 | 430.8 | 1.98 |
| B7 | 270.4 | 1.95 |
| F2 | 466.0 | 2.19 |

**Appendix 6-**

(a)

(b)

(c)

(d)

(e)

(f)

Potato plant growth responses for BFBF1 and 100% CF treatments at different field locations after six weeks from seed sowing. (a) and (b)- growth responses for 100% CF and BFBF1 at Bandarawela field site, (c) and (d)- growth responses for 100% CF and BFBF1 at Bibile field site, (e) and (f)- growth responses for 100% CF and BFBF1 at Padukka field site.

Potato field location at Horana for BFBF1 and 100% CF treatments after six weeks from the seed sowing.

# CZAPEK DOX AGAR (M075) – HIMEDIA

Czapek Dox Agar is a semisynthetic medium used for the general cultivation of fungi

**Composition**

| **Ingredients** | **g/ L** |
|---|---|
| Sucrose | 30.000 |
| Sodium nitrate | 2.000 |
| Dipotassium phosphate | 1.000 |
| Magnesium sulphate | 0.500 |
| Potassium chloride | 0.500 |
| Ferrous sulphate | 0.010 |
| Agar | 15.000 |
| Final pH (at 25°C) | 7.3±0.2 |

**Directions**

Suspend 49.01 g in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.

**Quality Control**

**Appearance**

Cream to yellow homogeneous free flowing powder

**Gelling**

Firm, comparable with 1.5% Agar gel

**Colour and Clarity of prepared medium**

Light yellow coloured, clear to slightly opalescent gel with a slight precipitate forms in Petri plates

**Reaction**

Reaction of 4.9% w/v aqueous solution at 25°C. pH : 7.3±0.2

# CZAPEK DOX BROTH (M076) - HIMEDIA

Czapek Dox Broth is a semisynthetic medium used for the general cultivation of fungi.

**Composition**

| **Ingredients** | **g /L** |
|---|---|
| Sucrose | 30.000 |
| Sodium nitrate | 3.000 |
| Dipotassium phosphate | 1.000 |
| Magnesium sulphate | 0.500 |
| Potassium chloride | 0.500 |
| Ferrous sulphate | 0.010 |
| Final pH ( at 25°C) | 7.3±0.2 |

**Directions**

Suspend 35.01 g in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and dispense as desired.

**Quality Control**

**Appearance**

Cream to yellow homogeneous free flowing powder

**Colour and Clarity of prepared medium**

Light yellow coloured, clear to slightly opalescent solution in tubes

**Reaction**

Reaction of 3.5% w/v aqueous solution at 25°C. pH : 7.3±0.2

# Combine Carbon Medium (CCM)

**Nitrogen-Limited Medium:**

Glucose - 2.5g/L

Sucrose - 2.5 g/L

Mannitol - 3 g/L

Sodium Malate - 2.0 g/L

Sodium Lactate (ml, 60%, v/v) - 5 ml/L

$K_2HPO_4$ - 0.20 g/L

$KH_2PO_4$ - 0.60 g/L

$Na_2Mo0_4.2H_20$ - 0.002 g/L

$MgSO_4$. 7 $H_20$ - 0.20 g/L

$CaCl_2$ - 0.02 g/L

(If agar, use 10-15 g Phytagar/L)

Distilled water 1000 ml

Final pH (at 25°C) – 6.8 ± 0.2

Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and dispense as desired.

(Koomnok *et al.*, 2007)

## R-2A Agar (M962) - HIMEDIA

R-2 A Agar is used for heterotrophic plate count of water samples using longer incubation periods

**Composition**

| **Ingredients** | **g / L** |
|---|---|
| Casein acid hydrolysate | 0.500 |
| Yeast extract | 0.500 |
| Proteose peptone | 0.500 |
| Dextrose | 0.500 |
| Starch, soluble | 0.500 |
| Dipotassium phosphate | 0.300 |
| Magnesium sulphate | 0.024 |
| Sodium pyruvate | 0.300 |
| Agar | 15.000 |
| Final pH ( at 25°C) | 7.2±0.2 |

**Directions**

Suspend 18.12 g in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.

**Quality Control**

**Appearance**

Cream to yellow homogeneous free flowing powder

**Gelling**

Firm, comparable with 1.5% Agar gel

**Colour and Clarity of prepared medium**

Light yellow coloured clear to slightly opalescent gel forms in Petri plates

**Reaction**

Reaction of 1.81% w/v aqueous solution at 25°C. pH : 7.2±0.2

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