The effect of the plant growth promoting rhizobacteria (PGPR) on *Nicotiana benthamiana viral* susceptibility

By

Spiwe Nyamuvurudza

A dissertation submitted in partial fulfilment of the requirements of the degree of

Master of Science

In

Environmental science

School of Animal, Plant and Environmental Sciences

University of Witwatersrand, Private bag 3, Wits 2050

Supervisor: Karl Rumbold

March 2017

ABSTRACT

Plant growth promoting rhizobacteria (PGPR) promotes plant growth in a variety of modes of action and also suppresses several phytopathogens causing plant diseases. There is evidence that *Pseudomonas* strains are able to induce systemic resistance, thereby enhancing the defensive capacity of many plant species, and they do so without any negative impact on the environment. Currently, many agricultural systems rely more on the use of chemical pesticides to combat plants diseases. The chemicals have several negative impacts on both human health and the environment. Therefore, there is need to investigate the ability to fight plant pathogens of alternatives like the *Pseudomonas spp* that do not harm the environment. Several strains of this genus are yet to be tested to see if they induce systemic resistance. Previous studies showed that bio surfactants produced by *Pseudomonas koreensis* exhibited strong effect against oomycetes *P. ultimum* in tomato plants. Induced systemic resistance (ISR) potential of *P. koreensis* following exposure to viruses has not been fully demonstrated to date. This study sought to investigate whether this strain has an effect on viruses and if it is able to induce systemic resistance against viral pathogens. The study started by growing the model plant *N. benthamiana.* The second stage involved carrying out assays of tobacco mosaic virus (TMV) after inoculating this virus in three bio treatments: (i) seed treatment of *N. benthamiana* with *P. koreensis* (referred to as the early treatment), (ii) root treatment at the transplanting stage (late treatment) and (iii) the control. In bio treatments (i) seeds were first sterilized by dipping them into 70% alcohol for 3 minutes and 0.1 % $HgCl_2$ for 1 minute and washing them with distilled water. Each seed was then soaked into 20ml of bacteria suspension for 30 minutes and in (ii) a litre of *P. koreensis* culture was then poured onto the roots of 36 *N. benthamiana* plants. The bacteria suspension was added at 10^7 colony forming units per gram of soil to each tray. It was observed that disease severity was lower in the *P. koreensis* plant treatments than for the control. Results of this investigation have shown that

P. koreensis can induce systemic resistance in foliar parts when plant seeds or roots are inoculated with this strain. This was demonstrated by separation of plant growth promoting rhizobacteria (PGPR) bacteria and TMV. Seeds and roots were inoculated with bacteria while the leaves were inoculated with TMV. The early bio treatment had the lowest mean number of necrotic lesions, and exhibited the mildest effects from TMV compared to the late bio treatment and control. Plants in the late bio treatment were moderately affected while the control was severely affected ($P<0.0001$) <0.05. The early and the late bio treatment both had higher leaf surface area than the control; ($P<0.0001$) <0.05. The early bio treatment lost the fewest leaves, and the late bio treatment lost a moderate number while the control lost the highest number ($P<0.0001$)<0.05.The reduced symptoms exhibited by plants inoculated with *P. koreensis* is an indication that *P. koreensi*s has anti-viral activity against TMV. It was concluded that *P. koreensis* can reduce plant's viral susceptibility and result in ISR. It is hence proposed that *P. koreensis* can be used as a biological control (bio control) agent against viruses.

Key words: Tobacco Mosaic Virus (TMV), *Pseudomonas koreensis* (*P. koreensis*), induced systemic resistance (ISR)

DECLARATION

I, Spiwe Nyamuvurudza, declare that this work is entirely my own. It has not been presented at any other institution for any other qualification or conference. I did not get any assistance from anyone or any organizations and I have referenced other people's work referred therein.

Signature ….Date 31/03/2017……

ACKNOWLEDGEMENT

I would like to express my gratitude to my Lord and saviour for taking me through this process. All glory and honour be unto you.

I wish to express my sincere gratitude to my supervisor Prof Karl Rumbold for offering me an opportunity to study in an area I enjoy. I would like to extend my deepest appreciation for his patience, encouragement and all the valuable guidance and support throughout my study period.

My sincere gratitude also goes to Dr Chinheya and his team in the department of plant pathology at the Tobacco Research Board, Zimbabwe for their assistance.

This dissertation would not have been possible without the support of my laboratory colleagues and I would like to express my appreciation to you

I would also like to thank my mum, sisters and brothers for their support, you are much appreciated. This project would not have been a success if you have not stood by me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

DEFINITIONS AND TERMINOLOGY

NaCl	Sodium chloride
DPI	Days post inoculation
HCN	Hydrogen cyanide
^{o}C	Degrees Celsius
JA	Jasmonic
ET	Ethylene
ABA	Abscisic acid

CHAPTER 1

1.0 INTRODUCTION

Most agricultural crops are susceptible to a wide array of diseases (Axel *et al.*, 2012; Someya *et al.,* 2012; Tamar *et al.,* 2013; Burketova *et al.,* 2016). Such diseases reduce both crop yield as well as quality (Tamar *et al*., 2013; Burketova *et al.,* 2016). An average of 10 % of crops is lost to disease globally on an annual basis (Strange and Scott, 2005; Tapadar and Jha, 2013). It is said that crop losses result in 800 million people, on an annual basis, having inadequate access to food (Strange and Scott, 2005). Numerous epidemics affecting crops have been experienced worldwide, such as potato late blight which has caused havoc in Western Europe in the past five years, and rice blast which has repeatedly caused serious damage in China and India (Salvary *et al*., 2006). In a related situation, in America, corn growers lost one billion US dollars in just one season to corn blight during the year 2001 (Strange and Scott, 2005). Less severe losses have also been reported from many other parts of the world and although losses might individually be relatively insignificant, collectively the losses become substantial (Salvary *et al*., 2006).

Demand for food in most developing economies which are driven by agriculture is high and growing, but the relative expansion in food production has not kept pace with increasing demand (Strange and Scott, 2005). Worsening the problem of food shortages are the high levels of disease infestation of crops in many tropical and subtropical countries aggravated by prevailing climatic conditions (Roy, 2003; Strange and Scott, 2005; Tapadar and Jha, 2013).One of the major difficulties to be overcome by many crop scientists is the increase in resistant pathogen strains coupled with absence of adequate crop protection technology, especially within poor regions (Beddington, 2010; Godfray *et al*., 2010; Tapadar and Jha, 2013). The barriers to food production are increasing with producers expected to maximize

resource use and reduce negative impacts on the environment (Chakraborty and Newton, 2011; Narayanasamy, 2013).

The producers are expected to achieve lower negative environmental impacts without compromising the total food output and are required to avoid crop failure at all costs (Tapadar and Jha, 2013; Burketova *et al*., 2016). Several methods are available for management of diseases. In recent decades what has encouraged many agricultural crops to thrive despite many diseases is the increased use of chemical pesticides (Beddington, 2010; Godfray *et al*., 2010; Burketova *et al*., 2016). Although chemical pesticides have proved useful, they increase the risk of ground water contamination, as well as death of non-target beneficial microorganisms playing important roles in their ecosystems (Johnsen, 2001; Axel *et al*., 2012; Someya *et al*., 2012; Tamar *et al*., 2013). Increased use of chemical pesticides causes a number of problems, including evolution of pesticide-resistant pathogen variants (Axel *et al*., 2012; Someya *et al*., 2012, Tamar *et al*., 2013; Babu *et al*., 2015; Burketova *et al*., 2016). Emergence of more aggressive pathogen strains with increased virulence has recently given rise to increased use of chemical pesticides (Johnsen, 2001; Axel *et al*., 2012; Burketova *et al*., 2016). This has significantly damaged ecosystems through contamination of groundwater and harvested food crops by heavy metals (Johnsen, 2001; Agbodjato *et al*., 2016; Zohara *et al*., 2016). Heavy metals are then transferred to humans when they consume the agricultural produce (Agbodjato *et al*., 2016). In the medical field, these metals have been reported to cause cancer (Agbodjato *et al*., 2016). The effect that these chemicals have on health makes their use unsustainable (Cook *et al*., 2000; Cordero *et al*., 2012; Burketova *et al*., 2016). Pressure from society to abandon use of chemical pesticides has intensified; hence calls for the development of environmentally friendly replacements (Cook *et al*., 2000; Bailey *et al*., 2010; Axel *et al*., 2012; Zohara *et al*., 2016). Finding alternative agricultural practices that avoid the

use of such agrochemicals has therefore become an urgent matter (Cook *et al*., 2000; Tapadar and Jha, 2013; Agbodjato *et al*., 2016).

1.1 Virus pathogens and their control

Most agricultural systems rely heavily on chemical pesticides to combat all plant pathogens (Someya *et al*., 2013; Agbodjato *et al*., 2016; Zohara *et al*., 2016). It has been observed that some of the chemicals used to control viral diseases reduce plant growth (Agbodjato *et al*., 2016). Intensified pressure by society to stop the use of chemical pesticides has been accompanied by realisation of the need to return to environmentally friendly cultural practices (Axel *et al*., 2012). A further outcome has been focus on breeding of naturally occurring resistant genes and induction of systemic resistance in plants by some plant growth promoting rhizobacteria (PGPR) (Raupach *et al*., 1996; Cook *et al*., 2000). There is consensus among plant pathologists that application of PGPR to combat diseases may compensate for reduced plant growth caused by chemicals such as methyl bromide (Someya *et al*., 2013; Agbodjato *et al*., 2016). Therefore, extensive use of these alternative ways of controlling viral diseases has become very crucial.

Many viral diseases such as Tobacco Mosaic Virus (TMV) are difficult to control (Siang-Hee *et al*., 1997). Tobacco Mosaic Virus is a broad host range virus that reduces growth of a wide range of more than 350 plant species (Shen *et al*., 2012). The disease is difficult to control because of strong virulence and easy transmission via sap during agronomic work as well as lack of effective chemicals (Melton *et al*., 2000; Scholthof, 2000; Shen *et al*., 2012). What makes TMV infestation particularly serious is that once a field has been contaminated with the virus, decontamination is very difficult (Yang *et al*., 2012). As long as crops are cultivated continuously, the virus continues to replicate in infected plants (Melton *et al*., 2000; Gulser *et al*., 2008; Yang *et al*., 2012), thereby infecting newly transplanted seedlings (Gulser *et al*., 2008 Yang *et al*., 2012). This viral disease has been reported to cause significant

economic loss of several crops worldwide and is transmitted to the host plants via soil (Siang-Hee *et al*., 1997; Yang *et al*., 2012). The only known chemical to date proven effective in preventing the spread of TMV in the soil is methyl bromide (Yoneyama, 1988a; Yang *et al*., 2012). However, this chemical was banned by the Montreal Protocol (Yang *et al*., 2012). Even if this chemical could be improved to reduce its environmental impacts, it nevertheless causes reduced plant growth (Agbodjato *et al*., 2016). It is becoming increasingly important that effective means of control are found because human population increases especially within the tropics mean demand for food is increasingly at risk of outstripping supply (Bailey and Mupondwa, 2006; Bailey *et al*., 2010). The prevailing climate within the tropics has worsened the problem because the warm wet conditions are conducive to growth and multiplication of many plant pathogens (Strange and Scott, 2005). The search for effective methods of controlling TMV over the past 100 years has produced mixed results and research outcomes have been reasonably successful in some situations (Yang *et al*., 2012). The improved quality of crop yields has been amongst the positive effects brought about in the quest for reliable control methods (Cook *et al*., 2000). However, each of these control methods such as breeding for resistance and chemicals has some drawbacks (Gulser *et al*., 2008).

1.2 Motivation

Many agricultural systems rely heavily on the use of chemicals. This has raised dissention due to the negative impacts such chemicals have on both the environment and people's health. Concerns raised by scientific and public role-players have contributed to the advent of strict chemical regulations in South Africa and elsewhere and the banning of some dangerous chemicals including via international conventions and protocols. A further issue is that diseases spread from crops to natural ecosystems where it is very difficult to successfully apply chemicals due to increased scale (Axel *et al*., 2012).

Intense societal pressure for sustainable agricultural systems has resulted in calls for alternative and more sustainable ways of controlling pests and diseases. Tamar *et al*. (2013) pointed out that there is a pressing need for the development of safer and more sustainable control methods. New agricultural strategies have emerged for ensuring high crop yields while at the same time protecting human health and the environment (Cordero *et al*., 2012). Several researchers have pointed out that the use of microorganisms or their secretions to prevent plant diseases offer an attractive low-impact alternative for the control of plant disease (Raaijmaker *et al*., Axel *et al*., 2012; Tamar *et al*., 2013; Zohara *et al*., 2016). Results of these studies have prompted the public to advocate for more use of biological control (bio control) rather than chemical pesticides.

Although the public has made known their concerns about heavy reliance on chemicals, which has raised fear that the chemical residues will remain in foodstuffs and the environment for a considerable time, bio pesticides as a means for plant protection still represent an insignificant portion of the overall pesticide market which currently is dominated by synthetic chemicals (Axel *et al.,* 2012).Furthermore, there is still significantly greater production of chemical pesticides compared to the production of bio pesticides (Hofte and Altier, 2010). Bio controls have been proposed as a potential supplementary strategy (Tran *et al*., 2008).Bio controls are insignificant, they only contribute 1 % to the commercial pesticide market (Fravel, 2005; Gross and Lopper, 2009; Bailey and Mupondwa, 2010, Bailey *et al*., 2010). Given the negative impact of many synthetic chemicals, bio controls should not be regarded as supplementary strategies. The ideal situation would be to completely replace all synthetic chemicals with safe, environmentally friendly bio control methods produced from PGPR such as *P. koreensis*. Use of bio pesticides in agricultural systems is still limited and they have not yet reached viable levels to replace chemical pesticides (Hofte and Altier, 2010). Only 14 bacteria have been registered as bio products in America, six are from *Bacillus spp*

and only five are from *Pseudomonas spp* (Hofte and Altier, 2010). *Pseudomonas spp* are said to be better and more appropriate bio control agents because of their ability to use more exudates as nutrient source, abundance, and have a high growth rate (Mark *et al*., 2006; Hofte and Altier, 2010).Therefore it has been recommended that more studies are needed to determine the ability of more beneficial soil bacteria in controlling plant diseases and this will help us to select appropriate candidates for the production of commercial bio control products for use in the production of food crops (Mark *et al*., 2006; Hofte and Altier, 2010; Someya *et al*., 2012).

To avoid the use of hazardous chemicals, development of environmentally friendly strategies including bio control agents has taken the centre stage worldwide (Zohara *et al*., 2016). It has been reported that in disease-suppressive soils, expression of diseases is not significant and disease suppression ability of these soils has been attributed to the host of microbial populations inhabiting these soils (Hofte and Altier, 2010; Yang *et al*., 2012). Many strains of microbes selected and isolated from these disease-suppressing soils have been seen to help in decreasing plant diseases when they are used to treat plant seeds and roots. Therefore, it is important that more of the soil microbes inhabiting such disease-suppressing soil must be isolated and intensively studied so as to increase the bio control pesticide market. Some of the microbial species and their strains inhabiting such soils have been well documented. Much is known about the reaction that they trigger in plants involving enhanced protection from various pathogen attacks. *P. putida, aeruginosa*, strain CHAO and *Bacillus spp*, for example, have been well studied. The secretions of these species suppressing different pathogens like bacteria, viruses and oomycetes as well as their ability to induce systemic resistance has been widely studied (Van Peer *et al*., 1991;Tambong and Hofte, 2001; Keel *et al*., 2002; Van loon *et al*., 2003; Kloepper *et al*., 2004; Tamar *et al*., 2013, Toyota and Watanabe, 2013; Tran *et al*., 2008). However, opportunities for *P. koreensis* in fighting plant

disease have not yet been extensively studied. One of the few investigations done to date was a study to elucidate potential for *P. koreensis* to fight plant disease was done by Hultberg (2010). His study investigated the usefulness of a bio surfactant produced by *P. koreensis* against the oomycete *P. ultimum*. The results of this study showed a significant reduction in disease, and further that the bio surfactant did not affect the soil micro flora. The ability of *P. koreensis* to fight viruses has, however, not yet been investigated. An in-depth study of this species is important so that effective recommendations can be given to bio control manufacturing agents on what PGPR to use in controlling specific diseases. It is against this background that this study seeks to investigate if *P. koreensis* can decrease virus plant disease in *N. benthamiana* by inoculating its seeds and roots with these bacteria and mechanically infecting its leaves with a virus (TMV). This will be followed by TMV disease assays.

1.3.2 Aim

To determine the ability of the bacteria in the induction of systemic resistance in the plant against TMV.

1.3.3 Objectives

The aim will be achieved through execution of the following objectives:

1 Cultivate *N. benthamiana* and inoculate with *P. koreensis.*

2 Inoculate *N. benthamiana with* TMV inoculum for leaves infection.

3 Perform TMV virus infection studies on *N. benthamiana* for disease suppression evaluation.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction and theoretical framework

Plant roots release nutrient-containing compounds rich in carbon and nitrogen into the soil and this nutrient rich environment attracts a host of microorganisms which use the plant exudates and lysates as they grow (Hofte and Altier, 2010; Someya *et al*., 2012). It has been reported that these soil rhizosphere bacteria are essential for plant growth and development (Adesemoye *et al*., 2008). Furthermore, the rhizosphere bacteria have been seen to help plants respond physiologically to adverse changes in environmental factors (Narayanasamy, 2013). In other words, they help plants to acclimatise effectively in response to environmental stress, offering protection against extreme biotic and abiotic stresses (Landa *et al*., 2004; Narayanasamy, 2013).

It has been reported that plants treated with certain types of rizhosphere bacteria have shown improved growth (Jahanian *et al*., 2012). These bacteria are said to actively encourage efficient absorption of minerals and water by plants (Gholani *et al*., 2007). They also promote growth by producing growth stimulating compounds (Babu *et al*, 2015). It is important to note that most of the growth results from suppression of harmful disease-causing bacteria or viruses (Van Loon and Glick, 2004).

PGPR growth promotion mechanisms are relatively complex generally involving (i) competition between PGPR and pathogens; (ii) anti-microbial compound production by PGPR and (iii) reduction of plant pathogen populations which limit plant growth (Hultberg *et al*., 2010; Yang *et al*., 2012). However, some of these suppression mechanisms like production of hydrogen cyanide HCN have the potential to reduce plant growth (Bakker *et al*., 2003; Diollo *et al*., 2011). In most cases the net effect nevertheless produces improved plant growth, thereby increasing agricultural crop yields (Leeman *et al*., 1995; Pieterse *et al*., 2008). Several model

plants have been used to isolate and identify the soil microbes that can promote growth and enhance the defensive capacity of plants.

2.2 *N. benthamiana*

In investigating the potential of PGPR in controlling and fighting plant diseases, researchers have now adopted the model plant *N. benthamiana* for the study of host-pathogen interactions, especially viruses (Chakrabarty, 2007; Goodin *et al*., 2008). The plant is endemic to Australia and it is known as "*tjuntiwari"* and "*muntju"* by the indigenous people (Burbidge, 1960). It has a widespread distribution and is found in seasonally arid habitats that most other native species do not favour (Monjero *et al*., 2015). It was first collected by colonialists on the New Holland coast in eastern Australia (Burbidge, 1960). The species has 19 pairs of chromosomes and was adopted as a model plant mainly because it is readily infected by many viruses that do not cause any infection in other model plants (Goodin *et al*., 2008, Bombarely *et al*., 2012). The plant is affected by almost 500 plant viruses (Chakrabarty, 2007; Goodin *et al*., 2008,). Yang *et al*. (2004) in Goodin *et al*., (2008) investigated the reason behind *N. benthamiana*'s susceptibility to such a wide variety of plant viruses. They concluded that this is partly linked to a mutation occurring in an RNA- dependent polymerase gene (NbRdRPIm) present in its genome (Chakrabarty, 2007). Another factor in favour of *N. benthamiana*'s selection is its rapid growth. A further reason for its popularity as a model plant is that it can easily be agro infiltrated (Bombarely *et al*., 2012; Monjero *et al*, 2015). It also does not require special treatments to germinate, but can simply be sown on the surface of a good quality, sandy and well-drained potting mix and with periodic gentle watering (Goodin *et al*., 2008; Bombarely *et al*., 2012; Monjero *et al*, 2015). Germination of this drought-tolerant plant takes 7-21 days (Goodin *et al*., 2008; Monjero *et al*, 2015).

Although *N. benthamiana* is indispensible as a plant pathogen study model, its establishment in the greenhouse has proved challenging in previous studies (Monjero *et al*,

2015). A review by Monjero *et al*. (2015) was done in order to provide more information on how to get optimal establishment of *N. benthamiana*. Their study investigated appropriate media and optimal growing conditions necessary for getting suitable *N. benthamiana* plants for research purposes.

Results from the study by Monjero *et al*. (2015) have shown that soil: vermiculite ratios of 3:1, or 2:3 produced the best and most healthy *N. benthamiana* seedlings within a short period of time (21 days). Seeds were ready for inoculation with a virus by this stage. The other ratios were seen not to be practicable such as absolute soil or vermiculite. The seedlings grown in the latter ratios were unhealthy and not suitable for viral inoculation since they were stunted. Seedlings did not thrive in either vermiculite only or sand only, and it was concluded that a combination of the two is critical for optimal growth.

2.3 Bacterial strain used: *Pseudomonas spp*

One of the Rhizobacteria that has been broadly studied is from the genus *Pseudomonas* (Cordero *et al*., 2012) owing to (i) its excellent ability to fight pathogens using a variety of complementary mechanisms and (ii) its well-established capacity to improve growth in plants in the absence of pathogens (Cordero *et al*., 2012). *Pseudomonas spp* are gram negative with simple nutritional requirements, they are not affected by other microorganisms and grow well under normal conditions (Mark *et al*., 2006; Hofte and Altier, 2010). The antagonistic ability of Pseudomonas *spp* depends on the production of metabolites such as 2.4 diacetylphlo roglucinol (DPAG) pyrrolnitrin PPV, phenazine PCA, pyluteonn PLT, (HCN) and volatile compounds, as well as degradation enzymes (Maurhofer *et al*., 1994; Cordero *et al*., 2012; Annapuma *et al*., 2013). Diollo *et al*. (2011) pointed out that *Pseudomonas* produce indol derivate, peptides, glycolipid, lipids and aliphatic compounds that can be used in place of chemical compounds in combating plant diseases. In Diollo *et al*. (2011) and Pieterse *et al*. (2000)'s studies the potential of *Pseudomonas* to prevent the development of plant pathogens

has been demonstrated. Many more studies have revealed that *Pseudomonas* is able to induce systemic resistance in plants (Maurhofer *et al*., 1994); Tran *et al*., 2008; Singhai *et al*., 2011). This is attributable to intimate interrelationships between the bacteria and plants (Kirankumar *et al*., 2010; Singhai *et al*., 2011). Induced resistance is a state of increased defensive capacity initiated by identifiable triggers stimulating the plant's defences against pathogens that will otherwise successfully attack the plant (Wei *et al*., 1996; Murphy *et al*., 2000; Singhai *et al*., 2011). Some species of *Pseudomonas* protect the plant by activating defence gene encoding (Nielsen *et al*., 2002; Cordero *et al*., 2012).

Several species of *Pseudomonas spp* isolated from roots of food plants have proved useful against a variety of plant pathogens (Osburn *et al*., 1989; Park and Kloepper, 2000; Siddiqui and Shaukat, 2003; Sorensen *et al*., 2001). The species *P. putida* is capable of suppressing TMV in soil (Yang *et al*., 2012). CHAO also works well to suppress fungi, viruses and nematodes (Siddiqui and Shaukat, 2003). *P. fluorescens* strain CHAO suppressed Tobacco necrosis virus (TNV) (Maurhofer *et al*., 1994).Furthermore, *Pseudomonas* B-25 increased plant resistance against TMV (Kirankumar *et al*., 2010).

It has been demonstrated that *Pseudomonas spp* constitute <4 % of all the microbes found in the soil. Nevertheless, they constitute 20 % of the rizhosphere microbes that can be cultured (Sorensen and Nybroe, 2004).

2.3.1 Characteristics of *Pseudomonas koreensis*

Most strains of *P. koreensis* found worldwide exhibit the following characteristics which distinguish them from other *Pseudomonas strains:* motility is provided by more than one polar flagellum, they are catalase and oxidase-positive and show hydrolysis or arginine Tween 80 (Kwon *et al*., 2003; Anzai *et al*., 2000; Peix *et al*., 2009). Most strains liquefy gelatin but do not show acidification of glucose and reduction of nitrates to nitrites is negative

(Kwon *et al*., 2003; Hurltberg *et al*., 2010). Most strains show a positive licithinase reaction while the urease reaction is variable among strains (Behredt *et al*., 1999; Anzai *et al*., 2000; Kwon *et al*., 2003). Indole is not produced on tryptophan. Strains grow at 4 degrees Celsius (°C) but not at 37 °C, growth occurs at 5% NaCl but not at salinity levels of higher than 7% (Kwon *et al.,* 2003; Hurltberg *et al*., 2010). Research done by Hurltberg *et al*., (2010) indicated that strains utilize Tweens 40 and 80, for example: N-acetyl-D-gluco samine: L- arabinose; D arabitol, D fructose; D-glactose and D-glucose D- mannitol; mannose; methyl pyruvate monomethyl succinate; acetic acid; cis-aconitic acid; atric acid; D galactonic acid; lactone and D gluconic acid (Kwon *et al.,* 2003). While the strains can assimilate glucose carprate, malate and citrate they are unable to assimilate maltose, adipate or phenylacete (Behredt *et al*., 1999; Anzai *et al*., 2000; Kwon *et al.,* 2003; Peix *et al*., 2009; Hurltberg *et al*., 2010).

2.4 TMV

Tobacco Mosaic strain used in this study was found on tobacco k326 plants grown for resistance by the Tobacco Research Board of Zimbabwe.

2.4.1 TMV biology, disease cycle and epidemiology and signs and symptoms

TMV falls within the genus *Tobarmovirus* and it is rod shaped measuring 300mm× 15mm (Gulser *et al*., 2008). TMV RNA is positive and is a direct messenger (mRNA) translated through the use of host ribosomes (Melton *et al*., 2000; Scholthof, 2000). Translation has been reported to commence within minutes of being infected and it makes use of its protein movement to spread from cell to cell (Siang-Hee *et al.,* 1997; Gulser *et al*., 2008). In the end after a series of movements from cell to cell, it reaches the plant's veins and systemically spreads via the phloem to roots and tips of the infected plant (Siang-Hee *et al.,* 1997; Gulser *et al*., 2008). Incidence of the disease varies from plant to plant and over time (Melton *et al*., 2000; Scholthof, 2000).

TMV has been studied extensively. Studies have shown that it can infect over 350 species resulting in two fold impacts as follows: (i) it lowers yields and (ii) reduces quality of output (Siang-Hee *et al.,* 1997; Gulser *et al*., 2008). Some of the important crop plants affected by TMV include tobacco, tomato and many other solanaceous plants (Siang-Hee *et al.,* 1997; Gulser *et al*., 2008). The Solanaceae Family is the most affected by TMV and the loss experienced is drastic (Melton *et al*., 2000; Scholthof, 2000). Transmission of TMV is easy and for this reason it is very difficult to control (Siang-Hee *et al.,* 1997). It is transmitted mechanically and if infected leaves, contaminated tools or workers' hands rubbed against a healthy plant can spread the virus and TMV disease can also develop from contaminated seeds (Siang-Hee *et al.,* 1997; Gulser *et al*., 2008). The virus is stable and it has been reported to infect plants even after being stored at 4 ^{O}C over a long period of about 50 years (Siang-Hee *et al.,* 1997).Furthermore, it has been reported to be present in tobacco products because it can withstand the curing process (Melton *et al*., 2000; Scholthof, 2000).

The virus gives rise to different symptoms depending on the type of plant infected, age of plant, virus strain and genetic background of the plant, as well as environmental conditions (Siang-Hee *et al.,* 1997; Gulser *et al*., 2008). TMV causes mottling mosaic pattern of light and dark green areas (figure 2 A & B), necrosis (figure 2C & D), stunting, leaf curling and yellowing of plant tissue. Symptoms can also be expressed as deformed fruits, delayed fruit ripening and non-uniform in the colour of fruits (Melton *et al*., 2000; Scholthof, 2000).

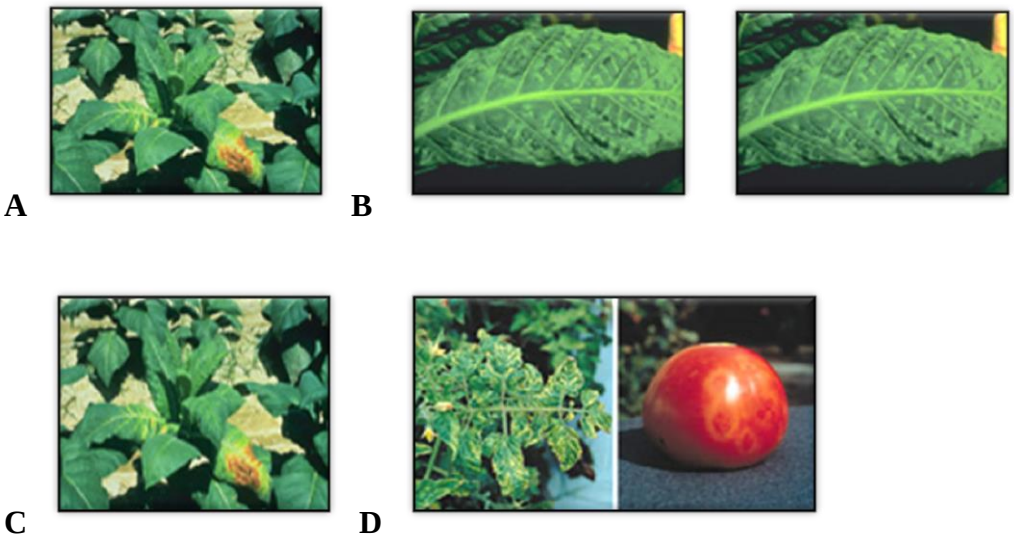


Figure 1. Signs of TMV infection: The signs and symptoms vary depending on the size of the plants at the time of infection. TMV causes mottling mosaic pattern of light and dark green areas (A) and stunting, leaf curling and yellowing (B and C), of plant tissue, deformed fruits (D) (Melton *et al*., 2000).

The symptoms appear in 7 to 14 days after infection, veins clearing are the initial symptoms of a plant newly infected by TMV and as soon as vein clearing appears, mosaic systems start to appear (Siang-Hee *et al.*, 1997; Melton *et al*., 2000; Scholthof, 2000). Mosaics are expressed as dark green areas known as "*islands*" without the virus and light green areas which have the virus (Siang-Hee *et al.*, 1997). Young and newly infected leaves usually become deformed and wrinkled and mosaics patterns only appear in new parts of the plant, although it does not mean that the virus is restricted only to these parts (Siang-Hee *et al.*, 1997). The virus has been found in many parts including corollas, roots and leaves even when there are no visible signs of infection (Melton *et al*., 2000; Scholthof, 2000).

2.4.2 Effects of TMV and control

Infection by TMV occurs easily due to the ease of spread of the virus. Many losses of crops have been reported the world over due to TMV infection and agriculturalists have not only experienced losses in crop volumes but also reduction in quality of produce (Siang-Hee *et al.*, 1997). In the year 2000, North Carolina lost 1.4 % in yield of tobacco which translated to 10.7 million dollars loss (Melton *et al*., 2000).

Time of infection has a bearing on TMV disease severity. It has been concluded by many studies that the younger the plants infected, the higher the loss in total output of the crops (Melton *et al*., 2000).

Several strategies can be used to manage the disease. Observing good sanitation and cultural practices has been seen to provide great help in managing the disease and is a good measure to avoid its spreading (Siang-Hee *et al.*, 1997; Melton *et al*., 2000; Scholthof, 2000) . Other effective methods used include methyl bromide and use of cross protection (Yoneyama, 1988). Cross protection involves inoculation of a very mild strain onto very young plants which would in turn protect the plants from future serious strains of TMV (Scholthof, 2000). Resistance has been highlighted as a key means for providing agronomists with the best way of managing TMV once a crop has been infected (Siang-Hee *et al.*, 1997). The other way that has been used to confer resistance is the use of N gene and it has been shown that N gene can reduce the incidence of TMV by a greater margin in tobacco (Siang-Hee *et al.*, 1997). N gene does this by initiating defence responses that will limit TMV replication and movement (Witham *et al*., 1996; Erickson *et al*., 1999) However, it has been discovered that plants that have this gene are of low quality and their yields will be lower than those that do not carry the gene (Erickson *et al*., 1999). It is also worth noting that, though N gene induces good quality resistance that can be reliable; its shortcomings have not been adequately dealt with (Melton *et al*., 2000; Scholthof, 2000). The implication of the short comings of these control methods used is that more research is needed to find effective methods to control the virus.

2.5. Root Colonization by PGPR

Many investigations have shown in the past that species of *Pseudomonas* easily colonize pericarps of sugar beet seeds. For this reason *Pseudomonas* has been seen to be effective in decreasing the occurrence of seed disease (Osbum, *et al*., 1989). It has also been demonstrated that the ability of bacteria to colonize roots is affected by environmental factors.

Lopper *et al*. (1988) showed that there is a positive correlation between root colonization and inoculum density. However; inoculum density did not have a significant effect on root colonization in some studies (Schmidt *et al*., 2004a). Schmidt *et al*. (2004b) observed that soil matrix and temperature greatly affected seed and root colonization by *Pseudomonads*. The population density of *P. fluorescens* was greatly reduced at high temperatures 25-35°C. However *P. aureofaciens* worked well at temperature above 22 °C (Mathre *et al*., 1994). Biotic and abiotic factors in the environment have both positive and negative effects on diversity and the way *Pseudomonas spp* are capable of fighting plant disease (Someya *et al*., 2012).

In the Field PGPR can be applied (i) to seeds, (ii) soil and (iii) aerial parts. Treating seeds with PGPR means that they are present from the beginning of plant growth and this is regarded as important in that it ensures successful colonization (Yang *et al*., 2012; Subashri *et al*., 2013). This further reduces competition with other inhabitants of the rizhosphere, therefore ensuring establishment of high population densities (Sorensen and Nybroe, 2004; Bouizgame, 2013). The inoculation of sugar beet seeds by *P. fluorescens-putida* lowered the *P. ultimum* colonization rate (Osbum *et al*., 1988).

Application of PGPR to soil is associated with many benefits. The low variation in soil environmental conditions ensures less stress and increases the survival rate of PGPR after application (Helbig, 2006). This is made possible by the buffering of excessive rainfall and dryness provided by the soil, thus maintaining a constant environment (Subashri *et al*., 2013; Narayanasamy, 2013).

Application of PGPR on aerial parts of plants is more difficult, colonization has limited success due to exposure to stresses such as too much dryness, heavy rainfall and wind and these adverse conditions make survival very problematic (Sorensen and Nybroe, 2004).

Nevertheless, there has been a significant reduction of angular leaf spots per leaf on cassava after foliar application (Keel *et al*., 2002).

2.6 How do plants defend themselves against diseases?

Plants can defend themselves against diseases through two different mechanisms, namely passive (constitutive) and active (inducible) mechanisms (Someya *et al*., 2012). The former occurs when an attack by a pathogen causes damage to the cuticle and the damaged parts secrete metabolites like resins which cause the development of disease in some pathogens (Someya *et al*., 2013; Narayanasamy, 2013). The latter is activated when plants are attacked by pathogens and resulting in changes like thickening of plant external walls, making it difficult for pathogens to enter the plant (Keel *et al*., 2002). This can also activate the development of hypersensitivity which involves the accumulation of toxic compounds and phytoalexins by the cell near pathogens (Keel *et al*., 2002).

Many studies have shown the positive effect of PGPR strains on plant development in soils infested with disease-causing organisms. Investigations by many scholars have shown several mechanisms that supress diseases activated by PGPR (Sorensen and Nybroe, 2004). PGPR has created several antagonistic ways to suppress disease-causing organisms' growth. In one instance, they have created competition for iron by production and secretion of lytic enzymes and in another they have triggered a reaction that increased resistance (Maheshwari, 2013; Narayanasamy, 2013).

Van Peer *et al*. (1991) investigated the antagonistic activity caused by *P. fluorescens*. Results of this study revealed that the plant was still protected even though bacteria were present around the roots and infection was in aerial parts. The disease-causing organism was slash-inoculated into the stem. Fungus and Rhizobacteria were not seen in contact with each other within the plant. Therefore, the investigators concluded that the protective effect released

was plant mediated. The resultant effect of these PGPR has been named Induced Systematic Resistance (ISR) (Pieterse *et al*., 2000).

2.6.1 ISR and SAR Expression and virus control

The effect attributed to induced systemic resistance (ISR) is like that of systemic acquired resistance (SAR). There is reduction in the severity of the disease and the actual number of plant infections is also decreased. (Maurhofer *et al*., 1994; Annapuma *et al*., 2013). This is caused by a decline in pathogen growth and in the establishment of pathogens on induced tissues (Van Loon, 2000). It is worth noting that neither ISR nor SAR offer total protection for the plant against pathogens (Annapuma *et al*., 2013). Nevertheless, slow multiplication of pathogens reduces the rate at which the disease develops and has the potential of saving the crop by avoiding significant drop in yield (Kirankumar *et al*., 2010).

SAR and ISR are effective over a wide array of diseases, and it has been found that ISR and SAR effectiveness overlaps in some of these diseases (Maheshwari, 2013; Narayanasamy, 2013). It has further been demonstrated that SAR and ISR provide improved plant protection when they are used together and are individually effective against certain pathogens and not effective against others (Van Wees *et al*. 2000; Ton *et al*., 2002). Hence it would be problematic to use results of one experiment of SAR and ISR to make general conclusion about their effectiveness in protecting different plant species from pathogens (Maurhofer *et al*., 1994). This indicates that species to species experiments with PGPR are necessary to reduce plant losses from diseases. Nevertheless, it has also been established that in certain plant species, both SAR and ISR are effective and therefore are an alternative to chemical pesticide use (Achuo *et al*., 2004).

ISR is a reaction that is created by PGPR starting from plant roots (Fig 2). A signal is given which spreads throughout the plant, thus improving defensive capacity in distant tissue

to further infection (Murphy *et al*. 2000; Toyota and Watanabe, 2013). Antagonistic activity of PGPR is the mechanism through which defence against pathogens is stimulated in upper parts of the plants (Kirankumar *et al*., 2010; Annapuma *et al*., 2013).

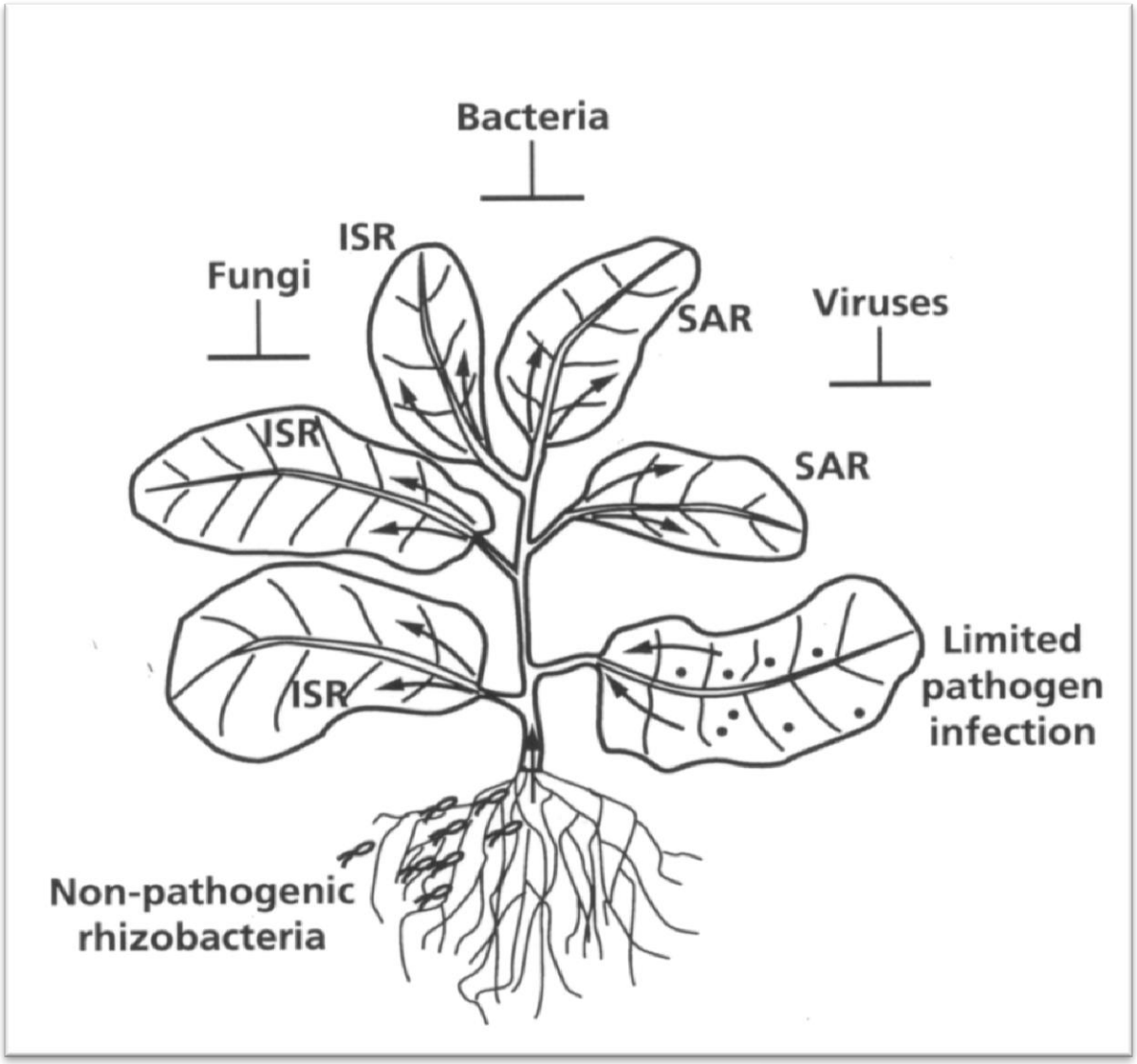


Figure 2. Schematic diagram showing how resistance is induced in plants: Non-pathogenic Rhizobacteria triggers a reaction that spreads systemically from the roots to the rest of plant parts enhancing their defensive capacity (Narayanasamy, 2013).

Research has shown that there are certain strains of PGPR that can trigger a reaction that enhances plant defensive mechanisms (Someya *et al*., 2013). In other words, it has been stated that occupation of the roots of plants by a range of non-pathogenic bio control bacteria

results in the development of enhanced defensive capacity against a wide spectrum of plant pathogens (Pieterse *et al.,* 2000; Toyota and Watanabe, 2013). ISR by seed or soil treatment with PGPR shields plants against various pathogens (Park and Kloepper, 2000; Pieterse *et al.,* 2000). Rizhosphere bacteria-mediated ISR has been reported for many crops. Park & Kloepper (2000) investigated an assemblage of different PGPR with ISR activity on tobacco. Results revealed that provocation of PR- la promoter were significantly improved compared to the background activity in the water control. Raupah *et al.* (1996) determined if PGPR strains that induce systemic resistance in cucumber to fungal and bacterial pathogen could also do the same in cucumber and tomato against cucumber mosaic virus. Results revealed that seed treatment with PGPR reduced the number of plants diseased with mosaic virus. In tomato, disease development was decreased. The purpose of the research done by Maurhofer *et al*. (1993) was to determine whether strain CHAO is able to induce resistance against leaf necrosis caused by tobacco necrosis virus (TNV). It was found that there was a significant reduction in lesion number. Pieterse *et al*. (2000) explored the part played by jasmonic acid JA ethylene in ISR. Results revealed that it is not associated with amplified levels of JA ethylene or SA in systemic tissues.

Rhizobacteria determinants that can evoke or draw out ISR include flagella, polysaccharides, iron regulated metabolites antibiotics, volatiles, phenolic compounds and quorum-sensing molecules (Hoffland *et al*., 1996, Alizadeh *et al*., 2013). Studies have revealed that ISR is SA independent but it requires a complete response to ET and JA (Pieterse *et al*., 2000). ISR and SAR have an effect on plant growth

It has been seen that gradual increases in PR in SAR have a negative effect on the rate of plant growth (Heil, 2002). On the other hand, PGPR used to trigger ISR is noted to increase plant growth rate (Someya *et al*., 2013). The other advantage peculiar to PGPR is the protection offered to plants against soil pathogens which otherwise attack seeds before

germination, protection are also afforded to germinating seeds (Heil, 2002). It is further argued that increased plant growth has an added advantage of shortening the period of vulnerability of plant in that increased growth means that the plants rapidly become adults and resistance induced by PGPR would have had sufficient chance to become established and thus can reduce damage that may be caused by disease causing organisms (Annapuma *et al*., 2013). PGPR can be applied to seeds so that when they germinate the roots are colonized, thus providing protection from an early age (Leeman *et al*., 1995c).

The above-mentioned characteristics make ISR-inducing PGPR an important weapon against diseases caused by JA-and ethylene-dependent defences in sensitive organisms (Someya *et al*., 2013; Narayanasamy, 2013). It is worth noting that the combination of ISR and SAR is more effective because it ensures that pathogens that are sensitive to JA and ethylene and SA-dependent defences are destroyed (Annapuma *et al*., 2013).

2.7 Population density and disease suppression

For significant disease suppression, the population density of rhizobacteria is important. The success of PGPR in suppressing diseases is principally dependent on their ability to keep their population at a high and stable level and maintain their metabolic activity (Bouizgame, 2013; Narayanasamy, 2013). It has been demonstrated that many *Pseudomonas* populations may decrease considerably within a short period of time, thus reducing their ability to suppress diseases (Subashri *et al*., 2013; Bouizgame, 2013). For instance, the 100-fold reduction of strain CHAO –RIF in both soil and on cucumber roots completely destroyed its ability to suppress *P. ultimum* in cucumber (Keel *et al*., 2002). A dose-response study of ISR by *P. flourescens* WCS 374 in radish concluded that population density of 10^5 colony forming units per gram of root are essential (Raaijmakers *et al*., 1995).

Combinations of strains of rhizobacteria have been seen to provide better results (de Boer *et al*., 1999; Bouizgame, 2013; Someya *et al*., 2013). On the same note, if rhizobacteria are to be effective bio a control agent against soil-borne pathogens, better establishment on the plant is of paramount importance (Bouizgame, 2013). Significant suppression by *Pseudomonas* strain has been reported to require a threshold population of 1, 2 x 10^5 to CFLG 4, 6 X 10^5 CFUG (Raaijmakers and Weller, 1998; Subashri *et al*., 2013). Some studies have shown that some strains of *Pseudomonas* failed to suppress pathogens with antibiotic phenazine and this was caused by a lack of mobility of PGPR which later affects rizhosphere colonization. It has been reported that in circumstances where no bacteria are introduced, one genotype of bacteria does not reach sufficient population size; thus the ability of field plants to express ISR is questioned (Chin-A-Woeng *et al*., 2003; Bouizgame, 2013). However, in some dose-response studies, some types of rhizobacteria have proved to be effective in smaller quantities whereas others failed for instance, RSIII was seen to be successful in suppressing the progress of disease in radish and flax based on very low initial population densities compared to RSI77 (Maheshwari, 2013). Results like these indicate that disease suppression through ISR by certain strains has the potential for improvement (Chin-A-Woeng *et al*. (1997; Bloemberg 2007; Someya *et al*., 2013).

Mechanism of disease suppression, work separately even when they come from the same source (Subashri *et al*., 2013). For example, fluorescent *Pseudomonads* can activate ISR and antibiosis. It can then be argued that a combination of these two processes has a high potential of producing a desired bio control (Narayanasamy, 2013). It can further be argued that such processes complement each other. For instance, antibiosis initially reduces the strength of the pathogen population; afterwards the weakened pathogens are then pitted against a plant with enhanced defensive capacity (Fukui *et al*., 1994). This will result in a much reduced development of the disease.

Furthermore, it has been argued that the use of strains that activate SA JA or ethylene-dependent pathways will increase usefulness of bio-control (Someya *et al*., 2013). Moreover, ISR is regarded as the best of these defensive mechanisms with the key feature being that it protects plants against both soil-borne and aerial plant diseases (Murphy *et al*., 2013). Apart from this, ISR triggered by one strain is successful in protecting plants against a range of pathogens (Ton *et al.*, 2002). Harnessing of these findings has the potential of reducing the amount of chemicals needed to protect crop plants from diseases (Bakker *et al*., 2003).

2.8 Factors affecting disease suppressive mechanisms

Use of PGPR has had variable results across different circumstances limiting their use in the commercial agriculture (Hultberg *et al*., 2010). The absence in uniformity of results has been attributed to variation in both physical and chemical properties within each area where PGPR are applied and this variability is thought to have an effect both on colonization and the mechanisms through which PGPR suppress diseases (Someya *et al*., 2013; Bouizgame, 2013). Factors that impact usefulness of PGPR include method of application, as well as abiotic factors like pH, temperature, moisture, texture, inorganic and organic compounds (Fukui *et al*., 1994). It has been realised that method of application of PGPR to seeds might have an effect on their distribution and colonization (Bouizgame, 2013). Strains applied in low concentrations were not effective and those applied in high concentrations seemed to be more effective and low concentration has therefore been seen to be one of the major factors affecting root colonization and expression of disease suppression mechanisms (Bouizgame, 2013). Landa *et al*. (2001) showed that conditions favourable to the development of disease were experienced and the success of PGPR to suppress disease decreased. A later study by Landa *et al*., (2004) showed a positive correlation between increase in temperature and bacterial population and inhibition of disease was experienced at optimum temperatures for bacterial development and production of poisonous metabolites (Landa *et al*., 2004). A study using *P. fluorescens* B5

showed that root colonization at ≥4 cm underneath the seed deteriorated with low soil water matrix (-330×10^3 Pa), however, the entire population per seedling increased at -140< 10^3Pa (Bouizgame, 2013). In addition to total population, colonization declined significantly at high temperature (25-35°C) (Bouizgame, 2013). On the same note, antagonistic potential of B5 against *P. ultmum* had a negative correlation with increasing temperature and declining soil matrix potential (Schmidt *et al*., 2004).

CHAPTER 3 MATERIALS AND METHODS

3.1 Experimental site and description

The greenhouse experiments were conducted at the University of Witwatersrand's Braamfontein Campus in Johannesburg, South Africa. *N. benthamiana* was used as the host plant in the experiment mainly because it is readily infected by many viruses that do not cause any infection in *other* model plants. (Goodin *et al*., 2008). The greenhouse was divided into 6 sections comprising of the same area and on the same gradient. A total of 54 pots were used with 2 plants in each pot. One set of plants had their seeds inoculated with *P. koreensis* then sown into jiffies (early treatment). The other plants were sown into jiffies inoculated with sterile distilled water. Autoclaved soil: vermiculite in the ratio of 3:1 was placed in each pot when the seedlings were eight weeks old. *P. koreensis* was used to inoculate the soil and vermiculite before the seedlings were transplanted (late treatment). The experimental leaves were inoculated with TMV-infected tobacco leaves saps (25 mg/Ml v/w) when the plants were at 6-8 leaf stage. Watering procedures was carried out once a day without applying any fertiliser. The two treatment groups were based on 2 bio preparation treatments and one control. The experimental period was from October 2016 to15 March 2017.

3.2 Experimental design

A completely random block experimental design was used, and random allocation of treatment to experimental units was done so as to avoid confounding outcomes between treatments effects and other unknown factors (Leedy & Ormrod, 2013; Pirk *et al*., 2013). Each treatment consisted of 6 units each with 6 replications equating to 36 plants per treatment. The replicates were also aimed at increasing accuracy of experimental effects (McDonald, 2008). To see the true effect of the bacteria in reducing severity of viral infection, we needed to control as much variability as possible. Creating blocks in the greenhouse took care of any variability in experimental conditions in different areas (McCrum, 2008). The experiment was set up as follows: (Fig3).

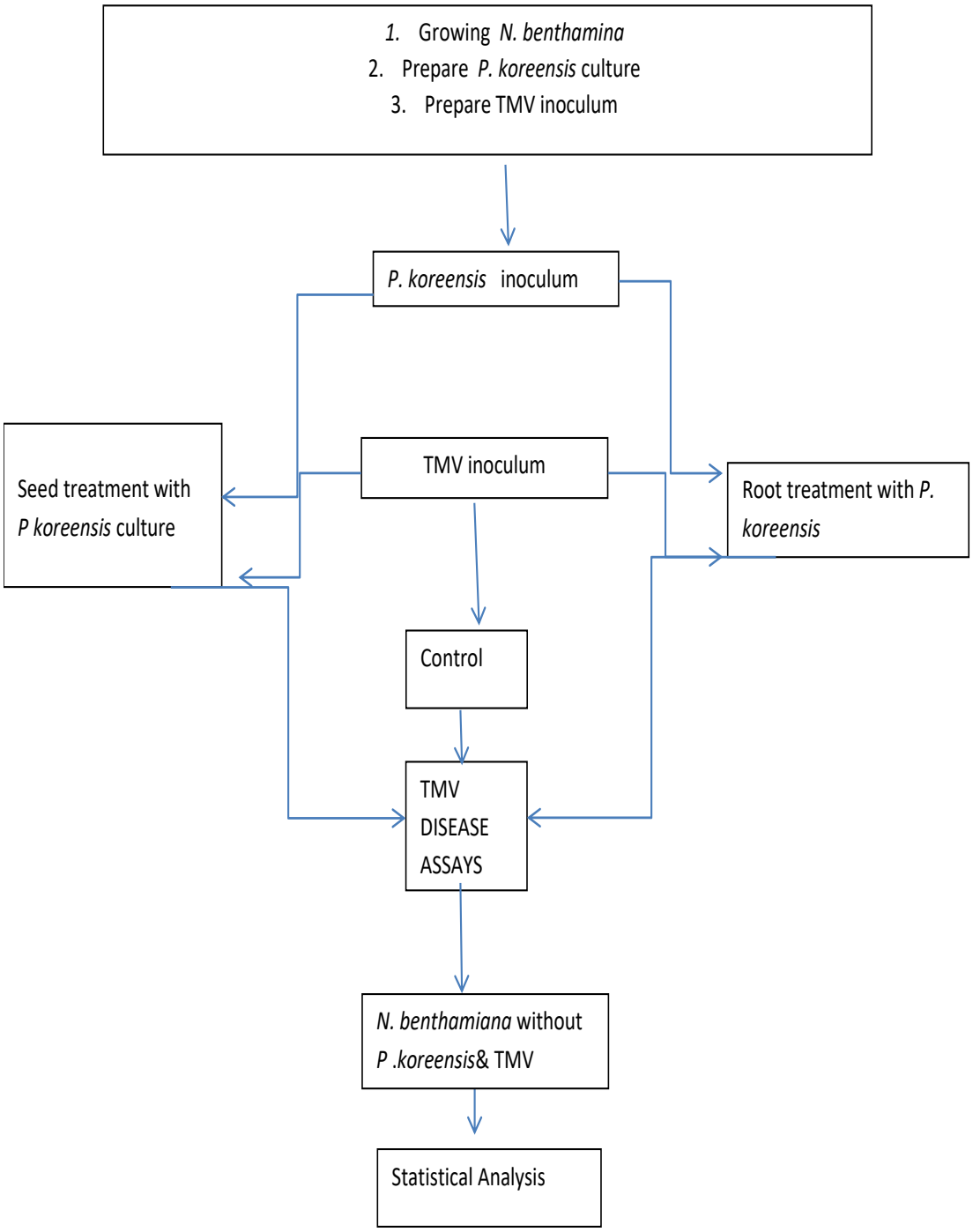


Figure 3 Schematic diagram showing the experiment set up

3.3. *N. benthamiana* cultivation

Two sets of plants were grown in two different trays; each tray contained 50 jiffies with 5 seeds per jiffy. The seeds were first sterilized with 70% alcohol for 3 minutes and 0.1 % $HgCl_2$ for 1 minute and washed repeatedly with distilled water (Chawla, 2002; Agbodjato *et al*., 2015). The seeds of *N. benthamiana* were sown in jiffies on the 30th of October 2016. The seedlings were grown for eight weeks at a humidity of 70% in the temperature regulated

greenhouse at a constant temperature of 25ºC and a photoperiod of 16 hours and 8 hours darkness throughout the growth period (Monjero *et al*., 2015). The seedlings were transplanted at approximately eight weeks after being sown into 15 cm and 8 cm pots filled with sterile soil: vermiculite (3:1) autoclaved twice at 121ºC for 20 minutes (Noumavo *et al*., 2013). The growth medium was chosen according to the recommendations of Monjero *et al*. (2015) which proved to be appropriate for the establishment of healthy indicator plants of *N. benthamiana* within the shortest possible period of time. A total of 54 pots were arranged on plastic trays. Water was regularly topped up in the trays so as to ensure that plants were always supplied with moisture.

At planting, one tray was inoculated with *P. koreensis* inoculum and supplied with water while the other was inoculated with the same amount of water as that of the *P. koreensis* inoculum. The trays were then maintained in a greenhouse at 25ºC.The experiment was monitored and data on germination and development was collected.

Seed germination percentage

The number of seed germinated was counted in each treatment 21 days after sowing. The total germination percentage was calculated as follows:

Germination percentage =

Total number of seeds germinated/Total number of seeds sown×100 (Mathivana *et al*., 2014).

3.3.1 *P. koreensis* inoculation

This experiment made use of an already prepared bacteria culture. The culture was prepared as follows: The stocked isolated bacteria was plated on Nutrient agar (sodium chloride, peptone 5g, meat extract 1g, 1000ml distilled water, agar-agar 20g, yeast extract 2g, meat extract 1g) and King B medium (1000ml distilled water, proteose peptone 20g, agar-agar

20g, glycerol 10ml, $K_2 H_2 PO_4$ 1.5g, $MgSO_4 * 7H_2O$ 1.5g).The plates were incubated at 28 °C for 24 hours (Szentes *et al*., 2013). The stocked bacteria culture was stored and incubated in a nutrient broth medium (Kings agar). The number of colony-forming units of *P. koreensis* culture in nutrient broth medium mixture and a 100-fold dilution of the *P. koreensis* was determined by means of a dilution plate count using a colony counter (Shen *et al*., 2013). A litre of *P. koreensis* culture was then poured onto the roots of 36 *N. benthamiana plants*. The bacteria suspension was added at 10^7 colony forming units per gram of soil to each tray. The second set of treatment involving soaking *N. benthamiana* seeds in *P. koreensis* culture before planting them. Each seed was soaked into 20ml of bacteria suspension for 30 minutes (Liu *et al*., 1995a; Liu *et al*., 1995b; Noumavo *et al*., 2013). The control pots were treated with sterile water equivalent to the volume of *P. koreensis* dilution.

A bacterial strain of *P. koreensis* IMBL1 used for the experimental procedures for this study was previously isolated from soil in Gauteng and identified through the use of 16s rRNA gene sequences as *P. koreensis* (Adereote *et al*., 2010).

3.4 *N. benthamiana* mechanically inoculated with TMV inoculum (leaf infection)

3.4.1 Preparation of Tobacco Mosaic Virus culture

In order to mechanically inoculate *N. benthamiana* seedlings TMV culture was prepared according to the method of Ding *et al*., (1998). TMV inoculums were prepared by grinding 0.5g -1g (1-4 leaves) tobacco leaf tissue infected with TMV provided by the Zimbabwean Tobacco Research Board in 10ml of cold 0.01M sodium phosphate buffer, ph. 7.0 on an ice cold sterilised mortar.

3.4.2 Mechanical inoculation

Healthy indicator plants were randomly placed in the different blocks once the plant reached 6-8 leaf stages (Pirk *et al*., 2013). Hands were washed thoroughly with soap and dried

before handling plants. Fully expanded leaves were dusted with diatomaceous earth and inoculated with 200µg of TMV per ml of 0.1M sterile sodium phosphate buffer pH7.0 (Sulzinski and Zzith, 1982; Sulzinski *et al*., 1994). A finger was dipped in sap and rubbed over the leaf while supporting each leaf with other fingers. A small hole was pricked in the apex of all inoculated leaves using sharp plant-label pegs (Hull, 2009). This was done to ensure that locally inoculated leaves could later be differentiated from non-inoculated leaves (Pazarlar *et al*., 2013). Label pegs were placed on each tray containing the pots inoculated with virus, and the date was recorded on the trays and labels were placed in front of each tray for ease of recording and so as to minimise the need to handle the plants (Ding *et al*., 1998; Mandal *et al*., 2008). In each experiment two plants were mock-inoculated with (0.05M sodium phosphate buffer pH7.0) for use as a negative TMV control (Fraser and Matthews, 1979; Ding *et al*., 1998).

3.5 Perform TMV virus infection studies on *N. benthamiana* inoculated with *P. koreensis for* TMV disease suppression evaluation

After inoculation, the plants were observed daily (early morning) and observations were recorded on data sheets. The day when first symptoms appeared was recorded and symptoms observed were described, the distinction between a local reaction and systemic reaction on inoculated and non-inoculated leaves was recorded (Sulzinski and Zzith, 1982; Sulzinski *et al*., 1994). When local lesions were present a record was taken of whether they are chlorotic (yellow), or necrotic (dead tissue), their diameter, colour in the centre and the approximate number per leaf (Sulzinski and Zzith, 1982; Sulzinski *et al*., 1994). This was determined by counting the number of lesions using a hand lens (Pazarlar *et al*., 2013). The total necrotic area was calculated by adding the areas of all the lesions present on the leaf. The area of one lesion was calculated as lesion diameter/2 × 3.14 (Pazarlar *et al*., 2013). An observation was also made and noted if lesions enlarged during the days following their first

appearance. The difference in leaf numbers between treatments was noted and respective leaf surface area was also measured. The leaf surface area was determined by laying the leaves to be measured on a 2 cm×2 cm grid and their outlines were traced (Sulzinski and Zzith, 1982; Sulzinski *et al*., 1994). The number of full squares was counted, and the area of partial squares was estimated (Pazarlar *et al*., 2013). The total surface area was calculated by adding full squares on the grid and the estimated total area of squares that were not full (Pazarlar *et al*., 2013). The area of the stem petiole was not included in the calculations. The plants were rated for symptom severity at day 20 and 35 after transplanting (Yang *et al*., 2012). The plants were kept in the greenhouse for 8 weeks after inoculation.

TMV Damage Scale: The plants were scored on day 20 and day 35 after inoculation based on severity and susceptibility of TMV infection by a1-5 scale according to (Yang *et al*., 2012; Pazarlar *et al*., 2013). The scale was used as follows: 1 was scored as healthy, slightly involutedly normal green leaves; 2 indicated completely involutedly green leaves; 3 indicated moderately to excessively damaged and dry leaves, together with involutedly green leaves, 50-80 % drying damage on most leaves; and 5 indicated drying damage on all leaves (Yang *et al*., 2012; Pazarlar *et al*., 2013). Each disease severity rating was also carried out for the mock-inoculated plants of the respective treatments as a standard (Yang *et al*., 2012; Shen *et al.*, 2014).

3.5.1 Evaluation of disease suppression by P koreensis

In order to determine the ability of *P.koreensis* PGPR, in the induction of systemic resistance in *N. benthamiana* against TMV infection, number of necrosis lesions on each leaf and the number of leaves on the plants (dependent variables) were counted. In addition, the necrosis area and the leaf area were measured and recorded. Furthermore to this, percentage number of plant infected under each treatment (disease ratio) and inhibitory effect and disease

severity was calculated (Shen *et al.,* 2014). Data on the disease ratio and inhibitory effect and disease severity was calculated using the following formulas by (Shen *et al.*, 2014).

- Disease ratio= (Infected plants/total plants) ×100%
- Inhibitory effect= [(local necrosis number of control - local necrosis number of Treatment/ Local necrosis number of control)] ×100%
- Disease severity ∑(disease grade× number of plants in each grade×100/ (Total number of plants ×highest disease grade

Means were then calculated and compared between the groups (independent variable).

3.6 Data collection and analysis

The data used in this study was collected by first cultivating the model plant *N. benthamiana* in a greenhouse at Witwatersrand University. The plants were then treated with a plant growth-promoting bacteria *P. koreensis* (36 plants were inoculated a week before virus inoculation and the second set of 36 had their seeds inoculated when they were planted) and 36 control plants were simply watered with distilled water). All the plants were infected with inoculum of TMV. Observations were made over a period of 35 days to assess the number of disease-caused necrotic and chlorotic spots, as well as leaf number and area. The response of *N. benthamiana plants* with different treatments: control, *P. koreensis* to TMV was determined in 6 replications. Each experiment included data for disease severity, leaf area and number as well as the percentage number of plant infected. The data was pooled for statistical analysis.

The statistical data analysis in this study was done in two stages. The first stage involved a descriptive analysis to describe the basic distribution of the data (Pirk *et al*., 2013). In the second stage data was subjected to ANOVA using SAS 9.4 software. The effects of the bio preparation treatments were considered significant at F value of P=0.05 (Nunes *et al*., 2015). In cases where a significant F value was obtained for the treatments, separation of the means was done by using multiple mean comparisons test (Bonfferoni, Turkey and Sheff) to

determine which groups of treatment differed from each other at P=0.05 (Kao a Green, 2008; Pirk *et al*., 2013).

In situations where the data was skewed and not following the normal distribution, and when standard deviation was increasing in direct proportion to the mean a log transformation was undertaken to make the data as normal as possible (Leedy and Ormrod, 2013; Pirk *et al*., 2013). In situations where log transformation was not successful we reverted to non-parametric tests (Kao and Green, 2008). On the same note, when equality of variances assumption was violated non parametric tests were conducted (Nunes *et al*., 2015). However, it is recommended in literature that if parametric and non-parametric test give similar results, one should rely on parametric tests because they are more powerful (Leedy and Ormrod, 2013). Therefore, in situations where this was the case we relied on the parametric tests results. In order to assess if there was a difference in the rate of germination of seeds under *P. koreensis* and those without, a two sample t test was conducted. The rest of the other parameters were subjected to ANOVA. ANOVA is done when means of the three groups are analysed (Nunes *et al*., 2015). This was a more appropriate method because it reduces type 1 error through its multiple comparison tests where difference in all groups is given at the same time (McCrum, 2008). Preforming out several t tests was going to increase the type 1 error rate (McCrum, 2008).

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Cultivation of *N. benthamiana*

Seed germination

Nicotiana. benthamiana seeds were treated with *P. koreensis* and were compared to seeds that were mock-inoculated with distilled water. It was determined that the PGPR had a significant effect on seed germination. In both treatments (one tray inoculated with *P. koreensis* and given distilled water, and the other provided only with a similar quantity of distilled water), seed emergence was noticed from day five in seeds treated with *P. koreensis* and day 6 in the control. The number of seeds inoculated with *P. koreensis* that had germinated each day was greater than those in the tray with control.

Percentage germination displayed different dynamics in the two treatments and ranged from 60 % to 100 % for the *P. koreensis*-inoculated seeds, and from 40 % to 100 % for the control seeds. Seeds under *P. koreensis* treatment had a mean percentage germination of 76.4 % and a standard deviation of ±13.211, while the control seeds had a mean percentage germination of 54.8 % and a standard deviation of ±14.462. The average percentage germination per jiffy was 21.4 % (95 % CL: 76.245-11.035), higher for the *P. koreensis* treatment than for the control seed treatment. (Table 1, Fig 4, Fig 5).

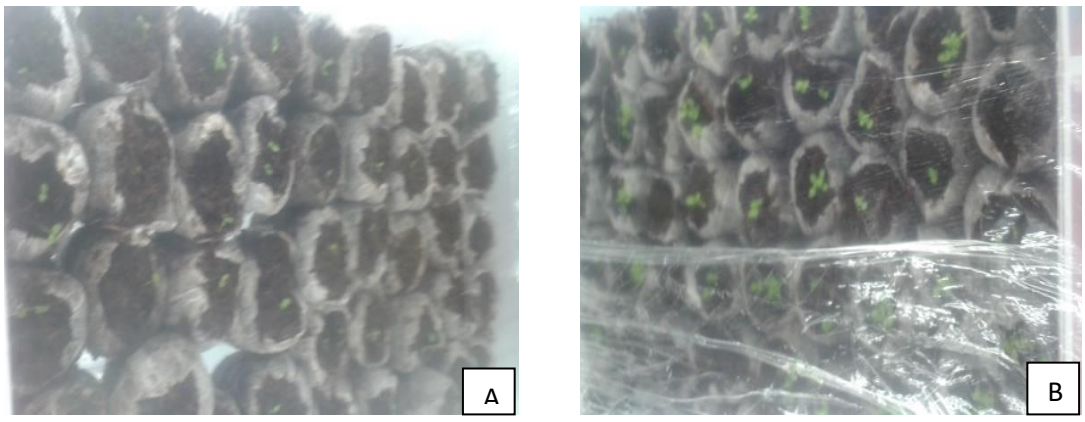


Figure 4. Rate of seed germination of two treatments: Seeds inoculated with *P. koreensis* and those without: The images (In A (un-inoculated) seeds that have germinated per jiffy are less than in B (inoculated) were taken on the 10th of November 2016 10 days after planting.

Tray A contains seeds that were treated with distilled water only and B contains seeds inoculated with *P koreensis* and provided with distilled water. On that day, the number of seeds that had germinated in each jiffy was higher in tray B than tray A. Tray B had more than one seedling per jiffy and in tray A most jiffies had only one germinated seedling.

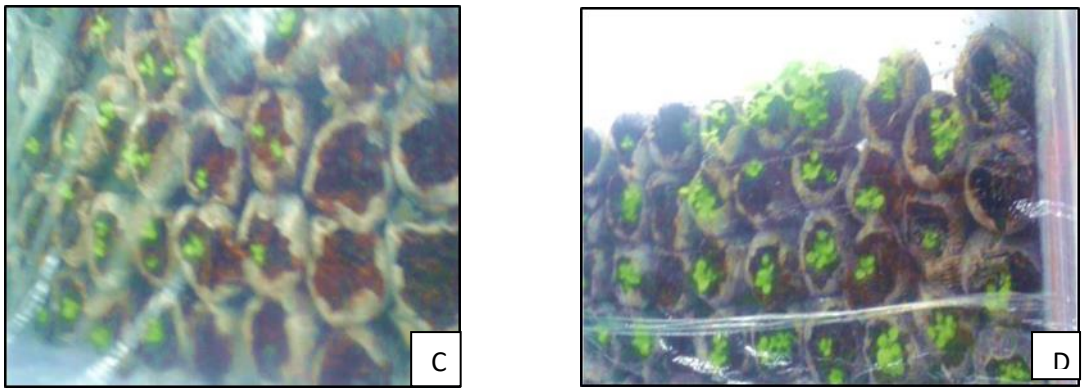


Figure 5.Rate of seed germination of seeds under two different treatments: Seeds inoculated with *P. koreensis* 15 days after sowing the images (C distilled water treatment & D *P. koreensis* treatment) were taken on the 15th of November 2016.

Tray C contains seeds treated with distilled water and tray D was treated with *P. koreensis* and distilled water. On this day jiffies in tray D had more than two seeds that had germinated some had up to 4 seedlings while jiffies in tray A had at most 2 or 3 seedlings.

At the transplanting stage, when the seedlings were transplanted into absolute vermiculite, they started to wither with some taking a long time (5 days) to recover while the remainder ended up dying. On the other hand, when the seedlings that were transplanted into soil to vermiculite 3:1 ratio, appeared healthy and were ready for inoculation in 21 days.

Several studies suggest that PGPR enhances growth, seed emergence and yield. In the cultivation of *N. benthamiana* we investigated whether *P. koreensis* promotes seed emergence by evaluating its effects on *N. benthamiana* germination rate and seedling development. The number and percentage of *P. koreensis*- treated seeds that germinated daily was greater than for the control. The number and percentage of germinated seeds was higher in the bacteria-inoculated seeds than for the control treatment. The rate of seed emergence also varied

according to the treatment of the seeds as follows: 2 new seeds emerged in jiffies with the bacteria each day and 1 or no seed emerged in those without.

The trends in the results indicate that PGPR has a positive effect on seed germination. It has also been shown that the growth medium that the seeds of *N. benthamiana* are planted and transplanted into is a key factor when attempting to establish healthy plants in the shortest period of time.

This study's results are similar to the results of Gholami *et al*. (2009). In the latter study, seed inoculation by a PGPR enhanced germination and seedling vigour. Jahanian *et al*. (2012) also found that inoculating seeds with PGPR resulted in significantly higher germination rate and shoot growth. Similarly, Adesemoye *et al*. (2008) compared plant growth promotion of *P. aeruginosa* and *Bacillus subtilis* in three vegetables and found out that both strains enhanced seed emergence. Both strains enhanced the germination rate over the controls by 50 % in tomato, 40 % in African spinach and 80% and 60 % in okra for *B. subtilis* and *P. aeruginosa* respectively. Improvement in growth ranged between 30 and 80 %. Noumavo *et al*. (2013)'s results have shown that treating seeds with PGPR promotes seed germination and percentage germination, and further improves root length, as well as shoot length in maize. Ashrafuzzaman *et al*. (2009) investigated the effects of several PGPR isolates on growth of rice seeds, and their results revealed that these isolates had a remarkable effect on the germination and growth of rice seeds. Germination increased by 2.3 to 14.7 %. Similarly, germination in the present study was enhanced by 21.4 % in inoculated seeds. The explanation given by Noumavo *et al*. (2013); Adesemoye *et al*. (2008); Gholami *et al*. (2009) is that PGPR facilitate an increase in the production of germination hormones like gibberellin which will activate enzymes like a amylase and many other enzymes specifically associated with germination (protease and nuclease) helping in the breaking down of starch and its

absorption. Mathivana *et al.* (2014) confirmed increased enzyme activity when seeds are inoculated with PGPR.

4.2 TMV leaf infection for mechanically inoculated *N. benthamiana*

Symptoms ontogeny

The types of symptoms induced by TMV on *N. benthamiana* were determined and the date when they were first noted was recorded. Initially the virus induced chlorotic lesions after 7 days past inoculation. (dpi). Necrosis lesions did not appear in the plants under *P. koreensis* treatment but were numerous in the control plants 20 dpi. Some plants under early treatment had minor to no chlorotic lesions for the duration of the experimental period. On young and emerging leaves of the control treatment TMV induced visible symptoms that varied both in size and type. However, curling of young and old leaves was more pronounced in the control and some of the late treatment plants. More green blisters were recorded in the control plants than for the early and late *P. koreensis* treatments.

Vein survey

Veins of all TMV inoculated plants were surveyed on a daily basis after inoculation to determine the first day when vein clearing was clearly visible. Vein clearing along the main veins of each leaf was first noticed on the control plants 11 dpi. No vein clearing was visible in plants under *P. koreensis* treatment until 15 dpi (Table 1).

Table 1. Ontogeny of TMV infection symptoms in leaves of plants under different treatments. Chlorosis appeared earlier than vein clearing.

	Leaf	
Treatments	Inoculated	Young systemic
Early *P. koreensis*	cl 12dpi	vc 15 dpi
Late *P. koreensis*	cl 12dpi	vc 17 dpi
Control	cl 7dpi	vc 11 dpi

cl chlorosis vc vein clearing

The time of appearance of the first TMV-induced symptoms for this investigation differed to the findings of other studies. Ding *et al*. (1998) observed that chlorotic lesions on the inoculated leaves appeared after three dpi. Vein clearing in that study appeared five dpi indicating that systemic spread of the virus was realised earlier in their study this means that local lesion appeared after three days and systemic spreading of the virus was seen after 5 days. In the current study the visible signs appeared only after 7 dpi in the control and 12 dpi in the plants under *P. koreensis* treatment. It is also important to note that at that early stage such symptoms were not yet pronounced and no systemic spreading of the virus was present in plants under *P. koreensis*. Therefore the late appearance of symptoms can be attributed to the presence of bacteria in plant roots.

4.3 TMV infection studies on *N. benthamiana* inoculated with P. *koreensis* for TMV disease suppression evaluation

Lesions

The initial signs of TMV disease appeared 7 dpi in the control plants while at that point the *P. koreensis* treatments did not yet exhibit symptoms. The plants that were exhibiting mosaic virus disease at that stage showed chlorosis (yellowing) and some green blisters although no necrosis (dead tissues) was observed. The lesions on the control were localised in the two inoculated leaves and had not yet spread systemically to other plants parts. The Necrosis lesion area ranged from 0.01mm to 0.64mm, with a mean of 0.04mm and a standard

deviation of ±0.104 in early treatments plants. The results for late treatment plants for this variable ranged from 0.08mm to 0.07mm, with a mean of 0.04mm and a standard deviation of ± 0.025. In the control plants, the necrotic area ranged from 0.03mm to 0.2 mm with a mean of 0.0mm and a standard deviation of ±0.042. The mean necrosis lesion area increased to 0.098mm larger for the control plants than the early and late bacteria treatments. However, later in the experiment the size of the necrotic lesion did not differ in bacteria treatments (Table 3).

Disease severity rating 20 dpi

At 20 and 35dpi respectively, plants were rated for disease severity. At day 20 the control plants showed more severe symptoms than for the early and the late treatments, with the mosaic virus having spread systemically to other leaves. Necrosis was observed on the control plants and chlorosis started to increase in the plants inoculated with *P. koreensis* at the time of transplanting (late treatment). Chlorosis was still minimal in the plants whose seeds were treated at sowing with *P. koreensis*. The number of necrotic lesions ranged from 1 to 3 with a mean of 2 and a standard deviation of ± 0.793 in the early treatment plants. These values were lower for the late treatment plants, ranging from 1 to 4 with a mean of 2.556 and a standard deviation of ± 0.939. Necrotic number was highest in the control treatment plants, with the number of lesions ranging from 4 to 7 with a mean of 5.861 and a standard deviation of ± 0.961. The number of necrotic lesions on the leaves differed across plants treatments, with the average number of patches being 2 ($df = 2$, $P < 0.0001$) lower for the early treatment than for the late and the control plants (Table 3, Fig 7).

Disease severity 35dpi

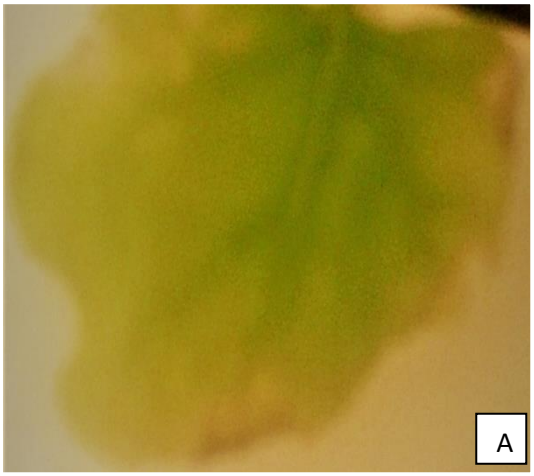
Plants were rated for disease severity on day 35 and it was found out that on average disease severity rating was 4.167 ($df = 2$, $P < 0.0001$) higher for the control than for early and

late *P. koreensis* treatments. Disease symptoms were also on average 2.417 (df=2, P<0.0001) higher for the late than for the early treatment. The disease severity ranged from 1 to 3 in the early treatment with a mean of 1.667 and a standard deviation of± 0.586. Disease severities in the late treatment ranged from 1 to 3, with a mean of 2.417 and a standard deviation of ±0.692. The control exhibited the highest disease rating ranging from 3 to 5 with a mean of 4.1667 and a standard deviation of ±0.737. Emerging leaves were also observed for signs of disease. It was determined that the size of necrotic area increased at a higher rate in the control than in the early and late *P. koreensis* treatment. When plants were evaluated for disease severity at day 35, the control and the late treatment plants had much more extensive necrosis (df 2, P< 0.0001) than both treatments under *P. koreensis.* At day 35, the control had some leaves that were over 80 % dry, while drying leaves were not observed on the early *P. koreensis* treated plants. The dead tissues on some control plants started falling resulting in holes in the leaves. On the same day most of the plants in the early and late treatments still had some healthy green leaves remaining. (Table 3, Fig 7).

A difference in number of leaves was also observed across the three treatments from day 35 dpi, whereas all the plants had on average equal number of leaves before TMV inoculation, by the 30th of January 2017. Leaf numbers of the early treatment ranged from 13 to 18, with a mean of 15.458 and a standard deviation of ± 1.769. The late treatment leaf numbers ranged from 12 to 15 with a mean of 13.333 and a standard deviation of ±1.129. The control leaf numbers ranged from 5 to 9 with a mean of 7.8033 and a standard deviation of ±1.176 (Table, 3, fig 7).

The average number of leaves was 15 (df=2, P<0.0001) higher for early *P. koreensis* treatment than for the late and the control treatments. The late treatment had an average leaf number of 13 (df=2, P<0.0001) higher than the control. However the number of leaves for the *P. koreensis* treatments did not differ from one another.

The new leaves in the control plants emerged with curling and most of the leaves on the control plants were in fact curled. Some control leaves grew asymmetrically (longer on one lateral edge than the other). The area of leaves ranged from 4.95 cm^2 to 7.73 cm^2 with a mean of 5.95cm^2 and a standard deviation of ±0.645 in the early treatment plants, while the area of the late treatment leaves ranged from 4.35 cm^2 to 6.65cm^2 with a mean of 5.55cm^2 and a standard deviation of ±0.687. Leaf area in the control ranged from 1.25 cm^2 to 4.50 cm^2 with a mean of 2.88cm^2 and a standard deviation of ±0.867. The average leaf area was 2.9 cm^2 (df =2, $P<0.0001$) lower for control plants than both treatments under *P. koreensis,* although it was the same for both *P. koreensis* treatments (Table 3, fig 6, fig 7).



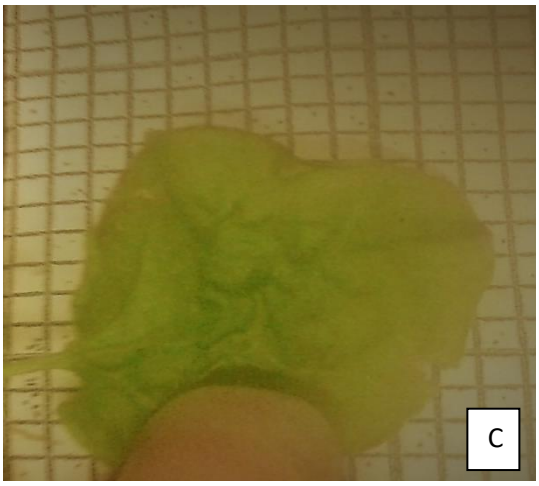


Figure 6.differences in leaf area between the treatments A is early, B late *P. koreensis* and C control. The images were taken on the 12th of March 2017. Leaf 'A' was taken from the early treatment *of P. koreensis*, while Leaf 'B' was from the control. Leaf 'C' was taken from the late treatment of *P. koreensis*. Plants from the two *P.koreensis* treatments had the same leaf area, but the control plants had a smaller leaf area.

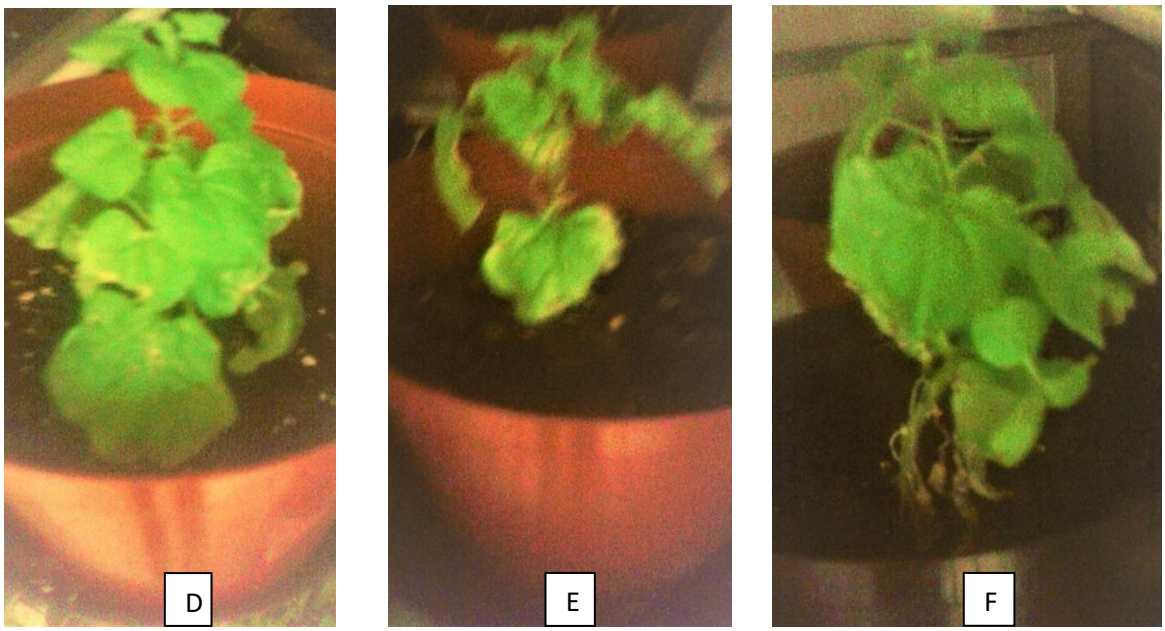


Figure 7.Plants under *P. koreensis* differences in (leaf area and number) Plant D was treated early with *P. koreensis*, plant E was a control and plant F had received late treatment with *P. koreensis*.

The necrotic area of the control was higher than both treatments under *P. koreensis*. At the end of the experiment it was observed that the number of leaves on the early and late treatment was higher than the control. In fact the control had lost more leaves than both treatments under *P. koreensis.* The control plants were stunted and their height was lower than those that had received *P. koreensis*. Therefore the control was more severely affected by TMV than the other treatments. Treatments under *P. koreensis* were affected to a similar extent.

Table 2 Effect of TMV on *N. benthamiana* under Early, Late *P. koreensis* treatment and the control necrosis area, number, leaf number, area and disease severity

Treatment	**Necrosis**		**Leaf**		**Disease severity**	
	Area (mm)	**No**	**Area (mm)**	**No**		
Control	0.10***	6 A	2.79	8 B	4.17	B
P. koreensis (late)	0.041	3 B	5.55	13 A	2.42	A
P. koreensis (early)	0.037	2 C	5.95	15 A	1.67	A

The means with different letters are significantly different from each other. With a probability of 5 % according to the Turkey, Bonferroni and Sheff test. Means with *** are significantly different from the others.

Extent of leaf necrosis per treatment differed significantly between the control and the early and the late *P. koreensis* treatments with the control having the highest average area and number of necrotic lesions. Necrosis area also differed in the two *P. koreensis* treatments. Leaf number and area was high for both *P. koreensis* treatments and lower in the control. Disease severity rating was higher in the control than for the *P. koreensis* treatments.

Per cent infection (disease ratio)

In order to determine the percentage number of plants affected by TMV, all TMV-impacted plants were counted for each treatment at day 11 and 20. The highest percentage of disease ratio was recorded for the control at day 11 (38.89 %). The other treatments did not yet have visible symptoms at that stage. Again, at day 20 the highest disease infection ratio was recorded for the control at 63.89%. By that stage the late treatment exhibited a low ratio and visible symptoms for the early treatment were just beginning to emerge. (Table 3).

Table 3. Per cent infection of *N. benthamiana* treated with different bio preparations and challenged with TMV.

Treatment	11 days after TMV inoculation		20 days after TMV inoculation	
	TMV infected/total plants	Rate%	TMV infected/total plants	Rate %
Early *P. koreensis*	0/36	0.00	4/36	11.11
Late P. koreensis	0/36	0.00	12/36	33.33
Control	14/36	38.89	23/36	63.89

***P. koreensis* TMV Inhibitory effect**

In order to determine the percentage inhibition rate for different *P. koreensis* treatments in the development of TMV, inhibitory effect was calculated according to the

formula developed by (Shen *et al*., 2014). It was observed that early treatment of plants with *P. koreensis* inhibits TMV development by 67%. Late treatment was also able to inhibit TMV to a lesser degree (by 50%) (Table 5).

Table 4 Rate of TMV inhibition by *P. koreensis*

Treatment	TMV inhibition effect %
Early *P. koreensis*	67
Late *P. koreensis*	50

TMV suppression

In order to determine if *P. koreensis* PGPR reduces TMV disease, several parameters were assessed. Investigations included calculation of the TMV inhibitory effect, disease severity ratio, leaf number dynamics, leaf area, and number of necrotic lesions.

In terms of disease dynamics, the major findings of this study have shown that severity of disease differs depending on treatment of *N. benthamiana*. TMV affected the control treatments earlier than the *P. koreensis* treatments. The size and area affected with necrosis was greater in the control than in the early and late *P. koreensis* treatments. Similar results were observed when a comparison was made between the two *P. koreensis* treatments with the late treatment showing more mosaic symptoms than the early treatment. The rate at which these three treatments lost their leaves due to dryness caused by the mosaic virus disease also differed. At the end of the observation period, the early treatment had more leaves than the other treatments. The leaf area was similar between the early and the late treatment, while the control plants leaf area was lower. It was demonstrated that more plants were under stress in the control than the other treatments. These differences indicate that when plants are exposed to viruses in the presence of PGPR (*P. koreensis)* they have a lower level of impact

from the virus than those without PGPR (Table 2, fig 8). Overall results have shown that treating plants seed or roots with *P. koreensis* inhibits virus development, and that inhibition ability of the bacteria is enhanced by early application. It is therefore concluded that *P. koreensis* reduces the virulence of TMV thereby controlling and reducing its impacts. It is further recommended that using *P. koreensis* earlier reduces TMV impacts by a higher magnitude.

ISR determination

ISR can be determined by the reduction of disease severity and the decrease in number of plant infections (Yang *et al*., 2012). According to Whippi (2001) in Narayanasamy (2013) rhizobacteria also have the potential of slowing down disease development and severity. Reduction in severity and number of infected plants is caused by decline in the growth and establishment of phytopathogens in systemic induced resistance tissues (Van Loon, 2000).

To determine if *P. koreensis* induce systemic resistance, *P. koreensis* inoculation and TMV were separated. *P. koreensis* was inoculated either on plant roots or seeds while TMV was inoculated in plant leaves. Some leaves in plants treated with *P. koreensis* showed minimal symptoms in the leaves compared to the control treatment. This is an indication that *P. koreensis* triggered a reaction that caused protection in distant parts of the plants.

Studies done to date have only demonstrated that bio surfactants from *P. koreensis* reduce disease caused by oomycetes (Hultberg *et al*., 2010). As far as is known anti-viral activity has not yet been demonstrated. Raupah *et al*. (1996) set out to investigate if the PGPR strain that had induced ISR in cucumber against fungal and bacterial pathogens could do the same in tomato plants against cucumber mosaic virus. Results showed that seeds treated with the bacteria reduced number of plants infected with the mosaic virus and disease development was generally decreased in tomato. The current study also sought to find out if *P. koreensis*,

previously reported to be able to reduce oomycetes-caused disease, could also show antiviral activity against TMV. The results in this study have demonstrated that *P. koreensis* has the capacity for antiviral activity against TMV. Similarly, Shen *et al*. (2014) set out to evaluate the effectiveness of *P. fluorescens* CZ powder in controlling TMV when applied in a greenhouse. Their results has shown that a 100-fold dilution of this powder supresses TMV up to 88.3 %. The current study has similarly shown that early and late treatment of plants with *P. koreensis* inhibits TMV development by 67 % and 50% respectively. The implication of this result is that treating plants early ensures that they are protected at an early age. In other words, resistance is induced early and pathogens will meet an already protected plant. Therefore, development of the disease is inhibited. Also as demonstrated in this study, Yang *et al*. (2012) has shown that early treatment of plants is important as it reduces disease severity by higher margins than late treatments. Results of the current and previous studies reveal that early seed treatment with PGPR is the best way to ensure plant protection. This procedure means that PGPR are present from the beginning of plant growth. This is advisable for ensuring successful colonization (Sorensen *et al*., 2001). Induction of resistance is only guaranteed by successful colonization.

The first set of symptoms that appeared on the mechanically inoculated leaves was chlorosis, with necrosis appearing at a later stage. Appearance of blisters as well as vein clearing along the main veins also appeared at a later stage. These symptoms were then accompanied by wilting and leaf spots. Some of the plants were stunted and some were deformed. These results are similar to those observed by other studies investigating effects of PGPR on plant disease protection, as well as others investigating the effect of TMV on plant productivity. Pazarlar *et al*. (2013) investigated the effect of TMV on plant productivity and their results found the same initial symptoms as in this study. Yang *et al*. (2012) also reported

the same initial signs of TMV when they were testing to see if *P. putida* (PGPR) had the potential of reducing the amount of TMV in the soil.

TMV reduced the number of leaves in all treatments, which is in line with what was observed in other studies (Pazarlar *et al*., 2013). However, the reduction of leaves across the three treatments was different although the leaves had nearly equal numbers before TMV inoculation at the end of the experiment plants treatment had different leaves. The leaves of those plants under *P. koreensis* were higher than the control. This demonstrates that *P. koreensis* has antiviral activity against TMV.

Yang *et al*. (2012) had similar results; initial signs of tobacco mosaic disease appeared on day 9 in the control with no symptoms on *P. putida* -treated plants. The control plants showed severe symptoms by day 20 when tobacco plants were rated for symptom severity. Their study also revealed that severity of TMV symptoms were significantly lower in plants treated with *P. putida* than for the control plants. The results of this study are in line with findings of Yang *et al*. (2012). First symptoms appeared on day 7 in the control and none were noticed in plants treated with *P. koreensis.* Similar to the current study, symptoms at day 20 were more severe in the control.

Conclusions made in a study by Guo *et al*. (2011) in Yang *et al*. (2012) were that *P. putida* exhibits antiviral activity against TMV. From the analysis of several parameters, the conclusion of this study is also in line with this study. *P. koreensis* has exhibited anti-viral activity against TMV. The mean number of necrotic lesions and area of *P. koreensis* treatments was lower than that of the control.

Furthermore, bacteria under study have shown that they have the potential for suppressing the multiplication and spread of TMV throughout the plant. This result is in line with several other studies demonstrating that several strains of the *Pseudomonas* species

suppress plant diseases. Several isolates of PGPR also reduced damping off disease in cucumber and CHAO also works well to suppress fungi, viruses and nematodes (Siddiqui and Shaukat, 2003).

Van Peer *et al*. (1991)'s results revealed that the plant was still protected from the virus even though *Pseudomonas* was restricted to the root but infection was on aerial parts. Furthermore, Pieterse *et al*. (2014) slash-inoculated disease-causing agent into the stem of plants and yet bacteria were found around the roots. In this study, the bacteria and the virus were not seen to be in contact with each other and it can safely be concluded that the protective effect was plant-mediated. The Pieterse *et al*. (2014) results are similar to the current studies which show that even though the bacteria are restricted to being around the roots, they still provide protection to the plant as a whole. This is demonstrated by the lower mean numbers of necrotic lesions on leaves for *P. koreensis*-treated plants than the control plants. The conclusion of Van Peer *et al*. (1991) was that the significant reduction in necrotic spots was plant mediated. Van Wees *et al*. (2000) infected leaves with virulent Pst on plants grown in soil with WC5417 (PGPR). Results revealed that these plants enjoyed more protection than control plants. Park and Kloepper (2000) investigated a diverse collection of PGPR and their results showed that a signal was activated in the PGPR-treated plants. Plants merely provided with water did not experience signal activation. Maurhofer *et al*. (1993) investigated if strain CHAO can induce ISR against leaf necrosis caused by TMV. An indication that this does induce ISR was the relative reduction in lesion number in inoculated plants. The current study can also conclude that the reduction in number and size of necrotic area, as well as a low leaf loss in *P. koreensis*-treated plants was plant mediated. Bacteria had been inoculated onto the roots only, yet there was a relative reduction in number of necrotic lesions on the leaves situated far away from the roots.

However, results of this study differ to other studies regarding *P. koreensis* secretions. In this study we cannot conclude on whether *P. koreensis* secretions have an effect on the virus. Other researchers have shown that certain bacteria colonize the roots (thus triggering a reaction that induces resistance throughout the plant) but they also have a direct effect on the pathogen itself (Sorensen *et al*., 2001). Some bacteria colonize the pathogen thus stressing it to such an extent that it cannot multiply. Otherwise the mechanisms involved in suppressing the virus are not well understood. It is known that the bacteria trigger a reaction but we are not aware of the mechanisms there of. The possible mode of action that could have triggered ISR in this study is the production of phenolic compounds and quorum-sensing molecules (Hoffland *et al*., 1996).It could also have been caused by increased production of salicylic acid in leaves and the production of siderophore pyoverdine(Maurhofer *et al*., 1994).

4.5 Reasons study results are similar to previous investigations

It is proposed that the reason why the results of the present study do not contradict other researchers' studies is that their recommendations were carefully observed in this study. The method developed by Monjero *et al*. (2015) for establishing *N. benthamiana* plants was carefully observed. This resulted in having healthy plants ready for inoculation in 21 days. Several scholars have recommended that treatment of seeds gives more favourable results. It is argued that seed treatment protects them from soil borne pathogens, and results in increased growth. This in turn shortens the vulnerable period when they are more susceptible to infection or damage. Seed treatment is thought to assist in allowing the plant to mature more rapidly and this might be one of the reasons why disease severity was higher in plants whose roots were inoculated with *P. koreensis* at transplanting compared to those whose seeds were inoculated at sowing. It is further argued that application of inoculum to seeds means that when roots develop they are immediately colonized and hence protection is offered at a tender age. This means that plants grown from bacteria-inoculated seed had enhanced defensive

capacity well before TMV infection took place. This would explain why it took longer for the bacteria-inoculated seeds to develop mosaic symptoms compared to the other two treatments. It has also been pointed out that root colonization ability is crucial because the PGPR success in controlling plant diseases is compromised if they do not manage to colonize the roots.

Furthermore, Dose response studies have concluded that population density of 10^5 colony-forming units per gram of roots are essential for plants to develop improved self-protective capacity (Raaijmakers *et al*., 1995). The current study noted this and inoculated using up to 10^7 colony-forming units per gram of roots. This exceeds the minimum amount required (that is the amount of bacteria exceeded the amount needed for the bacteria to suppress diseases) and hence we obtained similar results to other researchers.

Separation (bacteria in roots and TMV on leaves) of the plant pathogen and bacteria allowed several previous scholars to conclude that enhanced level of disease-resistance was plant mediated, resulting from an ISR. The current study also separated TMV and *P. koreensis* in order to determine if the bacteria under study trigger a plant immune response against the virus. Conducting similar separation to other studies has allowed us to reach a similar conclusion.

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

PGPR have become a very important way of controlling plant diseases and are superior to chemical controls that cause harm to both the environment and human health. *Pseudomonas* species have been found to be valuable in controlling viral diseases in crops. For this reason, continued investigations of more strains of this species have been recommended. This study comprises a much-needed study to identify potential beneficial soil microbes for use as bio pesticides agents against plant viruses. This study sought to investigate if *P. koreensis* can reduce the effects of viral diseases on plants. The analysis has shown that *P. koreensis* does indeed have an effect on reducing viral susceptibility of *N. benthamiana*. Results drawn from this study have proved that *P koreensis* is yet another strain of soil microbes that can induce systemic resistance. Some species in the *Pseudomonas* genus have been used as commercial isolates for production of bio control agents. Continued investigation of such species is critical since this will help to identify opportunities for commercial production of more bio control products. Countless studies have revealed that *Pseudomonas* species have great potential as bio control agents, results of the current study have also proved that *P. koreensis* is one of such. Therefore, it can safely be concluded that *P koreensis* can be used as a bio control agent and studies like the current study are needed to accelerate the identification of many other beneficial soil microbes as agents to be used as bio controls of plant viruses and other socio-economically damaging plant pathogens.

5.2 Recommendation

Future studies should be designed in such a way as to elucidate mechanisms involved with suppression of diseases in *P. koreensis*. Many studies have demonstrated that many PGPR are not successful in the field. Therefore, a field trial is important to investigate the usefulness of the antiviral activity of *P. koreensis* in the field. We still need to investigate the effect of *P.*

koreensis on a variety of crops to determine plants species that can be protected by it. We also recommend that investigations be done to determine the reason behind a PGPR working in one plant species and failing in the other. It has also been revealed that if strains of PGPR are combined and used together in one treatment they produce better results. We therefore recommend that investigations be undertaken of other PGPR strains that can be combined with *P. koreensis* and will provide improved plant resistance to pathogens. Above all investigation of any metabolites change in the plant because of bacterial activity is also crucial.

REFERENCES

Achuo, E.A., Audenaert, K., Meziane, H. and Höfte, M., 2004. The salicylic acid-dependent defence pathway is effective against different pathogens in tomato and tobacco. *Plant Pathology* 53(1), 65-72.

Adesemoye, A.O., Obini, M. and Ugoji, E.O., 2008. Comparison of plant growth-promotion with *Pseudomonas aeruginosa* and *Bacillus subtilis* in three vegetables. *Brazilian Journal of Microbiology 39*(3), 423-426.

Andreote, F.D., Da Rocha, U.N., Araújo, W.L., Azevedo, J.L. and van Overbeek, L.S., 2010. Effect of bacterial inoculation, plant genotype and developmental stage on root-associated and endophytic bacterial communities in potato (Solanum tuberosum). *Antonie van Leeuwenhoek, 97*(4), 389-399.

Aexel, C., Zannini, E., Coffey, A., Guo, J., Wales, D.M., Arendt, E.K., 2012. Eco-friendly control of potato late blight causative agent and the potential role of lactic acid bacteria a review. *Plant Science* 33, 581-596.

Agbodjato, N.A., Noumavo, P.A., Adjanohoun, A., Agbessi, L. and Baba-Moussa, L., 2016. Synergistic Effects of Plant Growth Promoting Rhizobacteria and Chitosan on In Vitro Seeds Germination, Greenhouse Growth, and Nutrient Uptake of Maize (Zea mays L.).*Biotechnology research international*, 2016.

Alizadeh, H., Behoudi, K., Ahmadzadeh, A., 2013.Induced systemic resistance in cucumber and *Arabidopsis thaliana* by the combination of *Trichoderma harzianum* and *Pseudomonas* sp Ps 14.*Biological control* 65, 14-33.

Andersen, J.B., Koch, B., Nielsen, T.H., Sørensen, D., Hansen, M., Nybroe, O., Christophersen, C., Sørensen, J., Molin, S. and Givskov, M., 2003. Surface motility in

Pseudomonas sp. DSS73 is required for efficient biological containment of the root-pathogenic microfungi Rhizoctonia solani and *Pythium ultimum*. *Microbiology* 149(1), 37-46.

Annapurna, K., Kumar, A., Kumar, L.V., Govindasamy, V., Bose, P. and Ramadoss, D., 2013. PGPR-induced systemic resistance (ISR) in plant disease management. In *Bacteria in Agrobiology: Disease Management* (pp. 405-425). Springer Berlin Heidelberg.

Anzai, Y., Kim, H., Park, J.Y., Wakabayashi, H. and Oyaizu, H., 2000. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *International journal of systematic and evolutionary microbiology*, *50*(4), 1563-1589.

Babu, A.N., Jogaiah, S., Ito, S., Nagaraj, A.K., Tran, L.P., 2015. Improvement of growth, fruits weight and early blight disease protection of tomato plant by rhizosphere bacteria is correlated with their beneficial traits and induced biosynthesis antioxidant, peroxidase and polyphenol oxidase. *Plant Science* 231, 62-73.

Bagley, C.A., 2001. Controlling tobacco mosaic virus in tobacco through resistance. vtechworks.lib.vt.edu

Bailey, K.L. and Mupondwa, E.K., 2006. Developing microbial weed control products: commercialization, biological, and technological considerations. *Handbook of sustainable weed management*, 431-473.

Bailey, K.L., Boyetchko, S.M. and Längle, T., 2010. Social and economic drivers shaping the future of biological control: a Canadian perspective on the factors affecting the development and use of microbial bio pesticides. *Biological Control*, *52*(3), 221-229.

Bainton, N.J., Lynch, J.M., Naseby, D. and Way, J.A., 2004. Survival and ecological fitness of *Pseudomonas fluorescens* genetically engineered with dual bio control mechanisms. *Microbial ecology* 48(3), 349-357.

Bakker, P.A.H.M., Ran, L.X., Pieterse, C.M.J. and Van Loon, L.C., 2003. Understanding the involvement of Rhizobacteria-mediated induction of systemic resistance in bio control of plant diseases. *Canadian Journal of Plant Pathology* 25(1), 5-9.

Beddington, J., 2010. Food security: contributions from science to a new and greener revolution. Philosophical Transactions of the Royal Society of London B*: Biological Sciences* 365(1537), 61-71.

Behrendt, U., Ulrich, A., Schumann, P., Erler, W., Burghardt, J. and Seyfarth, W., 1999. A taxonomic study of bacteria isolated from grasses: a proposed new species Pseudomonas graminis sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *49*(1), 297-308.

Bombarely, A., Rosli, H.G., Vrebalov, J., Moffett, P., Mueller, L.A. and Martin, G.B., 2012. A draft genome sequence of Nicotiana benthamiana to enhance molecular plant-microbe biology research. *Molecular Plant-Microbe Interactions*, *25*(12), 1523-1530.

Bouizgarne, B., 2013. Bacteria for plant growth promotion and disease management. In *Bacteria in agrobiology: disease management* (pp15-47). Springer Berlin Heidelberg.

Burbidge, N.T., 1960. The Australian species of Nicotiana L.(Solanaceae). *Australian Journal of Botany*, *8*(3), 342-380.

Burketova, L., Trda, L., Valentova, O., 2015. Bio-based resistance induces for sustainable plant protection against pathogen. *Biotechnology Advance* 33, 994-1004.

Bouizgarne, B., 2013. Bacteria for plant growth promotion and disease management. In *Bacteria in agrobiology: disease management* (pp. 15-47). Springer Berlin Heidelberg.

Cakmakçi, R., Dönmez, F., Aydın, A. and Şahin, F., 2006. Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. *Soil Biology and Biochemistry* 38(6), 1482-1487.

Chakrabarty, R., Banerjee, R., Chung, S.M., Farman, M., Citovsky, V., Hogenhout, S.A., Tzfira, T. and Goodin, M., 2007. PSITE vectors for stable integration or transient expression of auto fluorescent protein fusions in plants: probing Nicotiana benthamiana-virus interactions. *Molecular Plant-Microbe Interactions*, *20*(7), 740-750.

Chakraborty, S. and Newton, A.C., 2011. Climate change, plant diseases and food security: an overview. *Plant Pathology*, *60*(1), 2-14.

HS Chawla, 2002. *Introduction to plant biotechnology*. Science Publishers.

Chin-A-Woeng, T.F., Bloemberg, G.V., Mulders, I.H., Dekkers, L.C. and Lugtenberg, B.J., 2000. Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for bio control of tomato foot and root rot. *Molecular plant-microbe interactions* 13(12), 1340-1345.

Cordero, P.K., Calvigliasso, A., Principe, A., Godino, A., Jofre, E., Mon, G., Fischer, S., 2012. Genetic diversity and antifungal activity of native Pseudomonas isolated from maize plants grown in central region of Argentina. *Systematic and Applied Microbiology* 35, 342-351.

Cook, R.J., 2000. Advances in plant health management in the twentieth century. *Annual review of phytopathology*, *38*(1), 95-116.

Cottingham, K.L., Lennon, J.T. and Brown, B.L., 2005. Knowing when to draw the line: designing more informative ecological experiments. *Frontiers in Ecology and the Environment* 3(3), 145-152.

Dashti, N.H., Ali, N.Y., Cherian, V.M. and Montasser, M.S., 2012. Application of plant growth-promoting rhizobacteria (PGPR) in combination with a mild strain of Cucumber mosaic virus (CMV) associated with viral satellite RNAs to enhance growth and protection against a virulent strain of CMV in tomato. *Canadian Journal of Plant Pathology*, *34*(2), 177-186.

de Boer, M., van der Sluis, I., van Loon, L.C. and Bakker, P.A., 1999. Combining *fluorescent Pseudomonas* spp. strains to enhance suppression of *fusarium wilt* of radish. European Journal of Plant Pathology 105(2), 201-210.

Diollo, S., Crepin, A., Barbey, C., Orange, N., 2011.Recent advances in biological control mediated through potato rhizosphere. *Biotechnology Advance* 23, 78-88.

Erickson, F.L., Dinesh-Kumar, S.P., Holzberg, S., Ustach, C.V., Dutton, M., Handley, V., Corr, C. and Baker, B.J., 1999. Interactions between tobacco mosaic virus and the tobacco N gene. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *354*(1383), 653.

Fraser, L. and Matthews, R.E.F., 1979. Efficient mechanical inoculation of turnip yellow mosaic virus using small volumes of inoculum. *Journal of General Virology*, *44*(2), 565-568.

Fravel, D.R., 2005. Commercialization and implementation of biocontrol 1. *Annu. Rev. Phytopathol.*, *43*, 337-359.

Fukui, R., Poinar, E.I., Bauer, P.H., Schroth, M.N., Hendson, M., Wang, X.L. and Hancock, J.G., 1994. Spatial colonization patterns and interaction of bacteria on inoculated sugar beet seed. P*hytopathology* 84(11), 1338-1345.

Gholami, A., Shahsavani, S. and Nezarat, S., 2009. The effect of plant growth promoting rhizobacteria (PGPR) on germination, seedling growth and yield of maize. *Int J Biol Life Sci* 1(1), 35-40.

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M. and Toulmin, C., 2010. Food security: the challenge of feeding 9 billion people. *Science* 327(5967), 812-818.

Goodin, M.M., Ziltin, D., Naidu, R.A., Lommel, S.A., 2008.*Nicotiana benthamiana* its history and future as a model for plant pathogen interaction. *Molecular plant microbe interactions* 8,1015-1026.

Green, S.K., 1984. Guidelines for diagnostic work in plant virology.

Gross, H. and Loper, J.E., 2009. Genomics of secondary metabolite production by Pseudomonas spp. *Natural product reports*, *26*(11), pp.1408-1446.

Gülser, C., Yılmaz, N.K. and Candemir, F., 2008. Accumulation of Tobacco mosaic virus (TMV) at different depths clay and loamy sand textural soils due to tobacco waste application. *Environmental monitoring and assessment*, *146*(1-3), pp.235-242.

Hammerschmidt, R., 1999. Induced disease resistance: how do induced plants stop pathogens?. *Physiol Mol Plant pathol* 55,77-84.

Haverkort, A.J., Struik, P.C., Visser, R.G.F. and Jacobsen, E., 2009. Applied biotechnology to combat late blight in potato caused by Phytophthora infestans. *Potato Research* 52(3), 249-264.

Helbig, J., 2006. Microbial control of plant pathogens in horticultural crops-an overview. *Microbial biotechnology in horticulture* 1, 83-123.

Hoffland, E., Hakulinen, J., van Pelt, J.A., 1996. Comparison of systemic resistance induced by a virulent and non-pathogenic *Pseudomonas* species. *Biological control* 7, 757-762.

Höfte, M. and Altier, N., 2010. Fluorescent pseudomonads as biocontrol agents for sustainable agricultural systems. *Research in Microbiology*, *161*(6), pp.464-471.

Howell, C. and Stipanovic, R.D., 1980. Suppression of *Pythium ultimum* induced damping off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic poluterin. *Phytopathology* 70(8), 712-715.

Hull, R., 2009. Mechanical inoculation of plant viruses. Current protocols in microbiology, pp.16B-6.

Hultberg, M., Alsberg, T., Khalil, S., Alsanium, L., 2010.Suppression of diseases in tomato infected by *Pythium ultimum* with a bio surfactant produced by *Pseudomonas koreensis Bio Control* 55,435-444.

Jahanian, A., Chaichi, M.R., Rezaei, K., Rezayazdi, K. and Khavazi, K., 2012. The effect of plant growth promoting rhizobacteria (PGPR) on germination and primary growth of artichoke (Cynara scolymus). *Int J Agric Crop Sci* 4 923-929.

Johnsen, K., Jacobsen, C.S., Torsvik, V. and Sørensen, J., 2001. Pesticide effects on bacterial diversity in agricultural soils–a review. *Biology and Fertility of Soils*, *33*(6), pp.443-453.

Kao, L.S. and Green, C.E., 2008. Analysis of variance: is there a difference in means and what does it mean? *Journal of Surgical Research* 144(1), 158-170.

Kloepper, J.W., Ryu, C.M. and Zhang, S., 2004. Induced systemic resistance and promotion of plant growth by *Bacillus spp*. *Phytopathology* 94(11), 1259-1266.

KRAUS, J. and Loper, J.E., 1992. Lack of evidence for a role of antifungal metabolite production by Pseudomonas fluorescens Pf-5 in biological control of Pythium damping-off of cucumber. Phytopathology 82(3), 264-271.

Kwon, Y.S., Song, H.D., Pula, R.K., 2003.*Pseudomonas Koreensis* sp nov., .*Pseudomonas umsongensis* sp nov., .*Pseudomonas umsongensis* sp nov., *Pseudomonas jinjuesis* sp nov.,

novel species from farm soils in Korea *International journal of systematic and evolutionary microbiology* 53, 21-27.

Keel, C., Ucurum, Z., Michaux, P., Adrian, M. and Haas, D., 2002. Deleterious impact of a virulent bacteriophage on survival and bio control activity of *Pseudomonas fluorescens* strain CHA0 in natural soil. *Molecular plant-microbe interactions* 15(6), 567-576.

Kirankumar, R., Jagadeesh, K.S., Krishnaraj, P.U. and Patil, M.S., 2010. Enhanced growth promotion of tomato and nutrient uptake by plant growth promoting rhizobacterial isolates in presence of tobacco mosaic virus pathogen. *Karnataka Journal of Agricultural Sciences, 21*(2).

Landa, B.B., Navas-Cortés, J.A., Hervás, A. and Jiménez-Díaz, R.M., 2001. Influence of temperature and inoculum density of *Fusarium oxysporum* f. sp. ciceris on suppression of Fusarium wilt of chickpea by rizhosphere bacteria. *Phytopathology* 91(8), 807-816.

Landa, B.B., Navas-Cortés, J.A. and Jiménez-Díaz, R.M., 2004. Influence of temperature on plant–rhizobacteria interactions related to bio control potential for suppression of *fusarium wilt* of chickpea. *Plant Pathology* 53(3), 341-352.

Lee, B., Farag, M.A., Park, H.B., Kloepper, J.W., Lee, S.H. and Ryu, C.M., 2012. Induced resistance by a long-chain bacterial volatile: elicitation of plant systemic defense by a C13 volatile produced by *Paenibacillus polymyxa. PloS one, 7*(11), 48744.

Leedy, P.D. and Ormrod, J.E., 2013. *The nature and tools of research. Practical research: Planning and design*, 10th ed. Pearson, New York, USA.

Leeman, M., Van Pelt, J.A., Hendrickx, M.J., Scheffer, R.J., Bakker, P.A.H.M. and Schippers, B., 1995. Bio control of *Fusarium* wilt of radish in commercial greenhouse trials by seed treatment with *Pseudomonas fluorescens* WCS374. *Phytopathology* 85(10), 1301-1305.

Liu, L., Kloepper, J.W. and Tuzun, S., 1995. Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth-promoting rhizobacteria. *Phytopathology*, *85*(8), 843-847.

Liu, L., Kloepper, J.W. and Tuzun, S., 1995. Induction of systemic resistance in cucumber against Fusarium wilt by plant growth-promoting rhizobacteria. *Phytopathology*, *85*(6), 695-698.

Loper, J.E., 1988. Role of fluorescent siderophore production in biological control of. *Pythium ultimum* research. *Phytopathology* 85(10), 166-172.

Maheshwari, D.K. ed., 2013. *Bacteria in agrobiology: disease management*. Springer Science & Business Media,Uttaraknand,India.

Mandal, B., Csinos, A.S., Martinez-Ochoa, N. and Pappu, H.R., 2008. A rapid and efficient inoculation method for Tomato spotted wilt tospovirus. *Journal of virological methods*, *149*(1), pp.195-198.

Mark, G.L., Morrissey, J.P., Higgins, P. and O'Gara, F., 2006. Molecular-based strategies to exploit Pseudomonas biocontrol strains for environmental biotechnology applications. *FEMS microbiology ecology*, *56*(2), pp.167-177.

Mathre, D.E., Callan, N.W., Johnston, R.H., Miller, J.B. and Schwend, A., 1994. Factors influencing the control of *Pythium ultimum*-induced seed decay by seed treatment with *Pseudomonas aureofaciens* AB254. *Crop Protection* 13(4), 301-307.

Maurhofer, M., Hase, C., Meuwly, J., Metraux, P., Defago, G., 1994. Induction of System Resistance of Tobacco Necrosis Virus by the root –Colonizing *Pseudomonas fluorescent* strain CHAO: Influence of the gac A Gene & of Pyover dine Production. *Bio Control* 55, 645-654.

Mazzola, M.A.R.K., Zhao, X., Cohen, M.F. and Raaijmakers, J.M., 2007. Cyclic lipopeptide surfactant production by *Pseudomonas fluorescens* SS101 is not required for suppression of complex Pythium spp. populations. *Phytopathology* 97(10), 1348-1355.

McCrum-Gardner, E., 2008. Which is the correct statistical test to use?. British *Journal of Oral and Maxillofacial Surgery* 46(1), 38-41.

McDonald, J H 2008: Handbook of biological statistics, Baltimore Sparky House Publishing,New York, USA.

Melton, T.A., W.A. Gutierrez, A. Broadwell, and J. Wilson. 2000. Plant pathology department extension-research flue-cured tobacco disease report. North Carolina Cooperative Extension Service, Raleigh, USA.

Meyer, J.B., Lutz, M.P., Frapolli, M., Péchy-Tarr, M., Rochat, L., Keel, C., Défago, G. and Maurhofer, M., 2010. Interplay between wheat cultivars, bio control pseudomonads, and soil. Applied and environmental microbiology 76(18), 6196-6204.

Monjero, K., Mbogo, E., Erungu, J. and Miano, D., 2015. Establishment of Nicotiana benthamiana in the green house. *Journal of agricultural science 5*(2), 59-62.

Murphy, J.F., Zehnder, G.W., Schuster, D.J., Sikora, E.J., Polston, J.E. and Kloepper, J.W., 2000. Plant growth-promoting rhizobacterial mediated protection in tomato against Tomato mottle virus. *Plant Disease*, *84*(7), 779-784.

Murphy, J.F., Reddy, M.S., Ryu, C.M., Kloepper, J.W. and Li, R., 2003. Rhizobacteria-mediated growth promotion of tomato leads to protection against Cucumber mosaic virus. *Phytopathology*, *93*(10), 1301-1307.

Narayanasamy, P., 2013. *Biological management of diseases of crops*. Springer, London, UK.

Nielsen, T.H., Sørensen, D., Tobiasen, C., Andersen, J.B., Christophersen, C., Givskov, M. and Sørensen, J., 2002. Antibiotic and bio surfactant properties of cyclic lipopeptides produced by *fluorescent Pseudomonas* spp. from the sugar beet rhizosphere. *Applied and environmental microbiology* 68(7), 3416-3423.

Noumavo, P. A., Kochoni, E., Didagbé, Y.O., Adjanohoun, A., Allagbé, M., Sikirou, R., Gachomo, E.W., Kotchoni, S.O. and Baba-Moussa, L., 2013. Effect of different plant growth promoting rhizobacteria on maize seed germination and seedling development.*American journal of plant science* 4, 1013-1021.

Nunes, C., Capistrano, G., Ferreira, D., Ferreira, S.S. and Mexia, J.T., 2015, March. One-way Fixed effects ANOVA with Missing observations. In 12th International Conference on Numerical *Analysis and Applied Mathematics. AIP Conference Proceedings* (Vol. 1648, p. 110008).

Osburn, R.M., Schroth, M.N., Hancock, J.G. and Hendson, M., 1989. Dynamics of sugar beet seed colonization by *Pythium ultimum* and *Pseudomonas species*: Effects on seed rot and damping-off. *Phytopathology* 79(6), 709-716.

Park, K.S., and Kloepper, J.W., 2000.Activation of PR-la promoter by rhizobacteria that induce systemic resistance in tobacco against. *Pseudomonas syringae pv tabaci*. *Biological control* 18, 2-9.

Pazarlar, S., GÜMÜS, M. and ÖZTEKIN, G.B., 2013. The Effects of Tobacco mosaic virus on growth and physiological parameters in some pepper varieties (*Capsicum annuum L*) Botanicae. *Horti Agrobotanici Cluj-Napoca* 41(2), 427.

Peix, A., Ramírez-Bahena, M.H. and Velázquez, E., 2009. Historical evolution and current status of the taxonomy of genus Pseudomonas. *Infection, Genetics and Evolution 9*(6), p1132-1147.

Pieterse, C.M.J., Van Pelt, J.A., Ton, J., Parchamann, S., Mueller, M. J., Bucha, A.J., Metraux, J.P., Van Loon, L.C., 2000. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonateband ethylene but is not accompanied by an increase in their production. *Physiological & Molecular Plant Pathology* 57, 123 -134.

Pirk, C.W., De Miranda, J.R., Kramer, M., Murray, T.E., Nazzi, F., Shutler, D., Van der Steen, J.J. and Van Dooremalen, C., 2013. Statistical guidelines for *Apis mellifera* research. *Journal of Apicultural Research* 52(4), 1-24.

Raaijmakers, J.M., Leeman, M., Van Oorschot, M.M., Van der Sluis, I., Schippers, B. and Bakker, P.A.H.M., 1995. Dose-response relationships in biological control of fusarium wilt of radish by Pseudomonas spp. *Phytopathology 85*(10), 1075-1080.

Raaijmakers, J.M. and Weller, D.M., 1998. Natural plant protection by 2, 4-diacetylphloroglucinol-producing Pseudomonas spp. in take-all decline soils. *Molecular Plant-Microbe Interactions* 11(2), 144-152.

Ramamoorthy, V., Raguchander, T. and Samiyappan, R., 2002. Enhancing resistance of tomato and hot pepper to *Pythium* diseases by seed treatment with *fluorescent Pseudomonads*. European *Journal of Plant Pathology* 108(5), 429-441.

Raupach, G.S., Liu, L., Murphy, J.F., Tuzun, S., Kloepper, J.W., 1996.Induced systemic resistance in Cucumber and tomato against cucumber mosaic cucumovirus using plant growth promoting rizhosphere. *Plant disease* 8, 891-894.

Roy, A.K., 2003. Mycological problems of crude herbal drugs: overview and challenges. Indian Phytopathology 56(1), 1-13.

SAS, S. and Version, S.T.A.T., 2003. 9.4 [Computer Program]. *SAS Institute, Cary, NC*.

Savary, S., Teng, P.S., Willocquet, L. and Nutter Jr, F.W., 2006. Quantification and modelling of crop losses: a review of purposes. *Annu. Rev. Phytopathol* 44, 89-112.

Schmidt, C.S., Agostini, F., Leifert, C., Killham, K. and Mullins, C.E., 2004. Influence of inoculum density of the antagonistic bacteria *Pseudomonas fluorescens* and *Pseudomonas corrugata* on sugar beet seedling colonisation and suppression of *Pythium* damping off. *Plant and soil* 265(1-2), 111-122.

Schmidt, C.S., Agostini, F., Leifert, C., Killham, K. and Mullins, C.E., 2004. Influence of soil temperature and matric potential on sugar beet seedling colonization and suppression of *Pythium* damping-off by the antagonistic bacteria *Pseudomonas fluorescens* and *Bacillus subtilis*. *Phytopathology* 94(4), 351-363.

Scholthof, K.B.G., 2008. Tobacco mosaic virus: the beginning of plant virology. *Biological control* 12, 333-344.

Scholthof, B.G., Adkins, S., Czosnek, H., Palukaihtis, P., Jaquot, E., Hohn, T., Hohn, B., Saunders, K., Candresse, T., Ahlquit, P., Hemenway, C., Foster, G.D., 2001. Top 10 plant viruses in molecular plant pathology. *Biological control* 12, 938-954.

Shen, L., Wang, F., Yang, J., Qian, Y., Dong, X., Zhang, H., 2014.Control of tobacco mosaic virus by *Pseudomonas fluorescens* CZ powder in greenhouses and fields. *Crop protection* 56, 87-90.

Siang-Hee, T.A.N., Nishiguchi, M., Sakamoto, W., Ogura, Y., Murata, M., Ugaki, M., Tomiyama, M. and Motoyoshi, F., 1997. Molecular analysis of the genome of an attenuated strain of cucumber green mottle mosaic virus. *Japanese Journal of Phytopathology*, *63*(6), 470-474.

Siddiqui, I.A. and Shaukat, S.S., 2003. Suppression of root-knot disease by *Pseudomonas fluorescens* CHA0 in tomato: importance of bacterial secondary metabolite, 2, 4-diacetylpholoroglucinol. *Soil Biology and Biochemistry* 35(12), 1615-1623.

Singhai, P.K., Sarna, B.K., Srivasta, J.S., 2011. Biological management of common scab of potato through Pseudomonas species and vermicompost. *Biological control* 57, 150-157.

Someya, N., Morohoshi, T., Ikeda, T., Tsuchiya, K., Ikeda, S., 2012. Genetic diversity Ecological Evaluation of *Fluorescent Pseudomonads* Isolated from the leaves and roots of potato plants. *Micro Environs* 27, 122-126.

Someya, N., Ikeda, S. and Tsuchiya, K., 2013. Pseudomonas inoculants as agents for plant disease management. In *Bacteria in Agrobiology: Disease Management* (pp. 219-241). Springer Berlin Heidelberg.

Sørensen, D., Nielsen, T.H., Christophersen, C., Soerensen, J. and Gajhede, M., 2001. Cyclic lipoundecapeptide amphisin from *Pseudomonas sp*. strain DSS73. Acta Crystallographica Section C: Crystal Structure Communications 57(9), 1123-1124.

Strange, N. and Scott, P.R., 2005. Plant disease: A threat to global food security. *Phytopathology* 43, 83-116.

Subashri, R., Raman, G. and Sakthivel, N., 2013. Biological control of pathogens and plant growth promotion potential of fluorescent Pseudomonads. In *Bacteria in Agrobiology: Disease Management* (pp. 77-110). Springer Berlin Heidelberg.

Sulzinski, M.A., Jurkonie, D.D. and Adonizio, C.S., 1994. Tobacco mosaic virus subliminal infection of African violet. *Journal of the American Society for Horticultural Science 119*(4), 702-705.

Tamar, S., Singh, B.P., Khan, M.A., Kumar, S., Sharma, S., & Lal, M., 2013. Identification of *Pseudomonas aeruginosa* Strain producing Bio surfactant with anti-fungal activity *against phytophthora* infestans. *Potato Journal* 40, 155-163.

Tambong, J.T. and Höfte, M., 2001. Phenazines are involved in bio control of *Pythium myriotylum* on cocoyam by *Pseudomonas aeruginosa* PNA1. European *Journal of Plant Pathology* 107(5), 511-521.

Tapadar, S.A. and Jha, D.K., 2013. Disease management in staple crops: a bacteriological approach. In *Bacteria in Agrobiology: Disease Management* (pp. 111-152). Springer Berlin Heidelberg.

Ton, J., Van Pelt, J.A., Van Loon, L.C. and Pieterse, C.M., 2002. Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in Arabidopsis. *Molecular Plant-Microbe Interactions* 15(1), 27-34.

Toyota, K., and Watanabe, K., 2013. Research highlights Recent Trends in Microbial Inoculants in Agriculture. *Microbes Environs* 28,403- 404.

Tran, H., Kruijt, J., Raaijimakers, R. (2008). Diversity & activity of bio surfactant –producing *Pseudomonas* in the rhizosphere of black pepper in Vietnam. *Applied Microbiology* 104, 839-857.

Udaya Shankar, A.C., Chandra Nayaka, S., Niranjan-Raj, S., Bhuvanendra Kumar, H., Reddy, M.S., Niranjana, S.R. and Prakash, H.S., 2009. Rhizobacteria-mediated resistance against the blackeye cowpea mosaic strain of bean common mosaic virus in cowpea (Vigna unguiculata). *Pest management science, 65*(10), 1059-1064.

Van Loon, L.C., 1997. Induced resistance in plants and the role of pathogenesis-related proteins. *European journal of plant pathology* 103,753-765.

Van Loon, L.C., 2000. Systemic induced resistance. In Mechanisms of resistance to plant diseases (521-574). Springer Netherlands.

Van Loon, L. and Bakker, P.A.H.M., 2003. Signalling in rhizobacteria-plant interactions. In Root ecology (297-330). Springer Berlin Heidelberg.

van Loon, L.C. and Glick, B.R., 2004. Increased plant fitness by rhizobacteria. In Molecular ecotoxicology of plants (177-205). Springer Berlin Heidelberg.

Van Peer, R., Niemann, G.J. and Schippers, B., 1991. Induced resistance and phytoalexin ac cumulation in biological control of *Fusarium* wilt of carnation by *Pseudomonas sp*. strain WCS417r. *Phytopathology* 81(7), 728-734.

Van Wees, S.C., De Swart, E.A., Van Pelt, J.A., Van Loon, L.C. and Pieterse, C.M., 2000. Enhancement of induced disease resistance by simultaneous activation of salicylate-and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences 97(15), 8711-8716.

Wei, G., Kloepper, J.W. and Tuzun, S., 1996. Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. *Phytopathology*, *86*(2), 221-224.

Yang, J., Guo, C., Xilun, Z., Shen L., Qian, Y., and Wang, F., 2012. Inactivation of tobacco mosaic virus in soil by *Pseudomonas putida* A3-m strain to prevent virus mosaic disease.*Afr J Microbial* 65, 6301-6307.

Yoneyama, S., 1988. Control of P strain of tobacco mosaic virus in sweet pepper.(2) Effects of soil fumigation with methyl bromide in spring season. *Proc, Kanto Plant Prot, 35*, pp.53-55.

Zohara, F., Akanda, M.A.M., Paul, N.C., Rahman, M., S Islam, M.T., 2016. Inhibitory effects of *Pseudomonas SPP*. on plant pathogen *Phytophthora* capsia in vitro and in plant. *Biocatalysis and Agricultural Biotechnology* 5, 69-77.

APPENDICES

Appendix 1Statistical analysis Plant growth promotion evaluation

Seed germination

Descriptive statistics

: Number of seed germinated					
Treatments	N Obs	Mean	Std Dev	Minimum	Maximum
Bacteria see	50	2.16	0.74	1.00	3.00
Control	50	1.72	0.70	1.00	3.00
Percentage of seed		germination			
Bacteria see	50	76.40	13.21	60	100
Control	50	54.80	14.46	40	80

T test

Treatments	Method	Mean	95% CL Mean		Std Dev	95% CL Std Dev	
Bacteria see		2.1600	1.9501	2.3699	0.7384	0.6169	0.9202
Control		1.7200	1.5208	1.9192	0.7010	0.5856	0.8736
Diff (1-2)	Pooled	0.4400	0.1542	0.7258	0.7200	0.6318	0.8371
Diff (1-2)	Satterthwaite	0.4400	0.1542	0.7258			

Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	98	7.80	<.0001
Satterthwaite	Unequal	97.209	7.80	<.0001

Necrosis number

Descriptive statistics

Analysis Variable : Necrosis no					
Treatment	N Obs	Mean	Std Dev	Minimum	Maximum
Bacteria see	36	2.00	0.79	1.00	3.00
Bacteria tra	36	2.56	0.93	1.00	4.00
Control	36	5.86	0.96	4.00	7.00

ANOVA model

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	313.72222	156.86111	193.33	<.0001
Error	105	85.194444	0.8113757		
Corrected Total	107	398.9166667			

R-Square	Coeff Var	Root MSE	Necrosis number Mean
0.786435	25.9420	0.900764	3.472222

Multiple test comparison

Means with the same letter are not Significantly different.

Bon Grouping	Mean	N	Treatment
A	5.8611	36	Control
B	2.5556	36	Bacteria tra
C	2.0000	36	Bacteria see

Necrosis area

Summary statistics

Analysis Variable : Necrosis area

Treatment	N Obs	Mean	Std Dev	Minimum	Maximum
Bacteria see	36	0.04	0.10	0.01	0.64
Bacteria tra	36	0.04	0.03	0.01	0.10
Control	36	0.10	0.04	0.03	0.20

ANOVA model

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	45.2808285	22.640414	35.08	<.0001
Error	105	67.7657329	0.6453879		
Corrected Total	107	113.0465614			

R-Square	Coeff Var	Root MSE	Necrosis area log Mean
0.400550	-24.27307	0.803360	-3.309678

Multiple test comparison

Comparisons significant at the 0.05 level are indicated by ***.				
Treatments Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
Control - Bacteria tra	1.1649	0.7073	1.6224	**
Control - Bacteria see	1.5058	1.0450	1.9667	**
Bacteria tra - Control	-1.1649	-1.6224	-0.7073	**
Bacteria tra - Bacteria see	0.3410	-0.1230	0.8049	
Bacteria see - Control	-1.5058	-1.9667	-1.0450	**
Bacteria see - Bacteria tra	-0.3410	-0.8049	0.1230	

Krusskal Wallis test

Kruskal-Wallis Test	
Chi-Square	51.6860
DF	2
Pr > Chi-Square	<.0001

ANOVA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	118.500000	59.250000	130.29	<.0001
Error	105	47.7500000	0.4547619		
Corrected Total	107	166.2500000			

R-Square	Coeff Var	Root MSE	Disease severity rating Mean
0.712782	24.52220	0.674360	2.750000

Multiple comparison test

Means with the same letter are not significantly different.			
Bon Grouping	Mean	N	Treatment
A	4.1667	36	Control
B	2.4167	36	Bacteria tra
C	1.6667	36	Bacteria see

Leaf number

Descriptive statistics

Analysis Variable : Leaf number					
Treatment	N Obs	Mean	Std Dev	Minimum	Maximum
Bacteria see	24	15	1.77	13	18
Bacteria tra	24	13	1.13	12	15
Control	24	7	1.18	5	9

ANOVA model

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	909.75000	454.87500	235.77	<.0001
Error	69	133.12500	1.929348		
Corrected Total	71	1042.875000			

R-Square	Coeff Var	Root MSE	Leaf number Mean
0.872348	11.61541	1.389010	11.95833

Multiple test comparisons

Means with the same letter are not significantly different.			
Bon Grouping	Mean	N	Treatments
A	15.4583	24	Bacteria see
B	13.3333	24	Bacteria tra
C	7.0833	24	Control

Leaf area

ANOVA model

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	142.421319	71.210659	130.29	<.0001
Error	69	37.7122792	0.5465548		
Corrected Total	71	180.1335986			

R-Square	Coeff Var	Root MSE	Leaf surface area Mean
0.790643	15.51553	0.739293	4.764861

Multiple test comparisons

Means with the same letter are not significantly different.			
Bon Grouping	Mean	N	Trewatment
A	5.9517	24	Bacteria see
A	5.5537	24	Bacteria tra
B	2.7892	24	Control

APPENDIX 3 DEVELOPMENT OF *N BENTHAMIANA* AND DISEASE PROGRESS

Figure 8.Day 6 after planting non bacterized

Figure 9.Day 6 after planting bacterized seed

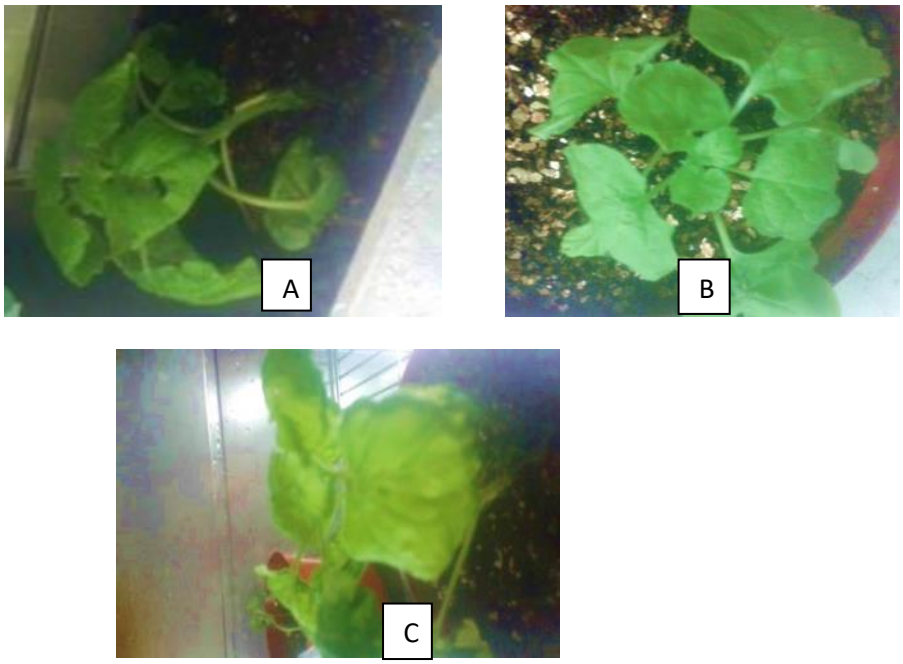
Figure10.14 days after planting

Figure 11 day 14 after planting

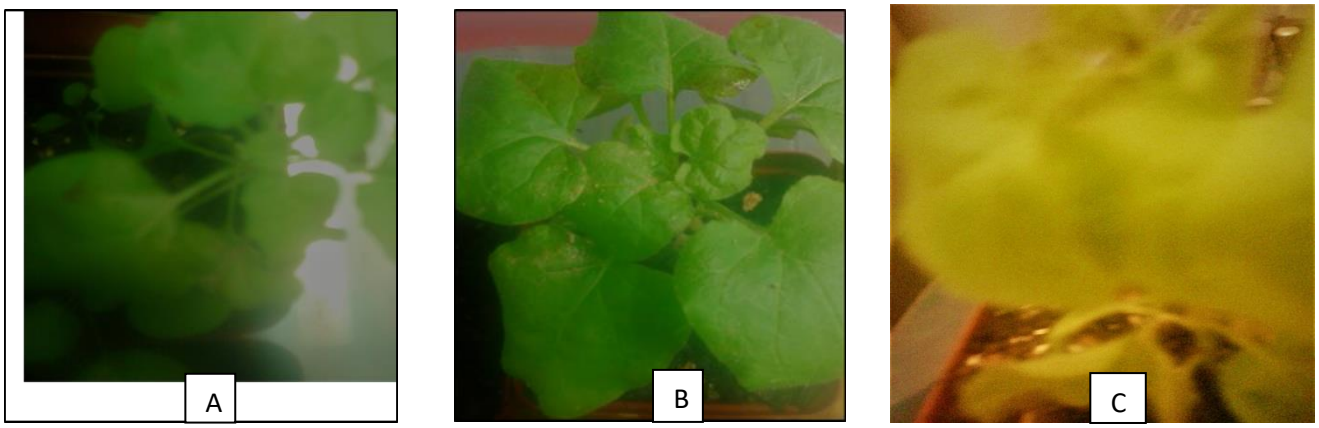
Figure 12.4 weeks after germination

Figure 13. 6 weeks after germination

Figure 14.6 Weeks after germination



Virus infection at 20dpi (20 February 2017) A is a late *P.koreensis* treatment , B is early *P. koreensis* treatment and C is the Control on this day the control is Showing clear signs of infection The early treatment necrosis area is getting bigger



Virus infection at 35 dpi (10 March 2017) A is a late *P.koreensis* treatment, B is early *P. koreensis* treatment and C is the Control on this day the control is Showing severe (leaves are curled) signs of infection The early treatment necrosis area is getting bigger. Nevertheless both the early and late treatment still has green leaves even though necrosis is clear.