Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Department of Ecology

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Abstract

It is important to investigate the potential of biological control measures in agriculture, especially where economic issues restrict the use of expensive inputs or when there are environmental concerns about toxicity of agrochemicals. The bacterium *Bacillus subtilis* has proved promising as a biocontrol agent (BCA) in suppressing various plant diseases and it has also been shown to promote plant growth in certain cases. During this study, the effect of *B. subtilis* on Fusarium wilt (caused by the fungus *Fusarium oxysporum*) on tomatoes, as well as its effect on the earthworm species *Pontoscolex corethrurus* was investigated. Furthermore, the combined effect of the BCA with *P. corethrurus* on tomatoes and *F. oxysporum* was investigated. This was done in three different experiments: 1) Laboratory study evaluating the effect on germination and growth of tomato seeds treated with *B. subtilis* received from University of Nairobi alternatively isolated root bacteria and infected with *F. oxysporum*, 2) Greenhouse experiment testing the effect alone and in combination of *B. subtilis* and *P. corethrurus* on *F. oxysporum* and tomato plants, 3) Toxicity experiment of *B. subtilis* on *P. corethrurus*. The results showed a positive effect of *B. subtilis* on plant dry biomass but did not increase the plant height. In addition, it showed tendencies of suppressing the disease caused by *F. oxysporum*, though, the results were not significant. However, the results were inconsistent and more research and experiments should be performed, evaluating the effect using different volumes and concentrations of the BCA. Furthermore, *B. subtilis* did not have a negative effect on *P. corethrurus* in this study. Since certain strains of *B. subtilis* have potential of working as BCAs, and are a promising and potentially environmentally friendly plant disease control bacteria, it is interesting to continue performing research on different crops and in different settings, as well as in combination with other BCAs. It is desired to develop a plant protection method that is easily available and applicable in all parts of the world, but with emphasis on small-scale farmers with limited means to access expensive chemical inputs.

Keywords: Biocontrol, BCA, *Bacillus subtilis*, Fusarium wilt, *Fusarium oxysporum*, Earthworms, *Pontoscolex corethrurus*, Tomatoes, Kenya, Tropical highland soil

Populärvetenskaplig sammanfattning

I den här studien har jag försökt att hitta en lättillgänglig och användbar metod för att bekämpa växtsjukdomar med biologisk bekämpning i småskaliga lantbruk i Kenya. För att skydda växter mot sjukdomsangrepp används i jordbruket både biologiska och kemiska bekämpningsmedel. Kemiska produkter skapar oro för miljöpåverkan och människors hälsa. Påverkan på mänsklig hälsa är särskilt påtaglig där läs- och skrivkunskaper är begränsade, vilket leder till att instruktioner inte följs, till exempel vad gäller skyddsutrustning och hantering. Kemiska produkter är även kostsamma och särskilt i utvecklingsländer är det svårt för lantbrukare att få pengar över för att köpa dessa medel. Växtsjukdomarna blir därför svårare att hantera och skördarna blir mindre. Vad som odlas på lantbrukarnas åkrar används inte bara för att föda familjen och gårdens djur, det är även en inkomstkälla då till exempel frukt säljs. En förlorad skörd blir alltså både en förlust i föda och inkomst. Därför är det viktigt att ta fram lättillgängliga metoder för att bekämpa växtsjukdomar, särskilt för människor i utvecklingsländer.

Fusariumröta är en svampsjukdom som angriper många olika växter, till exempel tomatplantor som den här studien fokuserar på. Sjukdomen angriper blad och rötter och får växten att vissna. Detta är en sjukdom som är svår att bekämpa, men vissa metoder inom biologisk bekämpning har hittats, till exempel användning av bakterien *Bacillus subtilis*. Bakterierna kan på olika sätt skydda växten, till exempel kan de kolonisera rötterna och förändra mekanismer i växten som gör att den bättre kan motstå sjukdomar. De kan även öka växtens produktion av biomassa. Men trots att bakterier är naturligt förekommande kan de eventuellt orsaka skada på andra organismer, så som daggmaskar som lever i jorden. Daggmaskarna konsumerar stora mängder jord och växtmaterial och får därmed i sig de substanser och organismer som finns där.

Därför har vi i den här studien, tillsammans med *Bacillus subtilis* påverkan på fusariumröta även valt att titta på hur bakterien påverkar daggmaskar, och specifikt en art som är vanligt förekommande i kenyanska jordar. Även daggmaskar i sig påverkar jordmiljön genom att gräva tunnlar samt tillgängliggöra växtnäring. De skapar därigenom även miljöer trivsamma för mikroorganismer så som bakterier, och har visat sig ha positiva effekter på växter. Därför har metoderna i den här studien valts för att kunna utvärdera en kombinerad samt enskild effekt av bakterierna och daggmaskarna på tomatplantor samt på sjukdomen fusariumröta, men även en direkt effekt av bakterierna på daggmaskarna för att utesluta skador på daggmaskarna.

Resultaten, om än aningen inkonsekventa, visar att *Bacillus subtilis* tenderade att bekämpa fusariumröta, och även att öka växternas biomassa. Bakterierna hade inte någon synbar negativ effekt på daggmaskarna. Daggmaskarna verkar även ha en positiv effekt på hur tomaterna växter, då tomaterna var högre i behandlingar där daggmaskar lagts till, vilket möjligen kan bero på deras aktiviteter som förbättrar mikroklimatet i jorden och förenklar näringsupptaget för växten. Studien bör dock upprepas och eventuellt modifieras aningen, för att man ska kunna säkerställa resultaten.

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Abbreviations

Al	Aluminum
B	Bacteria
BCA	Biocontrol Agent
Bs	*Bacillus subtilis*
Bt	*Bacillus thuringiensis*
CFU	Colony Forming Units
Cry	Crystal
Cyt	Cytolytic
DON	Deoxynivalenol
E	Earthworms
EUC	Embu University College
F	*Fusarium oxysporum*
IPM	Integrated Pest Management
ISR	Induced Systemic Resistance
JA	Jasmonic Acid
LBA	Luria-Broth Agar
MSA	Murashige & Skoog Agar
P	Phosphorous
PDA	Potato Dextrose Agar
PGPR	Plant Growth Promoting Rhizobacteria
SSA	Sub-Saharan Africa
T	Tomato plants
W	Water

1 Introduction

In Sub-Saharan Africa (SSA) various factors limit food production, insect pests and fungal diseases are among them (Badenes-Perez & Shelton 2006). Impact on human health (Oesterlund et al. 2014; Repetto & Baliga 1996) as well as on the environment has followed from the use of chemical pesticides, and getting access to pesticides is a challenge to resource poor farmers (Van Huis & Meerman 1997). Research and evaluations to find cost effective and environmentally friendly alternatives to chemical pesticides are taking place worldwide (Mathre et al. 1999). In Kenya, soil fertility decline, weeds, pests and diseases are among the major factors limiting crop production (Ayuke et al. 2012). To address some of these challenges, especially for pests and diseases, it is essential to investigate potential biocontrol methods in Kenya, where currently the use of agrochemicals is increasing. The authorities are taking measures against the use of agrochemicals by carrying out environmental impact assessment programs (Ntiba et al. 2001), and in many African countries, Integrated Pest Management (IPM) is considered the national crop protection strategy (Cherry & Gwynn 2007). PGPR (Plant Growth Promoting Rhizobacteria) can be used as a part of IPM and is a promising alternative to chemical pest treatment (Bhattacharyya & Jha 2012). Several species of *Bacillus* have proved capable for plant protection (Cavaglieri et al. 2005) of which *Bacillus amyloliquefaciens* is suppressing diseases as well as promoting plant growth (Abd El-Daim et al. 2014), and it has been found harmless to non-target organisms such as earthworms (Lagerlöf et al. unpublished).

A large microbial biomass and high soil organic matter content seem to be correlated with high productivity of agricultural fields (Murage et al. 2000), highlighting the importance of microorganisms in the soil. Furthermore, studies have shown that the microbial biomass tends to increase with practices such as agroforestry, compared to traditional agricultural practices, even though it takes many years to change soil conditions (Lagerlöf et al. 2014). Earthworms are, among other functions, important in the dispersing of microorganisms (Brown 1995). Furthermore, their castings are nutrient rich and contain beneficial bacteria that is easily available to plant roots, and can therefore be involved in suppressing plant diseases (Elmer 2009). Soil fauna is affected by soil management practices and generally increases in abundance in less disturbed environments (Ayuke et al. 2011; Lagerlöf et al. 2012), which is important to keep in mind in order to preserve soil fauna. In Kenya, about 9.5% of the country consists of arable land, and agriculture

comprises 75% of the population labor force (CIA 2014). The increasing population size together with the increasing use of pesticides and its health concerns make it important to investigate potential Biological Control Agents (BCAs) and its pathogen suppressive abilities, effect on plants as well as on soil fauna. Many studies have been focusing on the plant growth promoting and disease suppressive abilities of BCAs (e.g. Almoneafy et al. 2012), while few have combined such studies with experiments on non-target organisms.

2 Aims

The aims of this project were following:

- To present a literature review to get adequate background knowledge about the agricultural situation particularly in Kenya, current research about BCAs, the situation of the pathogen Fusarium wilt (*Fusarium oxysporum*) as well as the influence of soil fauna such as earthworms in the soil.
- To sample soil and isolate BCAs that are suitable and effective against *F. oxysporum* on tomatoes.
- Testing of isolated BCA from soil and *Bacillus subtilis* alone and in combination with *F. oxysporum* and earthworms on tomato plants to evaluate interactions, potential plant growth promoting effects and disease suppressive abilities.
- Testing if already isolated *B. subtilis* (which has previously proved promising as a BCA) will be toxic to earthworms.

The first hypothesis is that BCAs are present in the root zone of tomatoes especially in Fusarium wilt suppressive soils. The second hypothesis is that the bacterium that is native to the soil environment is harmless to soil fauna and has plant growth promoting effects.

To be able to determine the plant growth promoting effects and disease suppressive abilities, successfully isolated BCA as well as *B. subtilis* were tested in laboratory environment together with *F. oxysporum* on tomato seeds. Furthermore, a similar set-up was performed in a greenhouse where earthworms were included as well. The toxicity of the BCA was determined by performing a dipping experiment with earthworms in laboratory conditions.

3 Literature review

3.1 Agriculture and environmental impact

Due to the increasing population size worldwide, food production has to increase and thereby more land has to be converted into agricultural land, or intensification of already available land has to occur. The most productive fields are already cultivated, and when taking less productive land into cultivation the inputs might need to increase which might lead to environmental impacts such as a decrease in biodiversity and reduction of natural ecosystems. Furthermore, poorer water quality due to leaching from agricultural lands might follow. Therefore, improvements in technology and sustainable management methods are necessary (Tillman 1999). To date, the transformation of many complex ecosystems into simpler ecosystems has resulted in a subsequent reduction of biodiversity (Tscharntke et al. 2005).

Conditions for agricultural practices differ in different parts of the world. Where economy is not the largest constraint, high inputs such as chemical pesticides and fertilizers are commonly used (Kronvang et al. 1995). However, in systems where there are larger financial limitations and inadequate availability of products, biological control measures become more important (Van Huis & Meerman 1997) since expensive technologies are not available for the resource-poor small-scale farmers (Pretty 1999). According to Pretty (1999), sustainable agriculture has a positive feedback loop, and its effect on natural, social and human capital as well as the production of food, fiber and other products is generally positive. Pretty (1999) presents a number of success stories where resource poor farmers in SSA have improved their production by using already available means, there among biological control of pests, and the use of organic manure as fertilizers (Pretty 1999).

3.2 Agriculture in Kenya

Africa as a continent faces food security challenges, as about 27% of the world's undernourished people live there (CIAT 2014). Kenya currently holds a population of approximately 45 million people (CIA 2014), and this together with diminishing farm land, puts a lot of pressure on the agricultural land. Simultaneously, many crops yield below their potential. Improvements of yields as well as soil conserva-

tion measures are important in order to achieve sustainable agricultural production in Kenya (NAAIAP 2014). Generally, nutrient depleted soils are a threat for food security in SSA, where phosphorous (P) commonly is deficient (Ayuke et al. 2012), which is a major constraint against achievement of higher yields (Van Huis & Meerman 1997). Furthermore, land tenure and ownership limit the willingness of investing in soil-improvement measures (Van Huis & Meerman 1997).

To be able to improve agricultural extension services, NAAIAP (2014) characterized the major soil types in Kenya, an important tool when choosing the best possible management practices. NAAIAP (2014) came up with 25 major soil types in Kenya (NAAIAP 2014), deriving from many different types of parent materials (Ayuke et al. 2012). In Embu County, Nitisols are the main soil type and the soils vary from strongly acidic to neutral. For the acid soils, the use of manure is important, since it will reduce the toxic effect of aluminum (Al) and increase the availability of P (NAAIAP 2014). Soil chemical and physical degradation as well as biological degradation threatens the cropping systems in SSA (Ayuke et al. 2012) as 67% of the agricultural land is considered to be affected by land degradation (CIAT 2014). Depletion of organic matter and soil biodiversity is of main concern regarding biological degradation, factors leading to lower productivity and fertility (Ayuke et al. 2012). The biological importance is also apparent since the microbial biomass seems to be larger in high-productive fields compared to low-productive fields in Kenya's Central Highland (Murage et al. 2000).

Multi-cropping, the practice in which several crops are grown in the same field, is a common approach by small-scale farmers in Kenyan agriculture, and organic fertilizers (livestock manure) are commonly applied. Insect pests and pests associated with precipitation (such as fungal diseases) are major causes of crop losses. Chemical pesticides are used as a main pest control method (Badenes-Perez & Shelton 2006), although most chemicals are applied only by large-scale farmers (Ntiba et al. 2001). High value crops such as tomatoes (*Lycopersicon esculentum* L.) and important staple crops such as maize (*Zea mays* L.) are examples of crops typically grown in productive fields, while fodder crops and sweet potatoes (*Ipomoea batatas* L.) are more commonly grown in less productive fields (Murage et al. 2000). The production of crops in small-scale SSA cropping systems seems to differ depending on the location in the field where the highest crop production commonly occur close to the house with decreasing productivity further away from the house. This seems to be related to crop management practices, where fields regarded as less productive – often located further away from the house, are planted later. Moreover, the fields further away from the house seem to receive less nutrient resources as well as less weed removal practices (Tittonell et al. 2007).

3.2.1 Plant protection strategies in SSA

Resource-poor farmers in SSA commonly use biological plant protection methods. Location of the farm plot, crop rotation and intercropping, as well as the time of weeding, use of other toxic non-crop plants or traps are methods applied either intentionally or unintentionally. According to Van Huis & Meerman (1997), re-

source-poor farmers continuously experiment to find effective methods for plant protection. However, their agricultural practices usually give large variations in the yield outcome, and lack of financial resources and knowledge are restrictions for improving the situation. These agricultural strategies rely much on the farmers own experience as well as other farmers experience rather than on extension services (Van Huis & Meerman 1997).

In the Lake Victoria basin, the use of agrochemicals is becoming increasingly important, even among small-scale farmers, although most chemical pesticides are still applied by large-scale farmers (Ntiba et al. 2001), especially in coffee plantations (Ntiba et al. 2001; Repetto & Baliga 1996). It is difficult to introduce measures against over-use of pesticides since accurate data about pesticide usage is lacking. However, environmental impact assessment measures are now being undertaken by the Environment Authorities in the countries bordering the Lake Victoria basin (Ntiba et al. 2001). Even though Africa as a continent has the lowest use of pesticides of all continents (Repetto & Baliga 1996) for example due to lack of access to the inputs (Van Huis & Meerman 1997), inappropriate use of pesticides is an issue that poses health risks to the users. It might be due to illiteracy or unreadable instructions, as well as improper equipment when handling the chemicals (Repetto & Baliga 1996). Among small-scale farmers in Uganda, about 73% are using normal clothing when applying pesticides (Oesterlund et al. 2014). In the same study, it was shown that the main pesticides used belonged to class II (moderately hazardous), while the author suggests that earlier studies show an extended use of pesticides of class I (extremely to highly hazardous) in developing countries. However, results seem to vary between different regions (Oesterlund et al. 2014). In northern Tanzania, Ngowi et al. (2007) found that farmers used small amounts of class I pesticides, where insecticides comprised of 59% of the pesticides used, followed by fungicides (29%) and herbicides (10%) (Ngowi et al. 2007). According to Ngowi et al. (2007), it is necessary to bring attention to alternative, cost effective and environmentally friendly pest management methods especially since there seem to be many health issues connected to the pesticide usage, particularly in SSA (Ngowi et al. 2007).

3.2.2 Integrated Pest Management

It is desirable from a human point of view to increase the yield per unit area and input, while sustainable use of the natural resources is essential. Higher productivity and reduced negative impact have been seen in integrated, eco-efficient systems with livestock, crops and forestry (CIAT 2013). Integrated Pest Management (IPM) aims to use the best available methods for pest control. However, it does not necessarily mean a complete removal of chemical pesticides, although, the use of natural enemies is an important component of IPM (Van Huis & Meerman 1997). When deciding the crop protection method according to IPM, all available strategies should be considered, where risks to the environment as well as human health should be minimized. Disturbances to the agro-ecosystem should be as minimal as possible and natural pest management strategies should be encouraged (FAO 2015a). IPM can be seen as a method combining biological, chemical, physical as well as technological pest management methods (Munyua 2003).

There is not any pre-fixed IPM-package that is applicable to all farming systems, rather the strategy has to be adapted depending on factors such as agro-ecological and socio-economical settings (Van Huis & Meerman 1997). In Africa, many countries try to follow IPM where microbial BCA sometimes is included as the national crop protection strategy (Cherry & Gwynn 2007). This aims to promote food safety and good agricultural practices (Cherry & Gwynn 2007; FAO 2015a). However, implementation of IPM can be difficult; hence training of both farmers and extension workers in agro-ecology is essential to get the desired effect. Furthermore, the extension worker needs to consider the farmers knowledge and experiences, otherwise advice and recommendations might end up being poorly applied (Loehr et al. 2000). Policy making on pest management in a country is a vital part for the implementation of IPM. Supportiveness of the development of new knowledge and technologies as well as a conflict-free government regarding different agricultural methods are important (Munyua 2003).

3.3 Biological control using bacteria

Increasing pressure to reduce the chemical inputs, as well as financial issues hindering the use of costly agrochemicals are reasons to find alternative pest control methods (Lucas 2011). To reduce the dependency of chemical inputs, biological control offers an interesting and potentially environmentally friendly alternative (Bhattacharyya & Jha 2012). One type of biological control is the use of BCA. BCAs can provide protection through mechanisms such as predation, parasitism, antibiosis and competition for resources (Lucas 2011).

Currently, *Bacillus thuringiensis* (Bt) based products take up a major part of the market of BCAs, especially against insect pests (Lucas 2011). Bt is a gram-positive bacterium and is using Cry (Crystal) and Cyt (Cytolytic) protein toxins (Schnepf et al. 1998), which are pore-forming toxins (PFTs) (Parker & Feil 2005). The PFTs are formed during sporulation of the bacteria, Bt allocates up to 30% of its cellular proteins to produce the insecticidal proteins, which have been shown to be very specific targeting certain insect groups. The PFTs are further developed in the cell after which they are released during the end of sporulation in a cell lysis. When inserted into the membranes of insect cells channels are formed, which will eventually cause colloid-osmotic swelling and cell lysis, after which the insect dies (Parker & Feil 2005). Pest control products based on Cry proteins are cheaper to develop and register than synthetic pesticides, and so far, it has not shown any harm to other organisms other than the invertebrate pests that they are used for. Furthermore, compared to synthetic pesticides, the mode of action for the Cry protein is different, making it suitable for IPM-methods (Schnepf et al. 1998). Many species of *Bacillus* have proved to be promising BCAs (Cavaglieri et al. 2005). For example, the species *B. amyloliquefaciens* has been tested against fungal diseases, with promising plant protection results. Besides, they enhance plant growth. The lifestyle of the pathogen as well as the production of antifungal substances by the BCA are factors deciding the plant protection potential (Danielsson et al. 2007; Sarosh et al. 2009). *B. amyloliquefaciens* has further been tested in

combination with earthworms to evaluate if it is harmful to soil fauna, where the results show that it does not have any negative effects on the earthworm species tested (Lagerlöf et al. unpublished). This is an important aspect since many species of *Bacillus* produce chitinases (enzymes degrading chitin) (Shanmugam et al. 2011) while earthworms contain chitin in their cuticle and cetae (Laverack & Kerkut 1963).

Induced systemic resistance (ISR) is a defensive ability that plants develop when they are stimulated properly, by certain non-pathogenic rhizosphere bacteria. The mechanism depends on responses to ethylene and jasmonic acids (JA) (Bakker et al. 2003). PGPR can enhance plant growth under conditions that are stressful for plants (van Loon et al. 1998). Sarosh et al. (2009) have showed that root treatment of *Brassica napus* with *B. amyloliquefaciens* results in systemic gene expression in the leaves, where the production of JA has been observed. It is suggested that ISR in *B. napus*, in this case against the pathogen *Botrytis cinerea*, is induced by *B. amyloliquefaciens* (Sarosh et al. 2009). Furthermore, studies of the effect of heat stressed wheat plants treated with *B. amyloliquefaciens*, show that treatments using the bacteria seem to improve the plant fitness and thereby they can better withstand the stress (Abd El-Daim et al. 2014).

B. subtilis is a gram positive bacteria that has shown to have disease suppressive abilities (Shoda 1996), for example against *Fusarium* (Bhattacharyya & Jha 2012; Hariprasad et al. 2011). It can be formulated as a product that is easy to store, and it is robust and durable (Emmert & Handelsman 1999; Shoda 2000). Pathogenic nematodes on the common bean in Kenya have been shown to be suppressed by *B. subtilis* (Wepuhkhulu et al. 2011). Furthermore, in greenhouse studies *B. subtilis*, *B. amyloliquefaciens* and *Bacillus methylotrophicus* have demonstrated high pathogen inhibition effects, and plant height and biomass of plants treated with these bacteria were found to be larger than the control (Almoneafy et al. 2012). *B. subtilis* has also been shown to suppress *Fusarium verticillioides* on maize (Cavaglieri et al. 2005).

According to Lucas (2011), when using biological control for plant protection, the results might sometimes be unsuccessful. The methods may give inconstant results as well as being less efficient than conventional pesticides. Lucas (2011) states that it is unlikely that bio-pesticides will replace chemicals to a major extent, at least in large-scale agriculture, although BCAs may be of larger importance in small-scale and low-input systems. However, many chemicals are based on bioactive products, which reduce the difference between biological and chemical approaches (Lucas 2011).

3.4 Fusarium wilt

Fusarium wilt is a fungal disease caused primarily by *F. oxysporum* and it infects, among others, tomatoes and legume crops (PAN Germany, OISAT 2005). A plant infected with Fusarium first develops a yellowing (chlorosis) on its foliage after which the plant irreversibly wilts. Fusarium wilt is favored by fairly high tempera-

tures, especially in areas prone to drought (Allen et al. 1996). Different races affect different hosts, which depends on the virulence of the pathogen as well as the resistance of the host to the pathogen (Pegg & Langdon 1987). Outbreaks of Fusarium wilt in bananas currently result in large economic losses and they pose a threat to food production (FAO 2015b). Also the climbing bean, which is a popular bean variety in the Great Lakes Region, has been subjected to serious crop losses caused by Fusarium wilt (CIAT 2003). It spreads through water, soil as well as through contaminated equipment used in agriculture (FAO 2015b).

According to PAN Germany, OISAT (2005) there is currently no effective method available to protect crops against Fusarium wilt (PAN Germany, OISAT 2005), and to avoid the disease, use of resistant cultivars is the most effective method (Allen et al. 1996). It is also important not to rely on a single plant species (Buruchara & Camacho 2000). As such, there are continuous research trying to find and develop new resistant cultivars (CIAT 2003). Additionally, there are studies of Fusarium wilt on cotton showing that endophytic bacteria (bacteria living in the plant tissues without doing any harm nor gaining any other benefits from the host than habitat), have potential of working as BCA against the pathogen (Chen et al. 1995). Moreover, studies of Fusarium wilt using chitinolytic bacteria (bacteria that produce enzymes that have the ability to degrade fungal cell walls) have shown capabilities of suppressing the disease on cucumbers (Singh et al. 1999) as well as on tomatoes (Hariprasad et al. 2011). Besides, studies of Fusarium wilt on tomatoes have showed that already known biocontrol isolates, such as non-pathogenic *Fusarium* species, suppressed the disease (Larkin & Fravel 1998). Additionally, different species of *Bacillus* have shown to have antagonistic effects on Fusarium wilt on tomatoes, as well as promoting the growth of tomato plants. *Bacillus* species have been shown to use different approaches, such as producing chitinases or siderophores (low-molecular weight molecules that binds or chelates iron) (Shanmugam et al. 2011). It is projected that the occurrence of Fusarium wilt may increase due to future climate changes, which makes it important to come up with new crop varieties and disease management methods against the pathogen (Shabani et al. 2014).

3.5 Earthworms

Soil fauna is an important part of the soil and losses of biodiversity contributes to soil degradation such as depletion of nutrients, decrease in fertility, water scarcity as well as reductions in crop yield. Soil organism diversity can be altered by agricultural management practices, such as chemical inputs (Ruiz et al. 2008). Earthworms are sensitive to land management practices and are generally more abundant in natural ecosystems such as forests compared to intensive agricultural lands (Ayuke et al. 2011; Lagerlöf et al. 2012; Ruiz et al. 2008).

Earthworms are ecosystem engineers and highly affect the soil by their burrowing activities. They contribute to the mixing, moving and aeration of the soil (Ruiz et al. 2008). The presence of earthworms is generally viewed upon as a sign of a healthy soil (Edwards 2004; Murage et al. 2000). Furthermore, earthworms are

important concerning soil function, they increase the turnover of soil organic matter and nutrients, as well as increasing the rhizosphere activity (Brown 1995). Microflora has a limited ability to move through the soil and they may largely stay dormant, whereas earthworms have the ability to disperse and activate them. Different microorganisms may however have different abilities of surviving the passage through earthworms. Some earthworm species create "hot spots" of microbial activity, which might also be beneficial environments even for harmful organisms, though it seems like the benefits outweigh the negative effects (Brown 1995).

Elmer (2009) showed an increase of plant production as well as disease suppression in the presence of earthworms. In the same study, an increase of microbial abundance was observed where earthworms were added, which is believed to be the reason behind the disease suppression. It is suggested that earthworms create casts rich in beneficial microorganisms that plant roots can take advantage of (Elmer 2009). Edwards & Bater (1992) showed that the positive effect on plant growth depends on the earthworm species, where deep burrowing species seem to affect most (Edwards & Bater 1992).

The endogeic earthworms, i.e. earthworms that live and feed in the soil (Ruiz et al. 2008), such as the species *Pontoscolex corethrurus* (Glossoscolecidae, Oligochaeta) have been shown to enhance plant production, especially of maize, by changing soil physical and biogeochemical processes as well as microbial communities. Their presence have an effect on soil structure, especially aggregation, where they increase the proportion of macro-aggregates (Pashanasi et al. 1996). *P. corethrurus* is a strictly geophagous earthworm; it ingests large quantities of soil, and usually inhabits areas disturbed by humans, for example croplands, as well as marshy or swampy soils. It is common in the humid tropical climates and is tolerant to a range of physico-chemical conditions in soils. Furthermore, it has developed a mutualistic relationship with microorganisms, increasing the pH of the organic matter, making it easier for microorganisms to degrade even complex molecules (Lavelle et al. 1987).

Studies on food preferences by earthworms have shown that their feeding on fungal species differs depending on the ecological niche of the earthworms (Bonkowski et al. 2000). The same study showed that plant pathogenic fungi, such as *Fusarium*, were favored and frequently consumed, while basidiomycetes (which generally degrade recalcitrant polymers) were little preferred. This is also confirmed by Wolfarth et al. (2011), who showed that *Fusarium* biomass as well as the *Fusarium* toxin DON (deoxynivalenol) on wheat decreased in the presence of *Apporectodea caliginosa* (Wolfarth et al. 2011). This pattern can be assumed to depend on the fungal nutritional content, or the presence of antibiotics. However, the authors state that earthworms cannot be expected to graze selectively in their natural environment, since they co-consume such large amounts of soil and organic matter. They consume fungi together with other microflora that colonize plant litter (Bonkowski et al. 2000).

4 Materials and Methods

4.1 Study area

Sampling and laboratory work took place in Embu County (0°32'S, 37°37'E), 130 km north of Nairobi, Kenya from the 23rd of February until the 25th of April 2015. Average expected lowest and highest temperatures as well as average precipitation for those months in Embu are presented in table 1. The soils in Embu vary from strongly acid (pH 4.6) to neutral (pH 6.7) and consists mainly of Nitisols (NAAIAP 2014).

Table 1. Weather data for Embu, Kenya. Monthly average temperatures and precipitation year 2000-2012 (World Weather Online 2015)

Month	Average annual low temperature (°C)	Average annual high temperature (°C)	Average precipitation (mm)
February	14	27	27.2
March	15	27	111.3
April	16	25	269.1

4.2 Experimental design

Three types of experiments were conducted to get a comprehensive overview of the studied BCAs and their interactions with the pathogen, earthworms and plants.

1. The multiwell and toxicity experiments were done in laboratory. In multiwell dishes (Figure 2) the effect alone and in combination of the BCA *B. subtilis* and the pathogen *F. oxysporum* was tested on tomato seeds. The same experiment was also performed using a bacteria isolated from soil surrounding the roots of tomato plants sampled in Embu County, Kenya. The aim was to evaluate the germination of the seeds, and growing rate and survival of the emerging plants when exposed to different treatments. A water solution of the bacteria (*B. subtilis* or the isolated bacteria) and mycelia of *F. oxysporum* were added to the wells and the germination and growth rate of the tomatoes was observed.
2. The interaction experiment took place in a greenhouse that measured approximately 2 m × 5 m with a height of 2 m (Figure 1) in Embu University College (EUC). The purpose of the greenhouse was to allow optimized

sun-input, but at the same time as much as possible avoid interferences from pests, such as insects and birds that might feed on the plants. The sides of the greenhouse were covered with transparent fabrics of mosquito net type to allow air circulation while the top of the greenhouse was covered with transparent plastic to prevent uncontrolled rain water to enter. The experiment was investigating the effects alone and in combination of *B. subtilis*, *F. oxysporum* and *P. corethrurus* on tomatoes. A water solution of *B. subtilis* was used and *F. oxysporum* was added as mycelia. The aim was to investigate the combined effect in a relatively field-like situation.

3. The toxicity experiment assessed the direct effect on the earthworm *P. corethrurus* when exposed to *B. subtilis*. The aim was to evaluate if the bacteria had any negative effect on the earthworms. The earthworms were dipped in a water solution of *B. subtilis* alternatively distilled water and the effect on growth rate was observed.

Figure 1. Greenhouse exterior.

Preparations of materials as well as methods used are presented in the following sections.

4.3 Experimental units

4.3.1 Multiwell experiment

Multiwell dishes (costar® 12 well Cell Culture Cluster) that contained 12 wells with a height of 1.8 cm and a diameter of 2.3 cm were used for the laboratory experiments. The wells were filled to approximately halfway with Murashige & Skoog Agar (MSA) (Figure 2).

Figure 2. Multiwells filled with MSA and with added seeds.

4.3.2 Interaction and toxicity experiments

Plastic 5 liter buckets measuring 20 cm high and diameters of 13.5 cm at the bottom and 20 cm at the top were used for the toxicity and interaction experiment. Holes with a diameter of about 0.5 cm were made at the bottom to let excess water out, and a net was placed in the bottom to prevent earthworms from escaping. The toxicity test was kept with perforated lids to prevent excess evaporation but to let air enter, whereas the interaction test took place in open mesocosms. Transparent sides were covered with alumina foil to avoid uncontrolled processes and production of secondary metabolites (Figure 3).

Figure 3. Mesocosms for interaction experiment (left) and toxicity text (right).

4.4 Crop choice

4.4.1 Multiwell and interaction experiments

Tomatoes were selected as the experimental crop. Tomatoes are severely affected by Fusarium wilt in Kenya, highlighting the importance to find control measures against the pathogen. Seeds of the cultivar Rio Grande (Safari Seeds LTD), which

had been poison-treated with an insecticide and/or fungicide, were used in the multiwell and the interaction tests. In this experiment, the insecticide should not severely affect the results; however, a fungicide seed coating might make the infection of *F. oxysporum* more difficult, especially in the multiwell experiment where *F. oxysporum* was added before the seeds were germinated. However, seeds without the coating were not available which is why these were chosen. Plants of the same cultivar were collected for isolation of root bacteria.

4.5 Soil sampling

Soil sampling for the interaction and toxicity test was done in a crop field at the campus of EUC. The soil was kept in oven (120 °C) for at least 24 h to kill insects and insect eggs. Farmyard cow manure was prepared using the same procedure as for the soil. In the toxicity test, 900 g of soil and 90 g of cow manure were added in each mesocosm and thoroughly mixed together with tap water, while in the interaction experiment, 1000 g of soil and 100 g of cow manure were added and thoroughly mixed with tap water before tomato seeds were sown. The reason for the different amounts depend on the amount of soil available at the time. The cow manure was used to provide nutrients for plants and earthworms, where quite large amounts were added to make sure they had enough nutrients/food. Moisture content was measured in the initial stage as well as in the end of the experiments.

4.6 Test organisms

4.6.1 Soil fauna – *Pontoscolex corethrurus*

Earthworms of the species *P. corethrurus*, which is a geophagous earthworm consuming large quantities of soil, were sampled from wet soils near Rianjagi Coffee factory, 6 kilometers North East of EUC campus. The sampling was done by digging holes and picking the earthworms by hand. Individuals selected for the experiment had reached adulthood, determined by the presence of clitellum, or were of the same size as individuals with clitellum.

4.6.2 Pathogen – *Fusarium oxysporum*

Potato Dextrose Agar (PDA) was prepared according to the manufacturer´s instructions. The mixture was autoclaved in 121°C during at least 15 minutes. Plastic petri dishes were put under UV-light for at least 15 minutes to be sterilized before agar was added. *F. oxysporum*, a major cause of Fusarium wilt, was received through the University of Nairobi, and further cultured in an incubator in laboratories at EUC to receive mycelia for the experiments.

4.6.3 BCA – *Bacillus subtilis*

Since isolation of suitable BCAs is a time consuming process, already isolated *B. subtilis* received from the University of Nairobi in Kenya was also used. *B. subtilis* was tested alone and in combination with the pathogen *F. oxysporum* and earthworms on plants, as well as on earthworms alone. *B. subtilis* were grown in LB medium and kept on a magnetic stirrer until sporulation was reached. The suspension was heat-shocked for 5 minutes in 60 °C after which 50 ml of the suspension was moved to 25 Eppendorf tubes (2 ml per tube), and centrifuged in 5000 rpm for

8 minutes. The supernatant was removed and each pellet was suspended in 1 ml distilled water, receiving a total volume of 25 ml suspended pellet. Thereafter, a serial dilution was performed (Figure 4). 90 µl of the 10^{-4} dilution and 100 µl of the 10^{-6} dilution were spread on agar plates and incubated in 28 °C for 24 hours, during which time the bacteria colonies formed from each viable bacteria cell in the solution. Bacteria colony count was done using a colony counter to determine the concentration, and thereafter the stock solution was diluted to receive a concentration of 10^{7}CFU ml^{-1}, using the formula presented below, where C is concentration and V is volume (Equation 1).

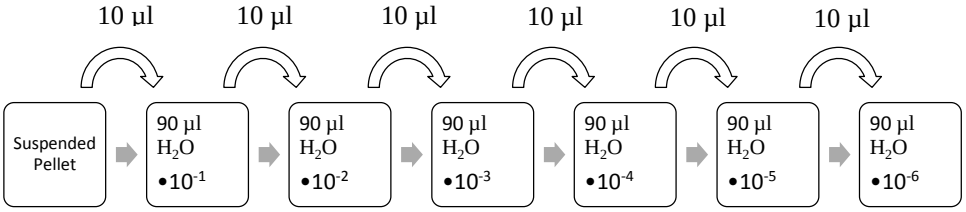

Figure 4. Schematic figure: Dilution series for determining bacterial concentration.

$$C_1 \times V_1 = C_2 \times V_2 \qquad \textbf{(Equation 1)}$$

4.6.4 Isolation of bacteria

Luria-Broth Agar (LBA) was prepared according to the manufacturer´s instructions. The mixture was autoclaved in 121 °C for at least 15 minutes. Plastic petri dishes were put under UV-light for at least 15 minutes to sterilize them before LBA was added. Soil surrounding the roots from the collected tomato plants was gently shaken off, and in the process, 10 g of the soil close to the roots, as well as some roots were collected and diluted in 95 ml of distilled water, after which it was filtered through cotton wool into an e-flask to remove large particles. It was further diluted in 10-fold dilution series by adding 1 ml of the solution in test tubes containing 9 ml distilled water. The test tubes were put on vortex between each dilution. This was repeated four times receiving dilutions of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} (Figure 5).

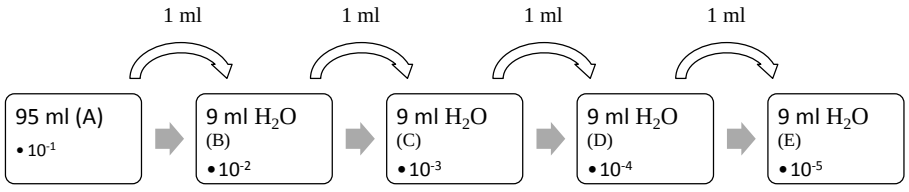

Figure 5. Schematic figure: 10-fold dilution series for isolating bacteria from soil, A=10 g of soil in 95 ml distilled water.

From C, D and E (Figure 5), 0.1 ml per test tube were removed and spread on agar-plates containing LBA-medium, each in two repetitions, after which the plates were kept in an incubator at a temperature of 22 °C. When bacterial colonies were visible, a small amount from one colony was moved to another agar plate using the streak-plate method. When clean colonies were obtained, the bacte-

ria were grown in LB-medium and kept on a magnetic stirrer and further prepared as described for *B. subtilis* (Equation 1, Figure 4).

4.7 Laboratory testing of bacteria – effects on plant growth and Fusarium infection

4.7.1 Isolated bacteria

Prior to the experiment, MSA was tested for its ability to support appropriate plant growth. In sterile multiwell dishes, tomato seeds were placed on the agar (one seed per well) in different combinations with the pathogen as well as the bacteria (Table 2). One day after tomato seeds were placed in the multiwell dishes, 50 µl of the bacterial solution with a concentration of 10^7CFU ml^{-1} was added to the wells. Mycelia of *F. oxysporum* were added simultaneously to the wells by using a sterile Pasteur pipette with a diameter of 2 mm, after which the dishes were placed in a window in order to get enough light for the emerging seedlings. Results were read one week after adding the bacteria and pathogen.

Table 2. Experimental set-up, each treatment in three replicates, each replicate consisting of four wells. To describe the different treatments, the following abbreviations will be used: B=isolated bacteria, F=*F. oxysporum*, E=earthworms and T=tomato plants

Multiwell test	
Treatment	Combination
1	Tomatoes + Bacteria + Fusarium
2	Tomatoes + Bacteria
3	Tomatoes + Fusarium
4	Tomatoes

4.7.2 B. subtilis

Tomato seeds were placed in multiwell dishes and left to germinate and grow during one week, after which *B. subtilis* and *F. oxysporum* were added, using the same method as for the isolated bacteria. Testing of *B. subtilis* was furthermore repeated using the same method as for the isolated bacteria, thus adding *B. subtilis* and *F. oxysporum* one day after placing the seeds on the agar. The same set up as in Table 2 was used. To describe the different treatments, the following abbreviations will be used: Bs=*B. subtilis*, F=*F. oxysporum*, E=earthworms and T=tomato plants.

4.7.3 Data analysis

Data analysis was performed using Minitab 17 Statistical Software. "2 proportions" statistical test was made to see if there was a significant difference between germination rate and growth rate between the different treatments, both for the isolated bacteria and *B. subtilis*. For significance, α=0.05 was used.

4.8 Interaction test – greenhouse experiment on interactions of bacteria, plants, Fusarium and earthworms

An interaction test was performed to evaluate the effect of *B. subtilis* on the pathogen *F. oxysporum*, the growth of plants and possible effects on non-target organisms such as earthworms (Table 3). Six seeds were planted per mesocosm with a spacing of approximately 5 cm between the plants, and 2 cm from the wall. Seedlings showing chlorosis were removed (but included when calculating the germination rate) since Fusarium wilt could not be excluded, and to avoid uncontrolled infection of the disease. After the seeds had germinated, four seedlings of as equal sizes as possible were left in the mesocosms for further testing.

Initial height of the plants was measured 16 days after the seeds were sown, 19 days after sowing of seeds, three earthworms of the species *P. corethrurus* were added per mesocosm. The worms were washed in tap water, quickly dried on tissue paper and weighed before being added to the mesocosms. One day after the earthworms were added, mycelia of *F. oxysporum* were introduced to the upper surface of the tomato plant leaves using a Pasteur pipette (2 mm in diameter). Three to four leaves per plant were infested, depending on the size of the plant. To make sure that the plants got infected, mycelia of *F. oxysporum* were once again introduced after one week by adding 3×3 mm agar pieces, specifically on plants not yet showing early infection symptoms. The same day as the second addition of *F. oxysporum*, 100 ml per mesocosm of the bacterial water solution (10^7CFU ml^{-1}) alternatively distilled water were added. The mesocosms were regularly watered to keep the soil moisture constant and plants were frequently checked and monitored for symptoms of *F. oxysporum*.

At the end of the experiment, plant height (28 days after adding the bacteria) as well as plant wet weight (29 days after adding the bacteria) and plant dry weight (30 days after adding the bacteria) were measured. Furthermore, for each plant; number of leaves, number of infected leaves, number of leaves with chlorosis, number of leaves with brownish symptoms, discolored veins and number of leaves with curly sides were noted (28 and 29 days after adding the bacteria). For chlorosis and brown discoloring, each leaf was given a score of 0-5 (0=0%, 1=20%, 2=40%, 3=60%, 4=80% and 5=100%) where it was later weighed into a percentage for the whole plant, depending on number of leaves given a certain point. For discolored veins and curl number of occurrences per plant was noted. Moreover, soil moisture (30 days after adding the bacteria) and soil dry weight (31 days after adding the bacteria), and earthworms were weighed (30 days after adding the bacteria).

Table 3. Experimental set-up, each treatment consisting of six replicates. To describe the different treatments, the following abbreviations will be used: Bs=*B. subtilis*, F=*F. oxysporum*, E=earthworms and T=tomato plants

Interaction experiment	
Treatment	Combination
1	Tomatoes + *B. subtilis* + Fusarium + Earthworms
2	Tomatoes + *B. subtilis* + Earthworms
3	Tomatoes + Fusarium + Earthworms
4	Tomatoes + *B. subtilis* + Fusarium
5	Tomatoes + Earthworms
6	Tomatoes + *B. subtilis*
7	Tomatoes + Fusarium
8	Tomatoes

4.8.1 Data analysis

Data analysis was performed using Minitab 17 Statistical Software. General Linear Model – one-way ANOVA was used to see if there was a significant difference between plant height, plant wet and dry weight as well as plant moisture content, earthworm biomass and Fusarium symptoms, while two-way ANOVA was used to see the effect of the different factors Bs, E and F. Tukey's test was used for comparison. For significance, α=0.05 was used.

4.9 Toxicity test – effects of bacteria on earthworms

A toxicity test was performed to evaluate the direct exposure of earthworms to *B. subtilis* (Table 4). Earthworms of the species *P. corethrurus* were washed in tap water, quickly dried on tissue paper and weighed before they were dipped into a water solution of *B. subtilis* with a concentration of 10^7CFU ml^{-1} during 20-30 seconds. Control groups of *P. corethrurus* were dipped in distilled water during 20-30 seconds. In each mesocosm, two individuals were added. Tap water was added regularly to the mesocosms to prevent the soil from drying out. Results were read after 19 days, by noting the weight of the earthworms.

Table 4. Experimental set-up, each treatment consisting of six replicates. To describe the different treatments, following abbreviations will be used: Bs=*B. subtilis*, E=earthworms and W=water

Toxicity test	
Treatment	Combination
1	Earthworms + *B. subtilis*
2	Earthworms + Distilled water

4.9.1 Data analysis

Data analysis was performed using Minitab 17 Statistical Software. General Linear Model was used to see whether there was a significant difference between final weight of the earthworms in the different treatments as well as the growth of the earthworms, where initial weight was used as a covariate. For significance, α=0.05 was used.

5 Results

5.1 Isolation of bacteria

During the isolation of bacteria from soil, two different species were obtained – species A and species B. Species A was preliminary identified as *B. subtilis* based on color and shape of growth while species B remained unidentified. When preparing a bacterial solution and plating the bacteria after serial dilution, species B appeared as species A making it probable that "species B" was a contamination. Probably clean colonies had not yet been obtained even if it appeared that way at first. During the second isolation of bacteria, petri dishes were left for too long in the incubator resulting in fly larvae feeding on the bacteria and agar, thus destroying the samples. But as far as could be determined by eye, in these samples "species B" was present.

5.2 Laboratory testing of bacteria

The multiwell experiments took place in laboratory environment, and as soon as the plants started to germinate, the lids of the dishes had to be removed to make room for the plants. Lack of light resulted in elongation, and fungal contamination disturbed the samples. *B. subtilis* received from University of Nairobi was first tested, and added to the wells after the seeds had germinated. Germination of the seeds was uneven; while some plants had already grown a few centimeters, others had just started to germinate. When results could be expected, the agar had already evaporated and dried out and the plants could not survive. Because of the different disturbances, no results could be read out of the first trial of the experiment.

When species A was tested, it was added simultaneously as *F. oxysporum*, which was one day after the seeds were placed on the agar. The multiwell dishes were from the beginning of the experiment placed next to a window to receive more sunlight. One week after adding bacteria and pathogen, germination rate and growth rate were observed (Figure 6). The data analysis for the first trial of species A, showed that the treatment T had significantly higher growth rate compared to T+F (P-value 0.039). There were no significant difference between any of the other treatments; however, the treatments with B (T+B and T+B+F) had a higher growth rate than the treatment T+F.

The laboratory experiment was furthermore repeated with species A as well as with *B. subtilis* (Figure 7 and Figure 8). For species A, the growth rate was significantly higher in the treatment T+B compared to T+B+F (P-value<0.0001), as well as in T compared to T+B+F (P-value 0.001). Furthermore, the growth rate was significantly higher in T compared to T+F (P-value 0.003) and in T+B compared to T+F (P-value 0.001) (Figure 7). Additionally, the data analysis showed similar results for *B. subtilis.* T+Bs+F had significantly lower growth rate than T+Bs (P-value<0.0001) as well as compared to T (P-value 0.001). Furthermore, the growth rate was significantly higher in T compared to T+F (P-value 0.003) and in T+Bs compared to T+F (P-value 0.001) (Figure 8). There was no significant difference in the germination rate between the different treatments in any of the trials (Figure 6, Figure 7 and Figure 8).

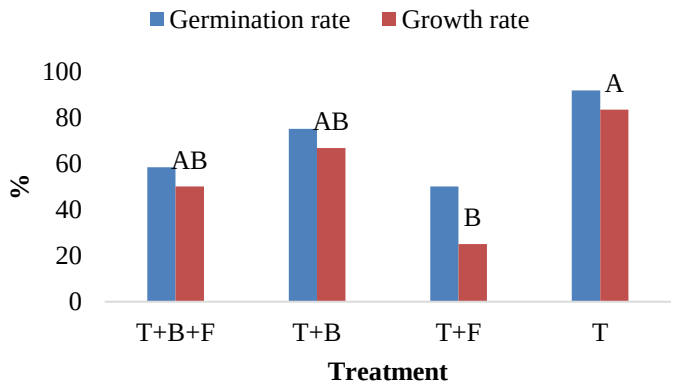

Figure 6. Germination rate (% of added seeds) and growth rate (% sown seeds resulting in viable plants) of tomatoes in trial 1, bacteria species A. Different letters indicate significance between treatments regarding growth rate. Each treatment consisting of 3 replicates (T=Tomatoes, B=Bacteria, F=*F. oxysporum*).

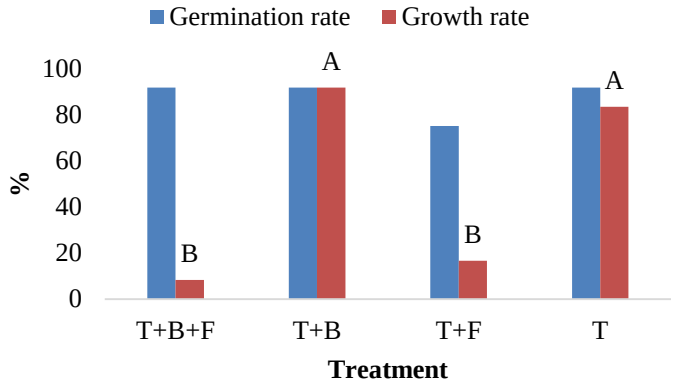

Figure 7. Germination rate and growth rate (see figure 6) of tomatoes in trial 2, bacteria species A. Different letters indicate significance between treatments regarding growth rate. Each treatment consisting of 3 replicates (T=Tomatoes, B=Bacteria, F=*F. oxysporum*).

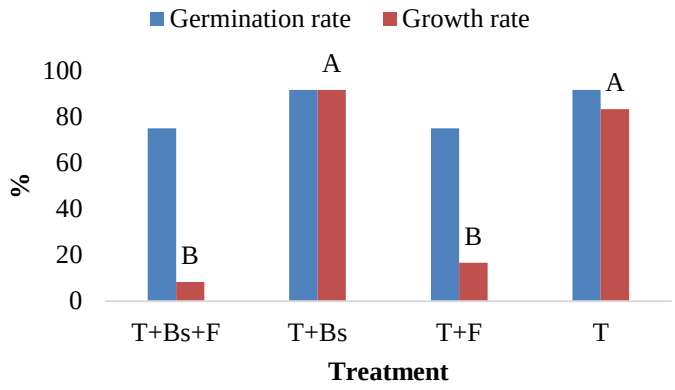

Figure 8. Germination rate and growth rate (see figure 6) of tomatoes in trial 2, *B. subtilis.* Different letters indicate significance between treatments regarding growth rate. Each treatment consisting of 3 replicates (T=Tomatoes, Bs=*B. subtilis*, F=*F. oxysporum*).

The infected plants did not show any different symptoms such as chlorosis compared to the uninfected plants. However, *F. oxysporum* was growing on the agar surrounding the seeds in the infected wells and lower growth rate could be observed where *F. oxysporum* was present. Figure 9 shows examples of results from the multiwell dishes.

Figure 9. Results from multiwell dishes.

5.3 Mesocosms – interaction experiment

5.3.1 Plant growth

The germination rate of the tomato seed was 91.4%. Plant initial height was measured 16 days after they were sown, as well as in the final stage of the experiment (47 days after sowing).

The plant final average height differed significantly between treatments with and without E (P-value 0.002), where plants were higher in the treatments with E. The difference in height (final average height-initial average height) was significantly higher in treatments with compared to without E (P-value 0.001). Plant final height as well as the difference between final average height and initial average height was also significantly higher in the treatments with T+Bs+F+E compared to T+Bs (P-value 0.036 and 0.011) as well as the treatment with T+Bs+E compared to T+Bs (P-value 0.024 and 0.010). Plant initial height was used as a covariate (Table 5).

Table 5. Initial plant height (16 days after sowing) and final plant height, each treatment consisting of 6 replicates (T=Tomatoes, Bs=*B. subtilis*, F=*F. oxysporum*, E=earthworms) Significant values are in bold. The one-way ANOVA refer to the test between the different treatments while the three-way ANOVA evaluated the factors Bs, E and F. Comparison with Tukey's test

Treatment	Initial Average Height (cm)	Final Average Height (cm)
T+Bs+F+E	7.3 ± 1.2	44.7 ± 3.2 ^{A}
T+Bs+E	7.6 ± 1.2	45.1 ± 4.7 ^{A}
T+F+E	7.7 ± 1.2	42.7 ± 3.0 ^{AB}
T+Bs+F	8.2 ± 1.3	42.6 ± 4.1 ^{AB}
T+E	7.3 ± 1.2	42.8 ± 4.4 ^{AB}
T+Bs	8.7 ± 1.3	41.7 ± 3.5 ^{B}
T+F	7.3 ± 1.0	41.5 ± 2.9 ^{AB}
T	8.1 ± 1.0	42.7 ± 4.1 ^{AB}
P-value		
One-way ANOVA		**0.012**
Three-way ANOVA		
Bs		0.147
E		**0.002**
F		0.955

The dry weight (Figure 10) was significantly higher in treatments with compared to without Bs (P-value 0.041) and significantly lower in treatments with compared to without F (P-value 0.020) (Table 6). There was no significant difference between the treatments as such.

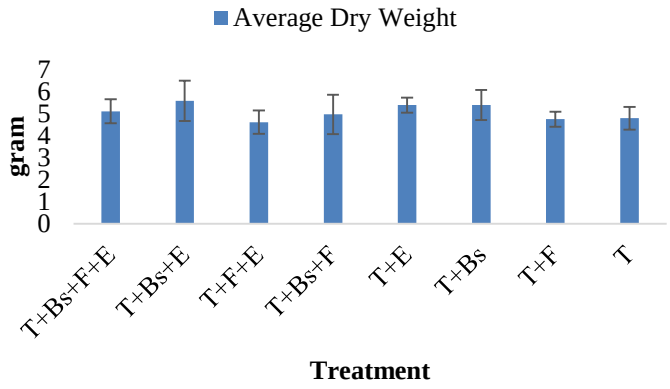

Figure 10. Final tomato plant dry weight, each treatment consisting of 6 replicates with 4 plants per replicate (T=Tomatoes, Bs=*B. subtilis*, F=*F. oxysporum*, E=earthworms).

Table 6. Statistical analysis on tomato dry weight. Impact of the different factors (Bs=*B. subtilis*, E=Earthworms, F=*F. oxysporum*). Significant values are in bold. The one-way ANOVA refer to the test between the different treatments while the three-way ANOVA evaluated the factors Bs, E and F

Factors	P-value
P-value	
One-way ANOVA	0.102
Three-way ANOVA	
Bs	**0.041**
E	0.282
F	**0.020**

Plant wet weight, moisture content and relative growth (height) are presented in appendix 1, 2 and 3.

5.3.2 Earthworm biomass

Only one day after weighing and putting earthworms in the mesocosms, ants were found in one of the mesocosms in treatment 1, feeding on at least two earthworms. The dead earthworms were removed to avoid more ants from coming, and the ants that could be found were removed. Afterwards, naphthalene balls were placed on the table in the greenhouse to repel insects. Weight data and mortality rate are presented in table 7. No significant difference in final average biomass or relative growth could be seen for the earthworms in the end of the experiment (Table 7). An effect can be seen from the presence of *F. oxysporum* on earthworm mortality rate and final weight, although not a significant difference.

Table 7. Earthworm average individual initial and final fresh weight, each treatment consisting of 6 replicates (T=Tomatoes, Bs=*B. subtilis*, F=*F. oxysporum*, E=earthworms). The one-way ANOVA refer to the test between the different treatments while the two-way ANOVA evaluated the factors Bs and F

Treatment	Average Initial weight (g)	Average Final Weight day (g)	Relative Average change (%)	Mortality (%)
T+Bs+F+E	0.39 ± 0.16	0.72 ± 0.32	84.6	44.4
T+Bs+E	0.35 ± 0.23	0.51 ± 0.29	45.3	50.0
T+F+E	0.42 ± 0.18	0.59 ± 0.23	41.7	16.7
T+E	0.28 ± 0.09	0.40 ± 0.10	41.1	66.7
P-value				
One-way ANOVA		0.237	0.302	0.109
Two-way ANOVA				
Bs		0.275	0.686	0.700
F		0.066	0.086	0.065

5.3.3 Fusarium symptoms

One week after infection, early symptoms were visible in some of the infected plants. The symptoms were mostly discolored leaves. For each plant, total number of leaves, number of leaves with chlorosis and other discoloring (mainly brown), curly leaves and leaves with discolored veins were noted. This was done for all treatments to determine if there was a difference between Fusarium treated and not-treated plants. Furthermore, a percentage of the total infection of chlorosis and brown discoloring of the plants was estimated, based on the scoring points 0-5 (Table 8) Example of symptoms can be seen in Figure 11.

The total number of leaves did not differ significantly between the treatments, although there were more leaves in the treatment T+Bs+E compared to T+F+E, but the difference was not significant. Furthermore, there were significantly more leaves in the treatments without compared to with F (P-value 0.007).

The percentage infected leaves did not differ significantly between treatments; neither did the estimated percentage chlorosis on the plants. However, there were more symptoms in the treatment with T+F+E as well as the treatment with T+E compared to the treatment with T+Bs+E, though not significantly more. Furthermore, the treatment with T+E had more symptoms than the control treatment with only plants. The discolored veins were significantly more abundant in the treatments with Bs compared to the treatments without (P-value 0.011). There were more folded leaves in the treatments with compared to without Bs, although the difference was not significant (P-value 0.054). Graphs of *Fusarium* infection are presented in appendix 4 and 5.

Figure 11. Leaves with brown discoloring (left) and plant with some chlorosis (right).

Table 8. Disease analysis. Number of leaves is an average per treatment, infected leaves is a percentage infected leaves of total number of leaves per treatment, brownish discoloring and chlorosis are percentage estimation of a plant presented as an average per treatment, discolored veins and folded leaves are percentage occurrence per treatment. Each treatment consisting of 6 repetitions with 4 plants per repetition (T=Tomatoes, Bs=*B. subtilis*, F=*F. oxysporum*, E=earthworms). Significant values are in bold. The one-way ANOVA refer to the test between the different treatments while the three-way ANOVA evaluated the factors Bs, E and F

Mean value						
Treatment	Number of leaves	Infected leaves (%)	Brown discoloring (%)	Chlorosis (%)	Discolored veins (%)	Folded leaves (%)
T+Bs+F+E	44.9	24.6	2.9	8.3	0.5	0.2
T+Bs+E	50.3	20.3	1.3	6.2	0.5	0.3
T+F+E	40.3	24.1	1.9	9.4	1.0	0.2
T+Bs+F	42.1	22.6	1.5	8.1	1.0	0.3
T+E	47.8	23.3	1.7	10.0	1.0	0.3
T+Bs	48.5	21.7	2.2	7.6	1.0	0.4
T+F	43.2	19.7	1.0	7.8	1.0	0.1
T	42.8	21.5	1.9	6.4	1.0	0.0
P-value						
One-way ANOVA	0.054	0.682	0.285	0.107	0.078	0.205
Three-way ANOVA						
Bs	0.087	0.917	0.356	0.221	**0.011**	0.054
E	0.328	0.256	0.449	0.165	0.083	0.694
F	**0.007**	0.479	0.887	0.239	0.128	0.360

5.4 Toxicity test

Earthworm weight was noted in the beginning as well as in the end (after 19 days) of the experiment (Table 9). At the end of the experiment, four earthworms were missing, two from each treatment and from different mesocosms. Two of them were found dead outside the mesocosms. The water content had been kept relatively constant.

At the end of the experiment, the biomass of E+W was larger, however, the initial average weight was also larger for that group. The relative average changes in biomass as well as the difference in growth (final average weight-initial average weight) were larger for the treatment E+W compared to E+Bs although not significantly different (Table 9).

Table 9. Earthworms subjected to *B. subtilis*, initial and final weight, each treatment consisting of 6 replicates (E=Earthworms, Bs=*B. subtilis*, W=water). No significant differences were found between the treatments. The one-way ANOVA refer to the test between the different treatments

Treatment	Average Initial Weight (g)	Average Weight day 19 (g)	Relative Average change (%)
E+Bs	0.60 ± 0.23	0.96 ± 0.38	58.78
E+W	0.71 ± 0.16	1.21 ± 0.24	71.75
P-value *One-way ANOVA*		0.129	0.218

6 Discussion

These experiments aimed to isolate BCA effective against Fusarium wilt, from Fusarium wilt suppressive soils. The soil sampling took place where there was no occurrence of Fusarium wilt, and in those soil samples according to the preliminary identification, *B. subtilis* was found – here called "species A". *B. subtilis* is known as a potential BCA (Almoneafy et al. 2012; Romero et al. 2004). Species A was relatively easy to find and to isolate, which is an advantage when looking for bacteria for potential biocontrol products. The aim was furthermore to test the bacteria on plants and in combination with a pathogen and earthworms, which was done in three different experiments. The isolated species A was tested on plants and against Fusarium wilt, though no tests were performed on soil fauna due to time limitations. Instead, already isolated *B. subtilis* received from the University of Nairobi was used for the toxicity test on soil fauna as well as in the interaction experiment with all combinations of bacteria, *F. oxysporum*, earthworms and plants. The three experiments intended to complement each other, to cover as many aspects and interactions as possible.

6.1 Isolation of bacteria

When isolating bacteria, only one species was found. There are certainly more species present in the soil, for example Gopalakrishnan et al. (2011) found 360 species when sampling rhizosphere soil, still, a large amount of species were probably not found (Pham & Kim 2012). Bacteria are selective to nutrient media as well as incubation temperatures. In this experiment, LBA was used as nutrient media as well as one incubation temperature, while Gopalakrishnan et al. (2011) used PDA as nutrient media and a higher temperature. Additionally, slightly different isolation methods were used which may give different results. In studies where more time is available, a suggestion would be to use different kinds of nutrient media as well as different temperature regimes to obtain as many species as possible. Perhaps the results might vary if doing the serial dilution in distilled water compared to a physiological salt solution. In this study, distilled water was used. Furthermore, soil sampling can be done in more sampling spots to obtain more variations, whereas in this study, only one soil sample at the time was used for serial dilution. However, the first hypothesis, which said that BCA are present in the root zone of a disease suppressive soil is confirmed. Furthermore, the aim of

this study was to find BCA-bacteria while no specific species was targeted, which is why sampling and serial dilution only were performed until a species that could be tested was obtained.

6.2 Laboratory testing of bacteria

For the first trial in the multiwell dishes, *B. subtilis* was tested on tomato seedlings in combination with *F. oxysporum*. The seeds were added to the MSA prior to inoculation of *B. subtilis* and infection of *F. oxysporum*, which in this environment proved challenging. No growth chamber was available; the experiments were kept in laboratory environment making them prone to different contaminations. Fungi other than *F. oxysporum* were growing in the wells and evaporation of the agar took place. The plants suffered from lack of light which resulted in elongation. Furthermore, when letting the plants pre-grow, they ended up being too large for the wells and after such time when results from the treatments were expected, the plants had already suffered from other conditions. When exposing tomato plants in laboratory environment to the isolated bacteria species A and *F. oxysporum*, the method was slightly changed to avoid some of the challenges as of the first trial. This time, the bacteria and pathogen were added one day after the seeds were placed in the wells, not letting the seeds pre-grow. The growth rate was slightly lower for the treatment containing only bacteria compared to the control, thus, no plant growth promoting effects could be observed in this trial, as could be seen for example in a study by Almoneafy et al. (2012). However, in treatments with both bacteria and *F. oxysporum*, the growth rate was higher compared to the treatment only having *F. oxysporum*. This suggests that the bacteria have an antagonistic effect against *F. oxysporum*. No symptoms of *F. oxysporum* could be observed on the plants during this period of one week; the period was probably too short for the plants to develop visible disease symptoms. Although, since the growth rate of the infected plants without bacteria was low it is likely that infection took place. Additionally, the mycelia of *F. oxysporum* could be seen in the wells, showing that the fungus was present. However, the growth rate was low even for the control plants, suggesting other disturbances as mentioned earlier.

In the second trial that could be done without most of the difficulties as described above, the bacteria did not seem to have any disease suppressive effects on *F. oxysporum* neither for species A nor *B. subtilis*. This is in contrast to previous studies of *B. subtilis* (Bhattacharyya & Jha 2012; Cavaglieri et al. 2005; Hariprasad et al. 2011; Shoda 1996; Wepuhkhulu et al. 2011), and also compared to the results from the first trial. During the period when the experiments were conducted, weather conditions changed and the temperatures got cooler, conditions that may explain some of the variations of the results. Perhaps the growth rate of the bacteria slowed down due to the temperature drops. Additionally, for the first trial, the bacterial solution was recently prepared while for the second trial, it had been stored for approximately two weeks. As stated by Lucas (2011) and Lucy et al. (2004), inconsistent results are an issue in biological plant protection. In this study, perhaps different concentrations or volumes of the bacteria would give other results, since only one concentration and volume were used,

especially because the results of the first trial with species A suggests that the BCA had some effect against the pathogen. *B. subtilis* is known as being easy to formulate as a product as well as easy to store (Emmert & Handelsman 1999; Shoda 2000). It would therefore be interesting to know if the storage time affected the results in this study. The solution was only stored for approximately two weeks, and if a product of *B. subtilis* is to be out on the market, it is desired that the product can be stored for a longer time period than that. With appropriate storage, there should not be any effect on a spore solution (Meijer, personal communication), nevertheless, something did affect the results in this study. There might always be different factors affecting the results, but perhaps it would be possible to repeat the experiment using one newly made bacterial solution and simultaneously use a stored solution to evaluate if that was the issue. Depending on those results, research on how to formulate a product in a way allowing it to be stored could be performed. As also mentioned, the temperatures changed and the season went from dry to rainy season, with temperature drops as a result. This could have been controlled if the experiments would have been kept in a growth chamber, although that was not possible at this time. However, when applying a product in the field the temperatures will vary, and the product has to be reliable even when the weather changes.

6.3 Interaction experiment

Even though all of the plants showed symptoms such as chlorosis on the leaves, other differences could be observed by eye, such as the tone of the green color as well as the size of the plants between the different treatments. The final average height was higher in treatments containing earthworms compared to the treatments without. Furthermore, analyzing the final plant height using plant initial height as a covariate showed that the height was significantly lower in the treatment with only *B. subtilis* compared to the treatment with *B. subtilis, F. oxysporum* and earthworms and also compared to the treatment with *B. subtilis* and earthworms. This makes it interesting to evaluate if the combined effect of *B. subtilis* and earthworms have a greater positive effect together than with the bacteria or earthworms alone. The wet weight (Appendix 1) as well as moisture content (Appendix 2) also differed between the treatments, where *F. oxysporum* tended to make the wet weight and moisture content lower and *B. subtilis* and earthworms seemed to make it higher. The dry weight did not differ significantly between the different treatments, still, it was significantly higher where *B. subtilis* was present and significantly lower where *F. oxysporum* was present. These results are partly in contrary to the results of Almoneafy et al. (2012) who showed an increase in plant height of tomatoes when treated with *B. subtilis* compared to the control. However, Almoneafy et al. (2012) also showed an increase in dry biomass, which could be observed in this study. Furthermore, plants in this study lacking *F. oxysporum* had more leaves. Perhaps plants treated with *B. subtilis* could put more energy producing biomass while the plants without *B. subtilis* used its energy for gaining height instead, making them higher but not increasing their biomass.

In this study, the mortality rate of the earthworms was high; the conclusions drawn by their presence should thus be interpreted with care. It is difficult to say if the mortality rate was randomly occurring between the treatments or if there was a reason behind. It appeared that the mortality rate was highest in the treatments without *F. oxysporum*. There are studies showing that earthworms favor *Fusarium* species as a food source (Bonkowski et al. 2000; Wolfarth et al. 2011). One reflection concerning the mortality in this study might therefore be the presence of *Fusarium.* The earthworms in the treatments with *F. oxysporum* had an extra source of food compared to the earthworms in the other treatments, perhaps favoring their environment. This could also be an explanation why the earthworms in the treatments with *F. oxysporum* were close to significantly larger than the earthworms in the other treatments, still, the mortality rate was high (67% in one treatment) and thus it might not be statistically correct to compare them. It would however be interesting to further investigate the effect of *F. oxysporum* on earthworms and to in more detail study their interactions.

As far as could be seen, two earthworms were killed by ants, but ants could not be observed in any other mesocosms after that incident. It also proved challenging to keep the soil moist enough, in the end of the experiment it appeared to be very dry, and according to Lavelle et al. (1987), *P. corethrurus* like relatively wet environments, preferably a soil moisture content of about 50%. In the beginning of the experiment the moisture content was approximately 40-50% while it was declining to approximately 20-30% in the end of the experiment. When watering, just enough water was added not to make it leach from the bottom to avoid the bacteria to be leached out. Though, perhaps the water did not infiltrate the soil but was going through the sides of the mesocosms, thus leaching. However, after the experiment was completed it came to our knowledge that root colonizing bacteria don't leach to a great extent (Meijer, personal communication), making this concern unjustified in this case, which is useful knowledge when repeating the experiment. The soil was very crowded with roots from the tomato plants in the end of the experiment, making the space limited for the earthworms. For future experiments, it might be a good idea to put plates below each mesocosm, thus the leached water will not be lost, perhaps keeping the soil more moist. Furthermore, larger mesocosms can be used to avoid the crowded environment, since tomato plants are relatively large.

Plants infected with *F. oxysporum* had significantly less leaves than plants without *F. oxysporum*. However, symptoms such as discolored veins occurred more often where *B. subtilis* as well as earthworms were present compared to without. Therefore, it seems like these symptoms might appear of other reasons than *F. oxysporum*. Perhaps other disturbances occurred in those treatments or perhaps the bacteria and earthworms themselves caused those symptoms. In fact, it was not possible to totally exclude insects from the greenhouse. Fusarium wilt has a number of ways of spreading (FAO 2015b), making it possible that it spread between the different treatments, even though care was taken during the experiment not to contaminate between the treatments. Furthermore, Whiteflies was present on the plants which are feeding on plant leaves. They are causing symptoms such as yel-

lowing and curl on the leaves (UC IPM 2014), that is similar symptoms as *F. oxysporum* gives. Furthermore, the larvae of the leaf-mining fly are causing symptoms as the discolored veins (Byers 2006). Perhaps it would be possible to prevent disturbances such as insects using an insecticide, although, since the aim was to test a BCA another treatment could interfere with the results. Thus, in this case, only the naphthalene balls were placed in the greenhouse to repel the insects by the smell, but nothing was added inside the mesocosms. The statistical analysis of discolored veins and curled leaves was performed using General Linear Model. Perhaps other statistical analysis would have been preferable in this case where only the occurrence or presence of the symptoms were noted.

In this experiment, effects could be seen from the application of *B. subtilis* where for example the dry biomass was larger were the bacteria was present. As for the multiwell experiment, it would be interesting here as well to try different volumes and concentrations of the bacterial solution. It would also be interesting to try combinations of PGPR to evaluate if a mixture gives more protection and growth-promoting effects since there are many microorganisms present in the soil (Gopalakrishnan et al. 2011). Studies have showed that a mixture of PGPRs tend to enhance plant growth and disease suppression, and give a more stable protection compared to when only one species of PGPR is used (Raupach & Kloepper 1998). It might be more realistic than only using one species of bacteria. Furthermore, the bacteria in this experiment were added about one week after the infection of *F. oxysporum*, perhaps it could have been added earlier, such as in the beginning of the experiment, or as a seed coating for the plant to be able to develop the antagonistic mechanisms prior to the infection of the pathogen. The plants were relatively old when the infection of *F. oxysporum* and adding of bacteria took place, which might have made them less vulnerable and susceptible to the treatments.

6.4 Toxicity test

There are studies showing the effect of earthworms on the microbial community (Brown 1995), but only a few studies have focused on the interactions and potential effect of bacteria on non-target organisms. The aim of this experiment was to investigate the effect of *B. subtilis* on earthworms native to the soils in Kenya. There are not many effective methods available for control of Fusarium wilt (PAN Germany, OISAT 2005), although, species of *Bacillus* have proved promising as BCAs (Shanmugam et al. 2011). Species of *Bacillus* produce chitinases, which is one mechanism behind its antagonistic effect against fungal diseases (Shanmugam et al. 2011). However, this might negatively affect earthworms which in their cuticle and cetae have chitin (Laverack & Kerkut 1963). Thus, it is interesting to investigate the effect of *B. subtilis* on earthworms prior to extended use of the BCA on crops in agricultural land. Lagerlöf et al. (unpublised) showed that *B. amyloliquefaciens* did not have any effect on the earthworm *A. caliginosa* and *Apporectodea longa*. However, studies of *B. subtilis* on soil fauna are sparse. Earthworms such as *P. corethrurus* are geophagous, thus consuming large quantities of soil. They are furthermore commonly inhabiting human disturbed areas, for example croplands (Lavelle et al. 1987), making them exposed to external inputs from the

agriculture, both due to their movements through the soil and through consumption of soil. It is therefore important to evaluate the potential effects of the BCA on them. In this experiment, the earthworms were not negatively affected by the exposure of *B. subtilis*, which was measured as effect on their growth rate. There was no significant difference in the weight increase of earthworms in the different groups. However, the earthworms dipped in distilled water had increased more in biomass, although not significantly more, yet it would be interesting to further investigate if this is because of *B. subtilis* or other factors. A repetition of the experiment with different concentrations of the bacterial solution could indicate if *B. subtilis* have a negative effect. The time period for the experiment could also be expanded, and perhaps more weighing and/or dipping occasions could be used. Nevertheless, the results obtained are in accordance with the second hypothesis which said that BCA native to the soil will not affect the earthworms negatively. Furthermore, as for the interaction experiment it would be interesting and potentially more realistic, to repeat this experiment but with a mixture of PGPRs. Thus the combined effect of the bacteria would be evaluated on earthworms.

6.5 Reflections

As combined results for the three experiments, it seems like the *B. subtilis* strain tested generally has a positive effect on plants, for example *B. subtilis* treated plants had a larger dry weight in the interaction experiment. Although, the results are inconsistent, and reasons behind that would be interesting to investigate. Perhaps the bacteria would work more efficient in combination with other PGPRs, as have been showed in previous studies (Raupach & Kloepper 1998). However, Raupach & Kloepper (1998) also argue that mixtures can be unfavorable to biocontrol, and that the effect can vary in different settings and hosts. Furthermore, even if one BCA is effective against pathogens or is promoting plant growth in one plant species, it might not work for other plant hosts and on other pathogens (Lucy et al. 2004). It is thus important to investigate the effects on different plants prior to application.

Due to lack of time, no analysis was made on the soil and/or roots of the tomatoes to see whether the BCA was still present in the mesocosms in the end of the experiment. Otherwise, it would have been interesting to know if there was a decline of the BCA after the inoculation as discussed by Lucy et al. (2004), or if the concentration was stable, or possibly increasing. Furthermore, it would be interesting to know if the microbial community changed with time and in that case, if it was different between the different treatments – it could for example be expected that it would be different in the presence of earthworms (Brown 1995) since they tend to improve the environment for microorganisms. It seems like *B. subtilis*, with some adjustments of methods when applying it on crops, have potential to work as a BCA. Tendencies of plant growth promoting and disease suppressive abilities can be seen, and if finding ways of making it more stable and reliable, it could be a good crop protection product. Since it was relatively easy to isolate and make a useful solution of, it can hopefully result in a relatively cheap product that can be readily available for small-scale farmers in SSA.

7 Conclusions

According to the three experiments performed in this study, the BCA *B. subtilis* tends to have a plant growth promoting effect, even though the results are inconsistent. It also shows antagonistic effects against the pathogen *F. oxysporum* on tomatoes. *B. subtilis* is furthermore harmless to earthworms of the species *P. corethrurus*, a common earthworm in the humid tropics. Further experiments evaluating the combined effect of *B. subtilis* and earthworms on plants and the pathogen would be interesting since the earthworm mortality was high in the interaction experiment. However, according to the results of the toxicity experiment, it does not seem like the mortality was caused by the bacteria. These results together with future results from similar studies will be of importance for the development of biological control measures in the agriculture, especially in small-scale agriculture in SSA.

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Appendix

1.

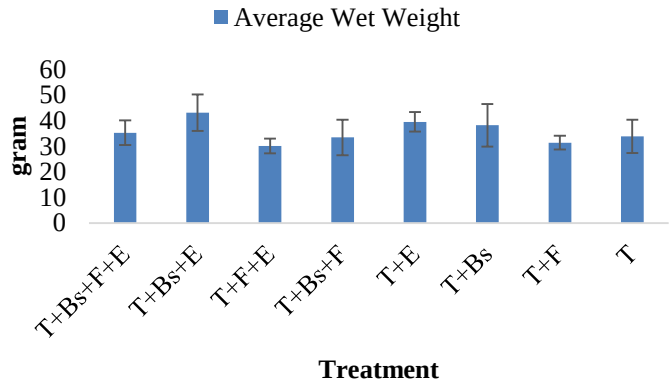

Figure 12. Average wet weight of plants in the interaction experiment

2.

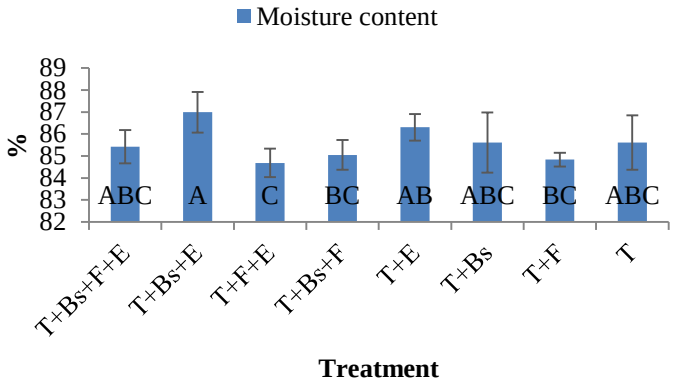

Figure 13. Moisture content of plants in the interaction experiment

3.

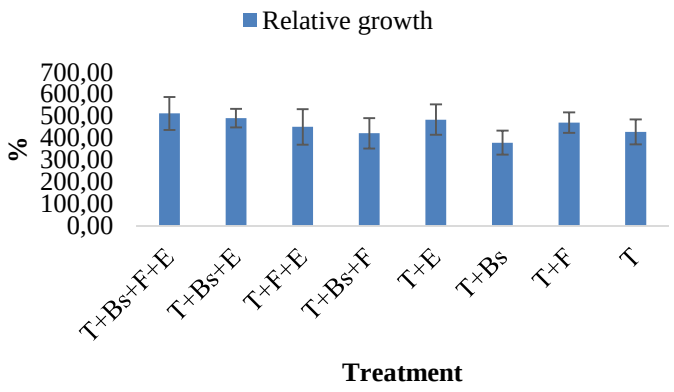

Figure 14. Relative growth (height) of plants in the interaction experiment

4.

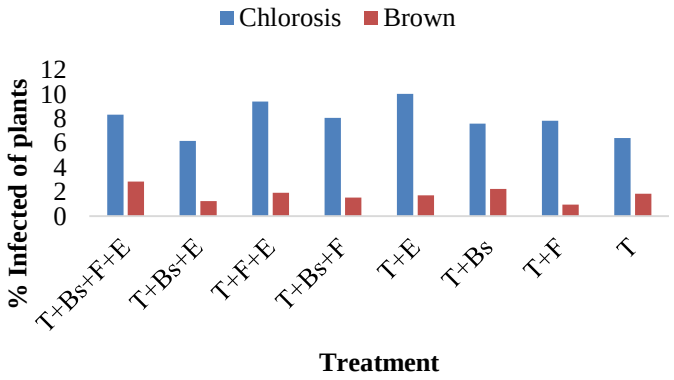

Figure 15. Fusarium infection, chlorosis and brown discoloring

5.

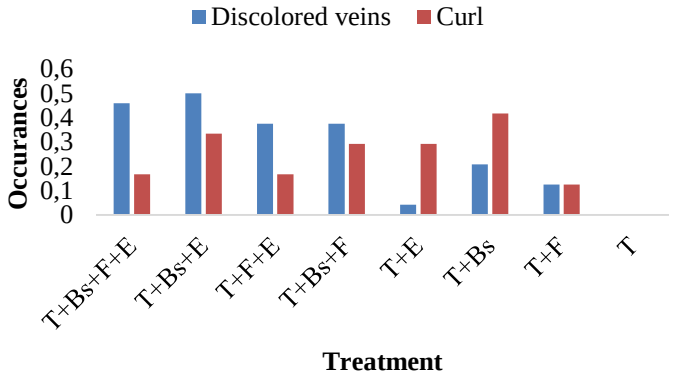

Figure 16. Fusarium infection, discolored veins and curly leaves