Potential of Biological Control for Tomato Complex Disease by Root-knot Nematode and *Fusarium oxysporum* f.sp. *lycopersici* using Entomopathogenic *Bacillus thuringiensis*

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QI JIAHELING

Bacillus thuringiensis を用いたサツマイモネコブセンチュウとトマト萎凋病による複合病の生物防除

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岩手大学大学院連合農学研究科

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（帯広畜産大学）

斉　佳鶴玲

Potential of Biological Control for Tomato Complex Disease by Root-knot Nematode and *Fusarium oxysporum* f.sp. *lycopersici* using Entomopathogenic *Bacillus thuringiensis*

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By

QI JIAHELING

United Graduated School of Agricultural Sciences

Graduate School of Iwate University

(Obihiro University of Agriculture and Veterinary Medicine)

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ACADEMIC ADVISORS

Dr. Daigo Aiuch

Obihiro University of

Agricultural and Veterinary Medicine

Hokkaido, Japan

Prof. Dr. Shu Hase

Yamagata University

Yamagata , Japan

Prof. Dr. Masanori Koike

Obihiro University of

Agricultural and Veterinary Medicine

Hokkaido, Japan

DECLARATION

I hereby declare that the dissertation entitled "Potential of biological control for tomato complex disease by root-knot nematode and *Fusarium oxysporum* f.sp. *lycopersici* using entomopathogenic *Bacillus thuringiensis*" for the degree of Doctor of Philosophy was compiled from the research works during 2014 April to 2017 March with my best ability and effort under the supervision of my academic advisors, wherein it was mainly guided by Prof. Dr. Masanori KOIKE, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Obihiro, 080-8555, Japan.

QI JIAHELING

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CONTENTS

要旨

トマトの栽培において、トマト萎凋病（植物病害）とネコブセンチュウ（植物寄生性線虫）による複合病は世界的に非常に問題になっており、大きな経済損失をもたらしている。現在、この複合病に対する防除方法は土壌消毒を中心とした化学的防除法や抵抗性品種を用いた方法等が実施されている。しかし、抵抗性を侵す新レースの発生や化学薬剤に依存しない環境保全型農業が求められており、代替防除技術の提案が必要とされている。そこで、本研究では *Bacillus thuringiensis*（以下 BT)を微生物防除資材をとして利用し、複合病の原因となるトマト萎凋病菌（*Fusarium oxysporum* f.sp. *lycospersici* 以下 FOL）とサツマイモネコブセンチュウ（*Meloidogyne incognita* 以下 RKN）をターゲットとし実験を計画した。

BT は自然環境から分離されており、人体や環境に安全かつ効果的な微生物殺虫剤で、世界で一番利用されている。最近、BT が害虫だけでなく線虫や土壌病害の被害を抑制する報告もなされている。このため、本研究では、BT のこれらの特性を生かし、植物の病害と植物寄生性線虫を同時に抑制する生物防除資材としての可能性を探ることを目的とし、以下の実験を計画した。

すなわち、実験1では BT によるトマト萎凋病菌の拮抗作用、ポット試験による発病

抑制効果と植物成長促進効果(PGPR)について検討した。次に、実験2では実験1による萎凋病抑制効果のメカニズムの一端を解明するため、BT の植物根面へのバイオフィルム形成能力を調査した。さらに、実験3ではBTのネコブセンチュウに対する殺線虫効果について検討した。最後に実験4では以上3つの実験結果を踏まえ、BT をもちいて、トマト萎凋病とサツマイモネコブセンチュウの複合病に対する防除効果をポット試験によりその防除効果の可能性を探った。

実験1:BT によるトマト萎凋病菌の拮抗作用、ポット試験による発病抑制効果と植物成長促進効果(PGPR)

FOL に対する BT の対峙培養では、*B. thuringiensis japonensis* (BT17)と *B. thuringiensis kurstaki* (BT18)の 2 系統が FOL に対して強い拮抗作用を示した。また、BT を育苗ポットに混和し生育させたトマト苗を FOL 汚染土に移植し、4週間後にトマト萎凋病の外部病徴を調査したところ、BT 処理区すべてに発病抑制効果が認められ、特に BT 17 と *B. thuringiensis CR371-H* (BT20)処理区の効果が顕著であった。これらの結果から、BT にはいくつかの系統でトマト萎凋病原菌に対する拮抗作用が認められ、ポット試験においても BT を根部に処理することにより萎凋病の発病を抑制することが明らかになったが、BT20 は FOL に対する拮抗性が認められなかったのでその効果は誘導抵抗性によるもの推察された。また、トマトの種子や苗に対する BT の PGPR

(Plant Growth Promote Rhizobacteria)としての効果を栄養細胞および培養ろ液を用いてロールタオル法により検討したところ、すべての系統において発芽促進(対照区に比べ 35.7～107.1%)、および幼苗伸長効果(87.7～381.1%)が認められ、これらの効果も発病を抑制する役割の一部を担っていると考えられた。

実験2:BT によるバイオフィルム形成能

BT6系統 *B. thuringiensis sotto* (BT15)、*B. thuringiensis israelensis* (BT16)、*B. thuringiensis japonensis* (BT17)、*B. thuringiensis kurstaki* (BT18)、*B. thuringiensis roskildiensis* (BT19)、*B. thuringiensis CR371-H* (BT20)と比較のためエスマルク DF(*B. thuringiensis*)、ジャックポット顆粒水和剤(*B. thuringiensis*)、ボトキラー水和剤(*B. subtilis*)、*B. simplex* モミホープ水和剤(*B. simplex*) を供試した。それぞれの培養液をマイクロプレートに分注し静置培養の後、CV 染色によりバイオフィルム形成量の定量化を行った(Sandrine et al., 2005)。また、土壌および液体培地中において、トマト苗と供試菌株を共存培養し根面のバイオフィルム形成を光学顕微鏡観察で評価した。その結果、静置培養の 1 日目から 2 日目にかけて、全ての菌株でマイクロプレート中の CV 染色量の増加が認められた。染色量の増加はマイクロプレート内のバイオフィルム形成量の増加と考えられた。それ以降は菌株によりバイオフィルム形成量の違いが認められた。次に土壌および液体培地条件下での共存培養し、トマト根面にはBT のコロ

ニー塊が付着しているのが確認された。以上の結果より、トマト根面に対しBTによりバイオフィルムが形成され、そのことがトマト萎凋病を抑制する可能性が示唆された。

実験3:BTのサツマイモネコブセンチュウに対する毒性

サツマイモネコブセンチュウに対するBTの毒性を調査するため、0.1ml2期線虫懸濁液(50J2/well)と0.9ml BTの培養ろ液、菌体(栄養胞子)を24wellマイクロプレートで共存培養した。24時間後、BTの培養ろ液、菌体処理区とも線虫の2期幼虫の致死率は高く、BTに殺線虫効果があることを確認した。さらに、BTと48時間共存培養したネコブセンチュウの卵のうを滅菌水に移し、7日後孵化した2期幼虫をカウントした。また、この卵のうを押しつぶし、中の正常卵と異常卵を数えたうえで、BTの栄養細胞、栄養細胞の代謝産物が線虫の孵化率を抑制することと異常卵の形成率が増加することを明らかにした。

実験4: BTによるトマト萎凋病とサツマイモネコブセンチュウの複合病抑制効果

以上の1～3の結果を踏まえ、BT17、BT18とBT20を選択し実験に供した。表面殺菌したトマト種子を催芽させた後に滅菌土に播種し、25℃温室条件で4週間生育させた。このトマト苗にBT3菌株の懸濁液(3×10^8 cfu/ml)を10mlずつ根圏に接種した。1週間後、5mlサツマイモネコブセンチュウ2期幼虫懸濁液(500 J2s/pot)を植物の根圏に接種した。さらに1週間後、BTとサツマイモネコブセンチュウを接種したトマト苗を

萎凋病原菌汚染土に移植し、4週間後に外部病徴含めた府複合病の被害調査(地上部生重、内部病徴、ネコブ指数)を実施した。実験には 11 処理区を設置した。すなわち、3系統(BT17、BT18、BT20) BT＋萎凋病処理区;3 系統(BT17、BT18、BT20)BT＋ネコブセンチュウ処理区;3 系統(BT17、BT18、BT20)BT＋複合病実験区;萎凋病だけ対照区;ネコブセンチュウ対照区;複合病対照区と無接種対照区を設けた。発病調査の結果、BT 処理区はすべて複合病の病徴を抑えた。

以上の結果から、BT17～20 の 3 系統は難防除病害であるトマトの萎凋病とサツマイモネコブセンチュウの複合病の発病を抑制できる微生物防除資材として有望であることが明らかになった。

Summary

In tomato production, the complex disease caused by Fusarium wilt (plant disease) and root knot nematode (plant parasite nematode) is a severe problem, and it also causes huge economic loss. Soil disinfestation and the utilization of resistant variety have been used in complex disease control. However, because of the development of resistance invaded new race, an alternative control technique is required. Soil disinfestation and the utilization of resistant variety were used in complex disease control. Therefore, this study was estimated to use *Bacillus thuringiensis* as an alternative agent to control the development of complex disease. *B. thuringiensis* is separated from natural environment, and it is an environmentally friendly bio-pesticide which is mostly used worldwide. Recently, several researches reported *B. thuringiensis* could also control the nematode and soil disease. Consequently, the current study was aimed to investigate the possibility of *B. thuringiensis* as a potential biological control agent against plant disease and plant parasite nematode, using four experiments.

EXP.1 clarified the antagonistic activity, wilt disease suppression effect, plant growth promoting rhizobacteria effect of *B. thuringiensis*. EXP.2 identified biofilm formation

ability on tomato root surface by *B. thuringiensis*. EXP.3 clarified toxicity of *B. thuringiensis* to root knot nematode. EXP.4 evaluated biocontrol potential of *B. thuringiensis* against complex disease.

EXP.1: The antagonistic activity of *B. thuringiensis japonensis* (BT17), *B. thuringiensis kurstaki* (BT18) against *Fusarium oxysporum* f.sp. *lycospersici* race2 (FOL) was examined by dual culture technique. Further, *B. thuringiensis* strains can suppress the development of wilt symptoms caused by FOL in tomato plants was confirmed. Inoculate six strains of *B. thuringiensis* suspension to the tomato seedlings in pot, and transplanted the treated tomato seedlings to FOL pollution soil, after 4 weeks the development of wilt symptoms and wilting score become less than control, especially *B. thuringiensis japonensis* (BT17) and *B. thuringiensis CR371-H* (BT20). Also, this study proved that *B. thuringiensis* strains are plant growth promoting rhizobacteria (PGPR) which can promote plant growth and it also can promote seed germination (35.7～107.1% than control) and shoot elongation (87.7～381.1% than control) by treating the tomato seeds with bacterial culture filtrate and bacterial suspension using roll towel method.

EXP.2: 10 under test strains, *B. thuringiensis sotto* (BT15), *B. thuringiensis israelensis*

(BT16), *B. thuringiensis japonensis* (BT17), *B. thuringiensis kurstaki* (BT18), *B. thuringiensis roskildiensis* (BT19), *B. thuringiensis CR371-H* (BT20), *B. thuringiensis* DF (BT DF), *B. thuringiensis GC-91* (BT *GC-91*), *B. subtilis MBI600*, *B. simplex CGF2856* were used in microtiter plate assay to evaluate *B. subtilis* biofilm formation. The plates were stained with crystal violet, and biofilm formation was quantified by measuring the OD_{595} for each well using a spectrophotometer (Sandrine et al., 2005). All tested strains showed a rise in absorbance from the first day to the second day, this result indicated the quantity of biofilm formation was increased. From second day to third day, some strains showed a decrease trend in absorbance. Also, in liquid and soil co-culture experiment, biofilm formations of bacterial strains treated tomato root surfaces were observed using an optical microscope. As a result, colony formations on tomato root surfaces by *B. thuringiensis* were confirmed under both culture conditions. We documented *B. thuringiensis* strains have the ability to colonize and form biofilms on plant root surfaces.

EXP.3: J2 suspensions (0.1 ml) were placed into wells of a 24-well tissue culture plate (50J2/well) containing 0.9 ml of culture filtrate and bacterial suspension. Liquid LB was used as the control. The effect of culture filtrate and bacterial suspension on the viability

of J2 was determined after 24h. *B. thuringiensis* strains significantly increased the percentages of J2 motility compare to untreated control. Also, we inoculated six strains of *B. thuringiensis* on *M. incognita* egg mass by co-culture method for 48h, and removed the egg mass into sterilized water. 7 days after inoculation, counted the quantity of hatched J2. Egg mass was crushed, normal eggs and abnormal egg in the egg mass were calculated. *B. thuringiensis* strains reduced the hatchability of egg compare to the untreated control and increased the abnormal egg formation.

EXP.4: According to the results of EXP.1~3, we choose BT17, BT18, BT20 used in pot experiment. Surface sterilized tomato seeds were grown at 25°C in greenhouse. After 4-week cultivate, tomato seedlings were treated with bacterial culture at 10 ml/pot (3×10^8 cfu/ml). After 7 days, 5 ml J2 suspensions were inoculated around plant roots (500 J2s/pot). After 7 days, the bacteria-nematode treated tomato seedlings were placed in the FOL-infested soil. Four weeks after pathogen inoculation, symptom expressions of the complex disease were evaluated. Eleven experiment groups were set: 3 BT strains (BT17, BT18, BT20) + FOL; 3 BT strains (BT17, BT18, BT20) + nematode; 3 BT strains (BT17, BT18, BT20)+ FOL + nematode; nematode control; FOL control; FOL+ nematode control; distilled water untreated control. As a result, *B. thuringiensis* strains

could significantly suppress the symptoms of complex disease; induce root gall; decrease wilting score and internal symptom; promote shoot length and weight.

This research showed the plant growth promoting effect, biofilm formation ability, nematicidal effect and complex disease suppression ability of *B. thuringiensis*. Overall, results obtained from these serial studies indicate that *B. thuringiensis* could control plant disease and plant parasite nematode effectively, as a potential biological control agent.

CHAPTER I.

GENERAL INTRODUCTION

Tomato (*Solanum lycopersicum*) is an important vegetable crop. In tomato production, a large number of diseases are happened. It is often hampered by vascular wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* (FOL) (W.C. Synder and H.N. Hans) and plant parasite nematode *Meloidogyne incognita*. FOL is a serious fungal pathogen of the tomato plant. And FOL is ubiquitous phytopathogen causing root rot, vascular wilt and damping off in many plant species (Vurro and Gressel, 2006). FOL can cause a huge loss of tomato quantity and quality, and it has the ability to colonize the roots of a large number of fireweeds and to produce resistant spore structures, so it can exist in most soils (Ailton et al., 2007). This wilt disease is soil borne and can persist for many years in the soil even if no host plants are grown. Fusarium wilt does not spread above the ground from plant to plant. Each plant is individually infected when the organism enters the root system (Larkin et al., 1998). This pathogen is difficult to control with traditional methods, such as preplant soil fumigation and other cultural methods, so it is a better way to choose biocontrol to control this disease (Archana et al., 2010).

In general, some effective means of control FOL include disinfection of the soil and planting material by using fungicidal chemicals, crop rotation with non-hosts of the

fungus, or by using resistant cultivars (Ana et al., 1997; Benhamou et al., 1998). Although wilt-resistant cultivars have been available for decades and provide some degree of control, the occurrence and development of new pathogenic races is a continuing problem. Indeed, there are no commercially acceptable tomato cultivars with adequate resistance (Jones et al., 1991). In addition, the resurgent interest in planting heirloom and other susceptible tomato cultivars has increased the incidence of Fusarium wilt. Since the current available control methods, such as chemical control, are either low efficient or difficult to apply, methods to control Fusarium wilts caused by FOL have been studied for a long time (Kotan et al., 2009). The chemical control measures destroy balances in the microbial community, which may cause the loss of abundance beneficial organisms, such as insect natural enemies, and may also lead to the evolution of resistant strains of the pathogen. Breeding resistant plant varieties can be difficult in the absence of dominant genes and development of new races of the pathogen that overcome host resistance (Jetiyanon and Kloepper, 2002). Furthermore, increasingly, there are more restrictions on utilization of fungicides due to public concern about harmful effects on the human health, animal health and environment, and residues in the food. Hence, there is a need for developing attractive management strategies that are

virtually efficient and environmentally safe (Sudhamoy et al., 2009). In contrast, the biological control as an alternative approach to control soil-borne plant diseases has attracted attention (Nagorska et al., 2007).

The other important tomato soil borne disease is plant parasitic nematodes (PPNs). Nematodes are the most abundant multicellular animals on the face of earth. Several hundreds of nematodes species are known to feed on living plants and cause a variety of plant diseases globally (Sikora and Fernandez, 2005). PPNs attack, feed on, and colonize lots of plant species, including potato, tomato, soybean and others, also cause annual crop losses of approximately $125 billion worldwide (Jung and Wyss, 1999; Williamson and Gleason, 2003; Chitwood, 2003). They are very difficult to control because many destructive PPNs are endo-parasites. Since they live underground (mostly live in the roots), this lifestyle can protect them even from chemical treatments (Bird et al., 2003).

Among the PPN, root-knot nematode (RKN) is the most economically important species causing dramatic yield losses to a broad range of crops, in particular vegetables (Trudgill and Blok, 2001). For instance, juveniles of the *M. incognita* penetrate the root and establish a permanent feeding site in different parts of the root (Wyss et al., 1992).

Juveniles stay in the feeding sites and take in nutrients from the plant tissues. After molts, juveniles develop into adults with a morphology change (Atkinson et al., 1996). The females have an egg-sac and lay eggs on the surface of roots, whereas the males move out of the roots (Williamson et al., 2003; Wyss et al., 1992). Large numbers of galls are visible of RKN-colonized plant roots, which interfere with the uptake of water and nutrients by the root system (Milligan et al., 1998). The lack of water and nutrients subsequently reduces the growth and overall health of the plant, increasing their sensitivity to other diseases (Castaagnone et al., 1992). Three species of root-knot nematode, *Meloidogyne arenaria, Meloidogyne javanica* and *Meloidogyne incognita*, are considered the most important on the basis of their geographical distribution all over the world and their large host range (Sassser et al., 1982). And the most widespread species in this genus is *M. incognita*. They are also involved with bacteria and fungi in many complex diseases and can also break down plant resistance system to pathogens (Taylor and Brown, 1976).

A number of methods for the management of root-knot nematode such as resistant varieties, soil solarization, chemical control, and biological control have been tried with different levels of successes to protect of tomato plants (Randhawa et al., 2001; Sakhuja

and Jain, 2001). Chemical management is effective, but expensive and these compounds tend to be highly toxic to people and the environment, it may lead to residue and soil pollution problems (El-Alfy et al., 2002). Tomato varieties resistant to root-knot nematode have been developed in some countries, but are not very popular due to their lower yields. An environmentally safe and economically reasonable root-knot nematode control practices needs to be available. Biological control is environmentally friendly and free from residual (Sumeet and Mukerji, 2000). In the past decades, many microorganisms have been extensively studied and show great potential to control root-knot nematodes, such as *Paecilomyces lilacinus* (Kiewnick and Sikora, 2006), *Pseudomonas* spp., *Pasteuria penetrans*, *Bacillus* spp. (Siddiqui and Mahmood, 1999), *Burkholderia cepacia* (Meyer, 2003) and *Arthrobotrys oligospora* (Morton et al., 2004). These microorganisms affect nematodes by various ways (Siddiqui and Mahmood, 1999; Terefe et al., 2009). For example, many bacteria including of *Bacillus*, *Pseudomonas* and *Clostridium* produce toxins that kill nematodes (Wei et al., 2003; Ali et al., 2002; Walia et al., 2000), while a few bacteria such as *Pasteuria penetrans* can directly parasitize nematodes (Chen and Dickson, 1998).

As we described before, root-knot nematodes and Fusarium wilt are soil-borne

plant parasites, both of them can cause severely economic losses worldwide to a broad range of plants. Plant diseases on average are responsible for up to 26% yield loss to global agriculture and sometimes there may be complete crop failure leading to 100% yield loss in a locality or a field (Khan et al., 2009). Fungal pathogens, *Fusarium* spp. and root knot nematodes, *Meloidogyne* spp. can also cause complex diseases in soil. Complex diseases often damage plants more severely and make more difficultly to control the disease than single pathogens alone. Both of the pathogens can damage plants independently, but cause more severely economic losses when they occur together (Bird, 1981; Kappelman et al., 1973; Minton, 1966). Infection of roots by root-knot nematodes makes soil-borne fungi infect with plants easily resulting in the development of root-rot and wilt diseases (Armstrong et al., 1976). Also, plants infected by Fusarium wilt and root-knot nematodes may lead to a breakdown of resistance to the interacting fungus and increase disease severity in susceptible cultivars (De Vay et al., 1997; Francl and Wheeler, 1993; Harris and Ferris, 1991a, b; Uma et al., 1997).

During the last few decades, chemicals have been largely used to control plant disease. However, most of the chemical pesticides are banned because of their adverse effect on human health and environmental and ground water pollution by leaving toxic

residual problem and harm beneficial microorganisms in soil (Stirling, 1991). Because of concern about the quality of the food and environment in recent years, there have been worldwide swings to use economical and environmentally safe methods to control plant diseases. The use of microorganisms offers an attractive alternative to manage plant diseases (Roberts et al., 2005). A number of microorganisms have demonstrated antagonistic activity against plant pathogens. It has been established that *Pseudomonas fluorescens* (Papavizas, 1985; Somasegran and Hoben, 1994; Peighami-Ashnaei et al., 2009) and *Bacillus subtilis* (Mukhopadhyay et al., 1992; Dawar et al., 2010) can effectively control the diseases caused by bacterial wilt diseases such as *Fusarium* spp., *Rhizoctonia* spp., leading to significant increase in the plant growth or yield of the plants. Similarly, the diseases caused by nematodes may also be controlled with *Pochonia chlamydosporia* (Kerry, 2000; Khan, 2008), *P. fluorescens* (Sikora, 1988; Pal et al., 2000; Khan, 2007; Singh et al., 2009) and *B. subtilis* (Khan et al., 2001).

Recently, an effective bio-insecticide prior study also has attracted considerable attention as a biological control agent against plant diseases (Zhou et al., 2008). Moreover, the bacterial wilt (*Ralstonia solanacearum*) disease-suppressing activity of *B. thuringiensis* was examined in tomato plants (Hyakumachi et al., 2013). And intensive

studies of nematicidal effects of *B. thuringiensis* have also been carried out, mainly aimed at development of bacteria effective against economically important parasitic nematodes such as *M. incognita* (Deviddas and Siddiqui Rehberger, 1992; Mahmood, 1995; Mohammed et al., 2008) and *Globodera pallida* (Racke and Sikora, 1992a, b). These prior researches may provide a possibility of *B. thuringiensis* to control the complex disease.

B. thuringiensis has been used as an effective bioinsecticide (Roh et al., 2007; Schnepf et al., 1998). The specificity of *B. thuringiensis* is showed highly beneficial in agricultural biotechnology. Unlike most insecticides, *B. thuringiensis* insecticides are highly toxic against target insects and friendly towards beneficial insects, non-target organisms such as humans and wildlife (Bravo et al., 2011). It is also not harmful to the environment. *B. thuringiensis* has been used as an alternative to chemical pesticides for decades by organic farmers to control insects. At present, *B. thuringiensis* is the only "microbial insecticide" in widespread use (Cherif et al., 2003, 2008; Dong et al., 2002).

The use of microorganisms with the aim of improving plant growth availability is an important practice and necessary for agriculture (Freitas et al., 2007). During the past decades, the use of plant growth promoting rhizobacteria (PGPR) for sustainable

agriculture has increased extremely worldwide. Significant increases in growth and yield of economically important crops in response to inoculation with PGPR have been repeatedly reported (Amara and Dahdoh, 1997; Asghar et al., 2002; Biswas et al., 2000; Bin et al., 2000; Chanway, 1998; Chen et al., 1994; Figueiredo et al., 2008; Gray and Smith, 2005; Gupta et al., 2000; Kloepper et al., 1980; Pan et al., 1999; Seldin et al., 1984; Vessey, 2003; Zhang et al., 1996). PGPR promote plant growth and suppress plant disease by colonizing plant roots, reducing plant pathogen populations in the soil, and maintaining a beneficial effect on plant growth (Jetiyanon et al., 2003). PGPR may increase plant yield by reducing plant pathogen populations in the soil, improving mineral nutrient uptake, phytohormone production, and maintaining a beneficial effect on plant growth (Emmert et al., 1999). PGPR may also interact with a variety of soil microorganisms that are normally present in the rhizosphere, in some cases acting as a biocontrol agent against pathogenic bacteria (Glick, 1995) (Fig. 1-1).

The sporulating gram-positive bacteria *B. subtilis* is a beneficial PGPR, which have also been used successfully as potential biological control agents (BCAs) to control plant disease (Kloepper et al., 2004). Similarly, some studies have also reported that *B. thuringiensis* was successful endophytic colonization in soybean, cotton,

cabbage and so on, even with concomitant production of Cry toxins; the efficient *B. thuringiensis* colonization of cabbage seedlings roots suggests this might be in fact the main route of its penetration in the plant (Argôlo-Filho et al., 2014). *B. thuringiensis* was able to colonize the roots of certain legumes, which resulted in an increase of nodulation and growth of the plants and *B. thuringiensis* produces toxins that can reduce pests or diseases attacks (Mishra et al., 2009). Therefore, the new view is that the insecticide *B. thuringiensis* can be used as PGPR to control plant disease.

PGPR can protect against fungal pathogen attack, promote plant growth, and play a role in the degradation of organic polymers in the soil (Archana et al., 2010; Betz et al., 2000). One of the mechanisms of PGPR protect plant from fungal pathogen attack is biofilm formation. Inoculate *B. subtilis* strains to Arabidopsis root which infected by *Pseudomonas syringae*, the mortality of Arabidopsis was reduced both in culture and in soil was observed, because of formation of an antimicrobial-producing biofilm formed by *B. subtilis* was confirmed (Ana et al., 1997). Biofilms are widely found structures in which microorganisms are protected against various stresses, allowing them to persist in adverse environmental conditions. Bacterial biofilms are formed when unicellular organisms come together to form a community that is attached to a solid surface and

encased in an exopolysaccharide matrix (Kunst et al., 1997) (Fig. 1-2). Furthermore, these microbial communities often interact with multiple species and their environment. The site of one such ecologically beneficial bacterial community is the root surfaces, where a rich microflora develops around the readily available nutrients released by roots (Kloepper et al., 1988). Also, both *B. subtilis* and *B. thuringiensis* can form biofilms at air-liquid interfaces (Cherif et al., 2003). If the formation of biofilms by *B. thuringiensis* is greatly found in plant root surfaces like *B. subtilis*, it may show that high bacteria density and more stably *B. thuringiensis* can exist in tomato root surfaces. Biofilm formation could also provide an evidence of *B. thuringiensis* as a PGPR.

Since *B. thuringiensis* has attracted great attention as a biological control agent and showed a potential activity to root-knot nematodes and bacterial wilt. The effect of using insecticide *B. thuringiensis* as PGPR to control plant soil disease such as complex disease was expected. However, there is little research to evaluate *B. thuringiensis* is PGPR, and the biocontrol efficiency by *B. thuringiensis* against *Fusarium oxysporum* f.sp. *lycopersici*, root knot nematode *M. incognita* and complex disease, so the objectives of this study were to:

- confirm whether *B. thuringiensis* could suppress the wilt disease caused by

Fusarium oxysporum f.sp. *lycopersici* (FOL), and to verify if *B. thuringiensis* can act as plant growth promoting rhizobacteria (PGPR), which can promote plant shoot elongation and seed germination.

- determine if *B. thuringiensis* strains have the ability to form biofilms on microtiter plates, colonize and form biofilms on tomato roots by liquid and soil co-culture methods.
- carry out to assay the systemic activity of *B. thuringiensis* against *M. incognita in vitro*, test the biocontrol efficacy in potted soil under greenhouse condition, and comprehensively evaluate nematicidal potential in PPN management.
- evaluate the biocontrol potential of selected *B. thuringiensis* strains with antifungal and nematicidal activities against disease complex caused by *M. incognita* and FOL in tomato plants.
- clarify the mechanisms of *B. thuringiensis* control complex, and the interactions among plant, pathogen, nematode and *B. thuringiensis.*

Fig. 1-1

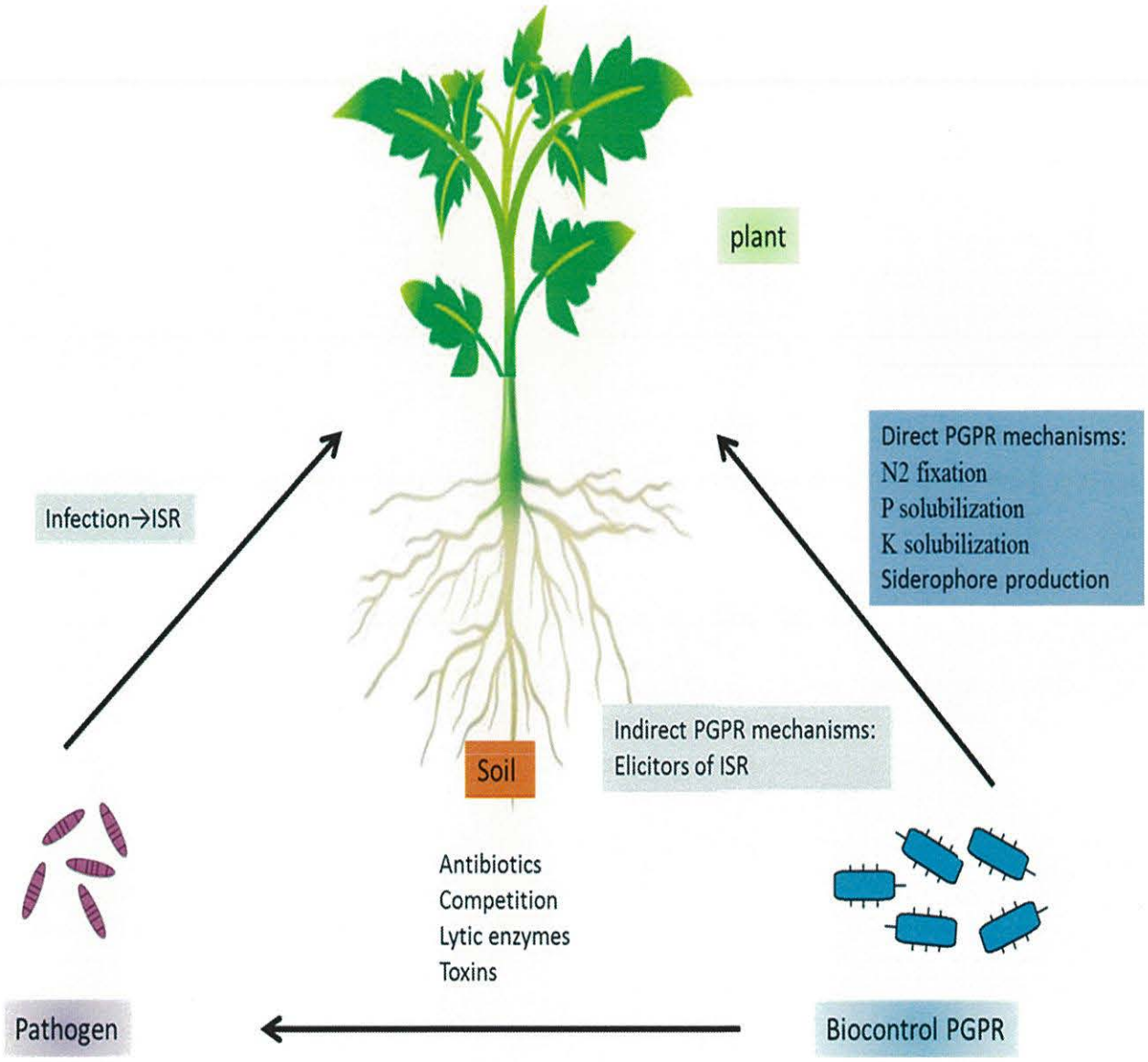

Fig. 1-1: Mechanisms of Plant Growth-Promoting Rhizobacteria

Fig. 1-2

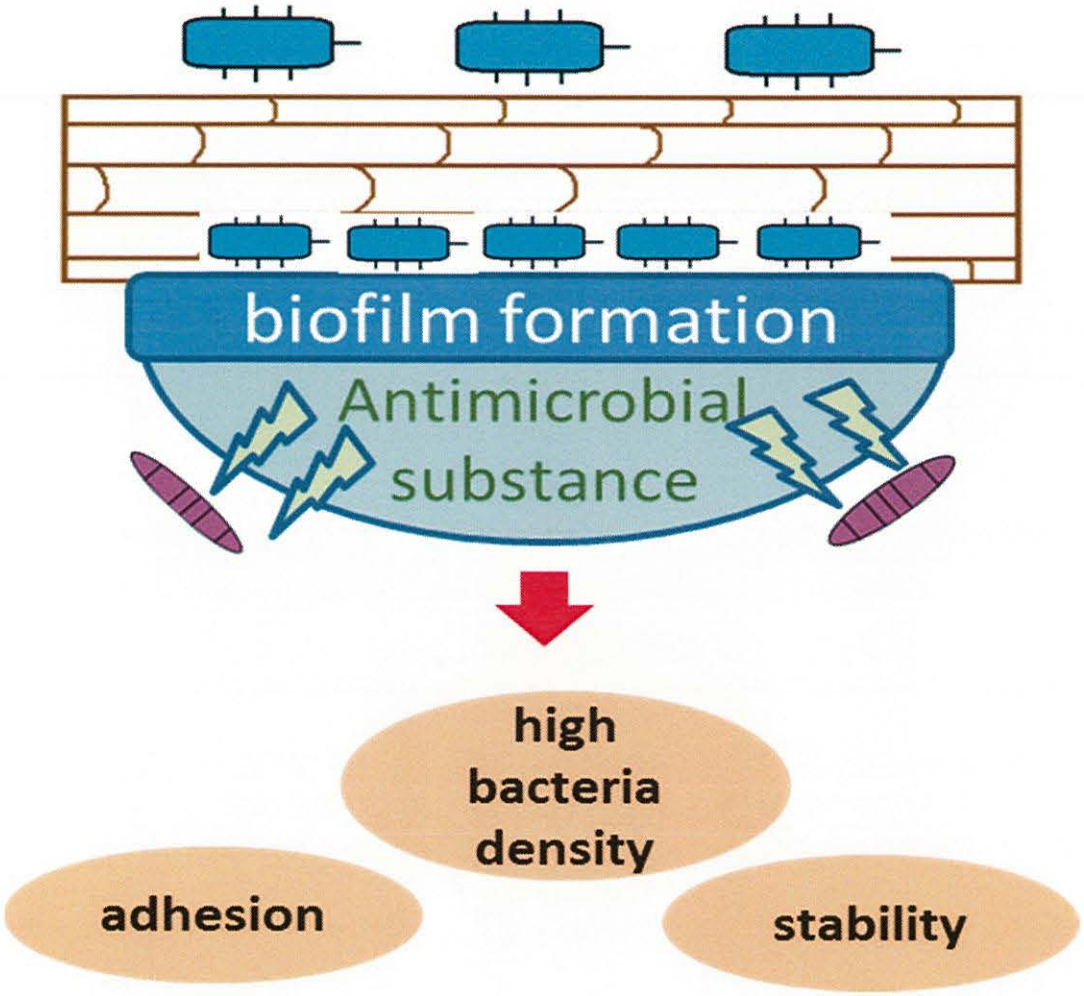

Fig. 1-2: Characteristics of biofilm

CHAPTER II.

Bacillus thuringiensis as Biological Control Agents for Tomato Fusarium Wilt

2.1 Introduction of chapter II

Bacillus thuringiensis has been used as an effective bioinsecticide because it produces the proteins Cry and Cyt, which are highly toxic to insects in certain situations. However, recently, *B. thuringiensis* was used as a biological control agent that can suppress plant disease. In this study, the antagonistic activities of *B. thuringiensis japonensis* (BT17) and *B. thuringiensis kurstaki* (BT18) against the fungal pathogen *Fusarium oxysporum* f.sp. *lycopersici* (FOL) were examined using a dual culture technique. Furthermore, *B. thuringiensis* strains suppressed the development of wilt symptoms caused by FOL in tomato plants. After inoculating six strains of *B. thuringiensis* suspension following inoculation of FOL, the development of wilt symptoms became less than control, especially with *B. thuringiensis japonensis* (BT17) and *B. thuringiensis CR371-H* (BT20). Furthermore, we proved that *B. thuringiensis* strains are plant growth promoting rhizobacteria (PGPR) that can promote plant growth. Seed germination and shoot elongation were promoted by treating the tomato seeds with a bacterial culture filtrate and a bacterial suspension.

2.2 Materials and methods

2.2.1 Fungal pathogen and bacterial strains preparation

Fusarium oxysporum f.sp. *lycopersici* (FOL) race 2 was used in the pathogenicity experiments. The isolate (FOL) was grown on potato dextrose agar (PDA) medium at 26°C for 7 days in a culture dish and then stored at 5°C. The isolate, recovered as needed from storage, was grown on PDA at 26°C for 7 days prior to inoculation.

The tested *B. thuringiensis* strains *B. thuringiensis sotto* (BT15), *B. thuringiensis israelensis* (BT16), *B. thuringiensis japonensis* (BT17), *B. thuringiensis kurstaki* (BT18), *B. thuringiensis roskildiensis* (BT19), *B. thuringiensis CR371-H* (BT20), (BT15: cry1Aa, BT16: cry4Aa, cry10Aa, cry11Aa, BT17: cyt1A, BT18: cry1Aa, cry1Ab, cry1Ac, BT19: cry21Ba, BT20: no cry gene) used for the present investigation were obtained from Research Faculty of Agriculture, Applied Bioscience Applied Molecular Biology Laboratory, Hokkaido University. For inoculum preparation, six strains of *B. thuringiensis* were inoculated in Luria Bertani broth (LB) and grown for 1 week at 30°C. For the preparation of *B. thuringiensis* culture filtrate (CF), the six strains of bacteria were inoculated in liquid LB broth and grown for 24 h with constant shaking

(150 rpm) at 28 ± 2°C. The culture obtained at the stationary phase was centrifuged at 6000 rpm for 10 min and the bacterial cells were resuspended in sodium phosphate buffer (100 mM; pH 7.0). The bacterial concentration was adjusted to 3×10^8 cfu/ml.

2.2.2 Antagonistic activity of *B. thuringiensis*

The antifungal activity of six strains of *B. thuringiensis* against FOL was evaluated by dual culture method on PDA, in triplicate. FOL (9mΦ colony) was inoculated in the middle of a PDA plate, and at the same time, the six strains of *B. thuringiensis* were inoculated at the edge of the PDA plate separately. A PDA plate that was only inoculated with the pathogen was treated as a control. The inoculated PDA plates were incubated at 28 ± 2°C. When the control PDA plates were full of mycelium, the length of a clear zone caused by *B. thuringiensis,* between the *B. thuringiensis* colony and FOL colony were observed.

2.2.3 Pot experiment

Subsequently, evaluation of the suppressive effect on FOL by six strains of *B. thuringiensis* in the soil condition (pot experiment) was conducted. Bacterial culture

filtrate and bacterial suspension were prepared. Tomato seedlings were grown at 25°C. The roots of 4-week-old tomato seedlings were treated with bacterial culture at 3 ml/pot (3×10^8 cfu/ml). FOL- infested soil was prepared. Sterile soil and maize flour were mixed completely at the rate of 2:1 (v/v) to make the culture soil. In a flask filled with 12 ml distilled water, 40 ml of culture soil was taken, and sterilized in an autoclave. The FOL was inoculated in the sterilized culture soil, and stored in a 25°C incubator for 7 days. Sterile soil (1 L) and 5% (v/v) FOL infested soil were mixed completely and put in a sterilized plastic box to make the FOL infested soil. The bacteria-treated tomato seedlings were placed in the FOL-infested soil (4 pots/box). Tomato seedlings treated with distilled water alone were used as a control. Three weeks after pathogen inoculation, the fresh weight, shoot length, and root length were measured and the wilting score was evaluated. Wilting score was evaluated based on leaf symptoms of wilting as follows: 0 = no wilt symptoms; 1 = <25% of wilting leaves; 2 = 26–50% of wilting leaves; 3 = 51–75% of wilting leaves; 4 = 76–100% of wilting leaves (Bora et al., 2004).

2.2.4 The activity of *B. thuringiensis* strains on promotion of tomato seed germination

To evaluate the PGPR effects of *B. thuringiensis* culture filtrate on seed germination, we performed bacterial treatment of tomato seeds. Bacterial culture filtrate and bacterial suspension were prepared. Seeds of tomato were surface sterilized with 1% sodium hypochlorite for 1 min and washed with sterilized water 3 times, then immersed in bacterial culture filtrate and bacterial suspension. After 24 h, the bacterial culture filtrate (or bacterial suspension) was thrown away and the seeds were dried for 12 h in sterile Petri dishes. Tomato seeds soaked in phosphate buffer alone were used as a control treatment. Bacterial culture filtrate (or bacterial suspension) treated tomato seeds were placed at 25°C (10 seeds/ dish). After 3 days, the seed germinations were evaluated after inoculation. Using same seed treatment method, pot experiments were carried out in a completely randomized design in a greenhouse at 25°C with five replicates. Bacteria treated seeds were sown in individual 9-cm-diameter pots containing sterilized soil (Shanmugam et al., 2011). For the pot experiments, plant fresh weight, dry weight, and height were measured 4 weeks after inoculation.

2.2.5 Root and shoot elongation

Bacterial suspensions were prepared. Seeds of tomato were surface sterilized with

1% sodium hypochlorite for 1 min and washed with sterilized water 3 times, then immersed in bacterial culture filtrate and bacterial suspension. After 24h, the bacterial culture filtrate (or bacterial suspension) was thrown away and the seeds were dried for 12 h in sterile Petri dishes (diameter = 90mm). Tomato seeds soaked in phosphate buffer alone were used as a control treatment. Bacteria - treated seeds were placed between folds of wet germination paper. A roll towel method involved rolling of the seeds in wet germination paper. First, a label with details regarding sample, number of replicate seeds and date was placed on the top left hand corner. Next, a wet paper towel was placed over the label. The seeds were then placed 2cm below the edge of the sheet in a row using forceps. A strip of paper was also wetted and placed over the seeds to keep them moist and to prevent them from being displaced while rolling. The wet paper towel was then rolled, using a rubber band to prevent unfurling of the roll. The rolled paper was subsequently placed in water at 25°C. After 2 weeks the shoot and root elongation were measured using a ruler with fine-scale measurements.

2.3 RESULTS

2.3.1 Antagonistic activity of *B. thuringiensis*

In the dual culture assays for antifungal activity, six strains of *B. thuringiensis* were tested. As a result BT17 and BT18 inhibited the mycelial growth of the pathogen FOL significantly, with an inhibition clear zone. These results show that BT17 and BT18 have antagonistic activity against FOL, which can inhibit the mycelial growth of FOL (Fig.2- 1).

2.3.2 Pot experiment

The results showed that tomato roots treated with *B. thuringiensis* culture filtrate suppressed the growth of FOL and the development of wilt symptoms to less than the pathogen control; the strains BT17 and BT20 had a significant effect (ANOVA, $F = 11.64$; $df = 7, 16$; $P < 0.01$) (Fig. 2-2a-b). A highly antagonistic activity of *B. thuringiensis* strain, BT17 was assessed with and without inoculation with FOL and compared with untreated controls in a pot. The bacterial treatment led to a significant reduction in the wilting score, from 83.3% to 25% (Fig. 2-2b), with less disease incidence compared to pathogen treatment alone. The bacterial treatments recorded a significant increase (ANOVA, $F=13.12$; $df=1, 41$; $P < 0.01$) in tomato plant weight from

11.2% to 220.1% (Table 2-1) relative to the pathogen control, and increased the tomato shoot length from 43.1% to 108% (Table 2-1) relative to the pathogen control, and root length from 13.7% to 65.6% (Table 2-1) relative to the pathogen control.

2.3.3 The activity of *B. thuringiensis* strains on tomato seeds germination promotion

All the six strains of *B. thuringiensis* increased the germination rate of tomato seeds compared to untreated control tomato seeds (Fig. 2-3a), with increases ranging from 35.7% to 107.1% (Fig. 2-3b). Among all the test strains, BT18 showed the highest seed germination rate. In the pot experiment, *B. thuringiensis* strains promoted plant length from 4% to 28.5% versus untreated control (Table. 2-2), promoted plant fresh weight from 3.8% to 108.4% versus untreated control (Table. 2-2), promote plant dry weight from 35.3% to 201.9% versus untreated control (Table. 2-2).

2.3.4 Root and shoot elongation

The results of the roll towel experiment showed that all of the *B. thuringiensis* strains promoted tomato shoot and root elongation compared to the untreated control (Fig. 2-4a), ranging from 87.7 to 381.1% (Fig. 2-4b). In particular, BT17 promoted

shoot and root elongation significantly compared to other strains and untreated controls (Fig. 2-4b).

2.4 DISCUSSION

B. thuringiensis has been used as an effective bioinsecticide because it produces the proteins Cry and Cyt, which are highly toxic to insects in certain situations (Betz et al., 2000). In recent years, many new functions of *B. thuringiensis* have been discovered that protect plants from pathogen infection. Recently *B. thuringiensis* was used as a biological control agent that can suppress plant disease. In this study, the antagonistic activities of BT17 and BT20 against FOL were examined by dual culture technique. It was confirmed that *B. thuringiensis* strains can suppress the development of wilt symptoms caused by FOL in tomato plants. In addition, this study proved that *B. thuringiensis* strains were plant growth promoting rhizobacteria (PGPR) which promoted plant growth, seed germination, and shoot elongation.

Compare to chemical control, biocontrol by use of PGPR shows a potentially attractive and efficient disease management approach. PGPR are soil bacteria that able

to colonize the root system of plants, and enhance plant growth and development under field conditions (Kloepper et al., 1988; Raupach et al., 1998). Indeed, PGPR are able to promote plant growth and reduce disease in crops (Jetiyanon and Kloepper, 2002). Since Gram-positive have the ability to form desiccation- and heat- resistant spores, it can be formulated into stable products readily (Emmert et al., 1999). Root colonizing *Bacillus* spp. is well-known by disease control and enhancement of plant growth (Kloepper et al., 2006). For example, *B. subtilis* is the best-characterized member of Gram-positive bacteria, and has become a paradigm organism (Kunst et al., 1997). *B. subtilis* is treated as an excellent biocontrol agent, because it has many characteristics, such as the promotion of plant growth, formation of viable spores, production of structurally diverse antibiotics, and a ubiquitous presence in soil (Ryu et al., 2004; Bais et al., 2004; Cenci et al., 2006; Liu et al., 2006).

In this study, six strains of *B. thuringiensis* (BT15, BT16, BT17, BT18, BT19, BT20) were tested. All six strains of *B. thuringiensis* could suppress the growth of FOL and the development of wilt symptoms in tomato plants (Fig. 2-2b). By treating the tomato roots with *B. thuringiensis* suspension, the wilt disease caused by FOL was significantly suppressed. But the suppressive activity might not be caused by the

competition for space, nutrients and ecological niches between *B. thuringiensis* and FOL. *B. thuringiensis* generally produces several compounds, such as antimicrobial substances that include b-exotoxins, antibiotics, degrading enzymes, bacteriocins, and a signal molecule in the bacterial quorum-sensing system (Cherif et al., 2003; Dong et al., 2002). *B. thuringiensis* strains were screened for their antibacterial, anti-insect, lactonases, chitinases, β-1, 3-glucanases, and zwittermicinA (Arora et al., 2003; Cherif et al., 2003; Stabb et al., 1994). Six tested strains had at least two major insecticidal toxins genes. Concerning fungal biocontrol, all the strains inhibited the growth of *Fusarium oxysporum* and *Aspergillus flavus* and four strains had all or most of the antifungal determinants examined, with strain *Bt* HD932 showing the widest antifungal activity spectrum (Raddadi et al., 2009). *B. thuringiensis* could produce compounds that occur outside of the cell, such as b-exotoxins and the antibiotic zwittermicin A (Zhou et al., 2008). The six strains of *B. thuringiensis* that were used in the experiments in this study might produce such compounds.

In all of the tested *B. thuringiensis* strains, BT17 and BT18 showed antagonistic activity against FOL by dual culture assays (Fig. 2-1) and inhibited the symptoms of wilt disease in pot (Fig. 2-2b). From these results, we conclude that *B. thuringiensis* can

product antimicrobial substances against the growth of FOL. However, in the pot experiment BT20 also inhibited wilt symptoms with a low wilting score and high plant weight (Table. 2-1), in contrast to the dual culture assays, where fungal growth was not inhibited by BT20. Therefore, there is less likelihood that antimicrobial substances were produced by BT20, unlike BT17 and BT18 that might directly suppress the growth and spread of FOL in tomato. It was inferred that BT20 suppressed the wilt disease in an indirect way. Moreover, all of the tested *B. thuringiensis* strains could promote seed germination (Fig. 2-3b) and root and shoot elongation (Fig. 2-4b) significantly. From this point, the mechanism of antagonistic to fungal plant pathogen and induced resistance should be clear.

PGPR enhance plant growth either directly or indirectly (Glick, 1995); direct mechanisms of plant growth promotion by PGPR can be demonstrated deficient plant pathogens or other rhizo-microorganisms and promoting the uptake of nutrients from the environment, while indirect mechanisms of plant growth promotion involve the ability of PGPR to lessen the deleterious effects of phytopathogenic organisms on crop yield (Ramamoorthy et al., 2001). PGPR strains can control plant pathogens through several mechanisms, including production of antimicrobial compounds, induced

systemic resistance (ISR), and competition for spaces and nutrients with pathogens (Kloepper et al., 2004; Munees et al., 2014; Van et al., 1998). Tomato roots treated with a cell-free filtrate of *B. thuringiensis* suppressed the development of wilt symptoms caused by bacterial wilt disease *Ralstonia solanacearum*, through the plant defense system (Hyakumachi et al., 2013). The co-activation of ET-dependent signaling pathway with the SA-dependent signaling pathway and suppression of JA-dependent signaling may play key roles in induced resistance of *B. thuringiensis* to *R. solanacearum* in tomato (Hyakumachi et al., 2014). In our data, it is also possible that some kind of elicitor compounds existed in BT20, because of non-antagonistic fungal activity and Fusarium wilt suppression effects.

Since PGPR are well known for their disease reduction and growth promotion abilities, biocontrol by use of PGPR shows a potentially efficient alternative disease management approach (Van, 2007). This study provides evidence for the first time that *B. thuringiensis* can be used as PGPR, which have bioactivity against a plant pathogen by suppressing the wilt disease and promoting plant seed germination, root and shoot elongation. However, why *B. thuringiensis* can promote seed germination and root and shoot elongation were not yet clear. The mechanism behind BT20 causing induced

systemic resistance should be further studied. To further understand the mechanism of *B. thuringiensis* - induced resistance to *Fusarium oxysporum*, it will be important to identify the specific substances present in the CF that can induce resistance to *Fusarium oxysporum* in tomato. Interestingly volatile compounds and lipopeptides produced by *Bacillus* spp. have been identified as elicitors in ISR (Ryu et al., 2004). Therefore, characterization of the substances able to induce disease resistance will provide new insights for further evaluation of the practicality of *B. thuringiensis* as an effective biocontrol agent.

Fig. 2-1.

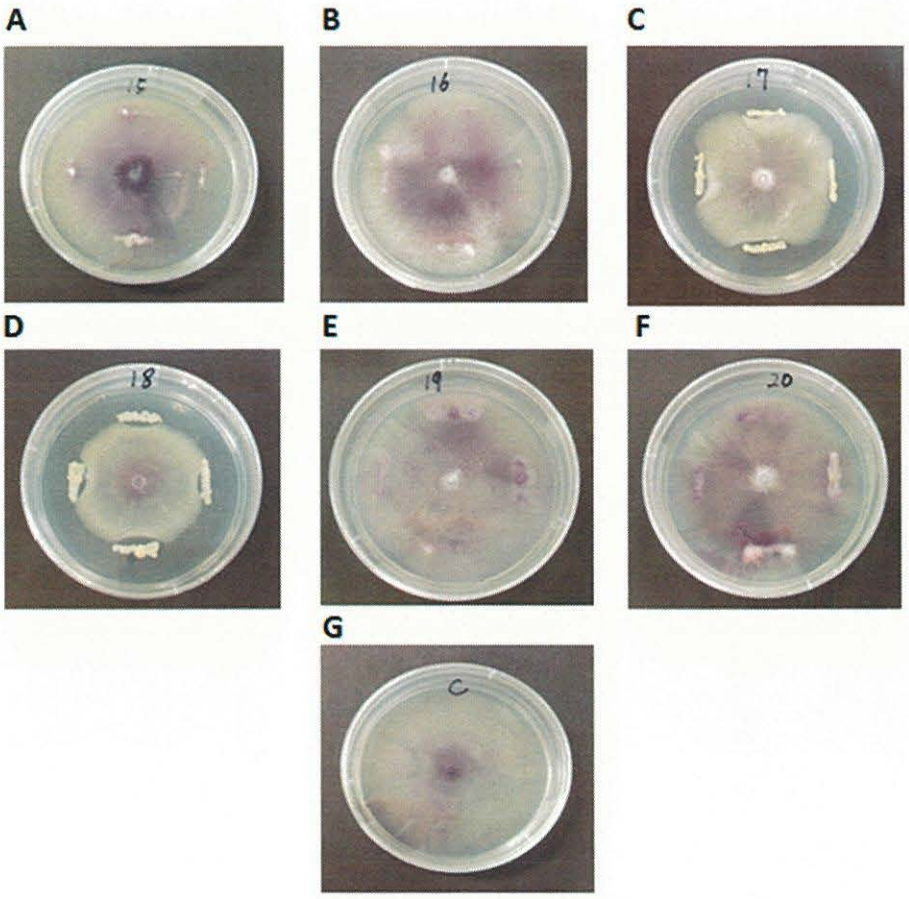

Fig. 2-1. Antagonistic bacteria selection was evaluated by dual culture technique on PDA. PDA plate that was only inoculated with the FOL was treated as a control. The clear zone caused by BT17 and BT18 against FOL were clearly shown in this picture (Fig. 2-1 C, D).

A: BT15 treament; B: BT16 treament; C: BT17 treament; D: BT18 treament; E: BT19 treatment; F: BT20 treatment; G: control.

Fig. 2-2a.

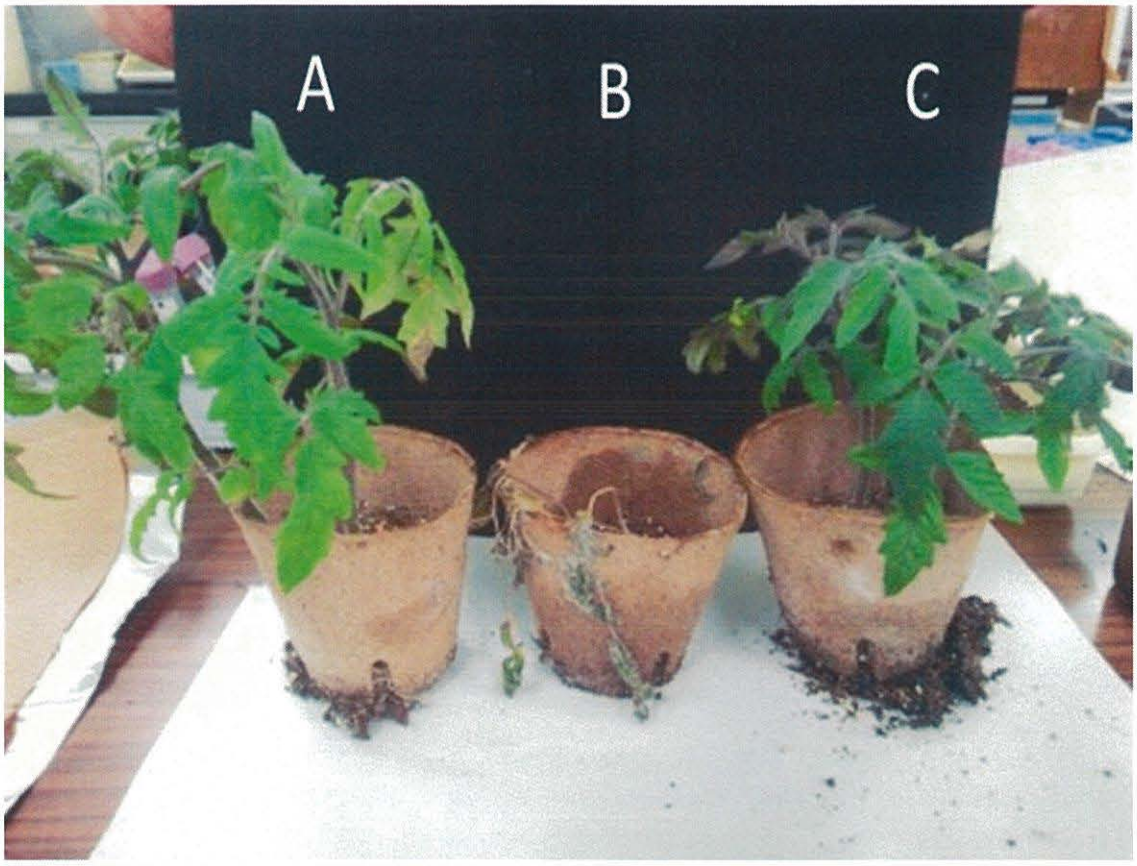

Fig. 2-2a. Development of *Fusarium* symptoms in tomato plants by treating their roots with *B. thuringiensis* strains. (A) Tomato seedlings treated with BT17 and FOL; (B) tomato seedlings treated with FOL alone; (C) tomato seedlings treated with distilled water alone. Wilting score in tomato plants with their roots treated with the suspension of *B. thuringiensis*. CF: pathogen control; C: untreated control.

Fig. 2-2b.

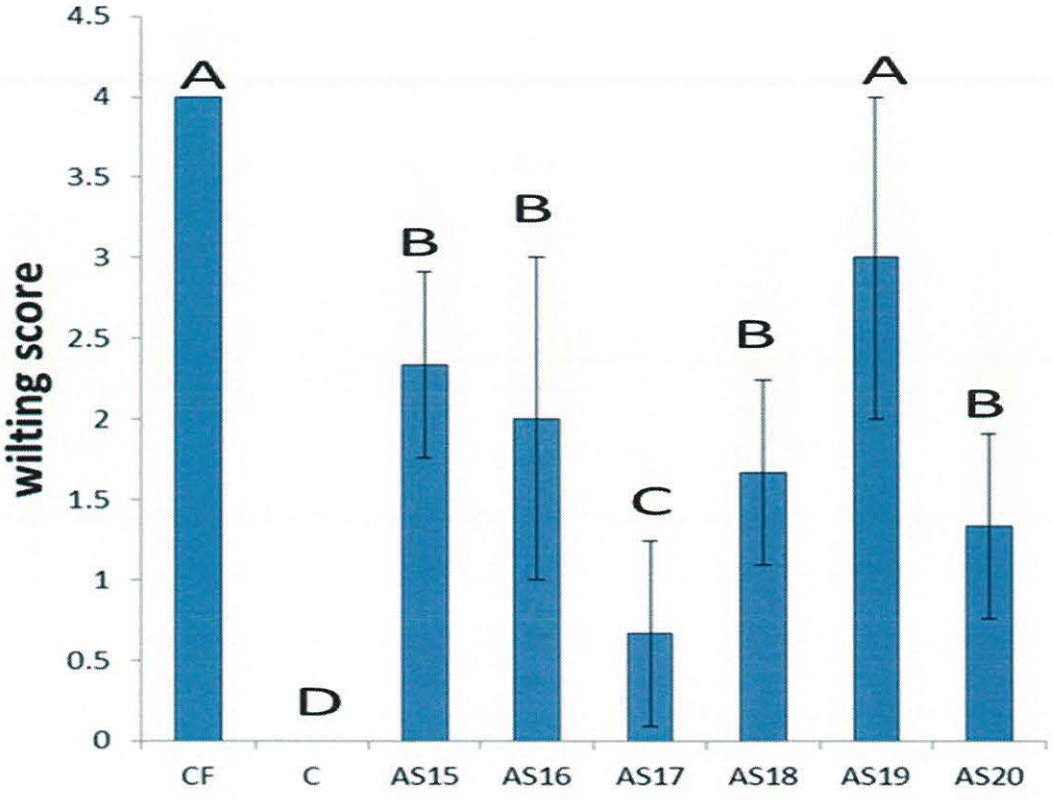

Fig. 2-2b. Development of *Fusarium* symptoms in tomato plants by treating their roots with *B. thuringiensis* strains. Wilting score was evaluated based on leaf symptoms of wilting as follows: 0 = no wilt symptoms; 1 = <25% of wilting leaves; 2 = 26–50% of wilting leaves; 3 = 51–75% of wilting leaves; 4 = 76–100% of wilting leaves.

Mean and standard deviation of three replicates per experiment are presented. Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

CF: pathogen control; C: untreated control

Fig. 2-3a.

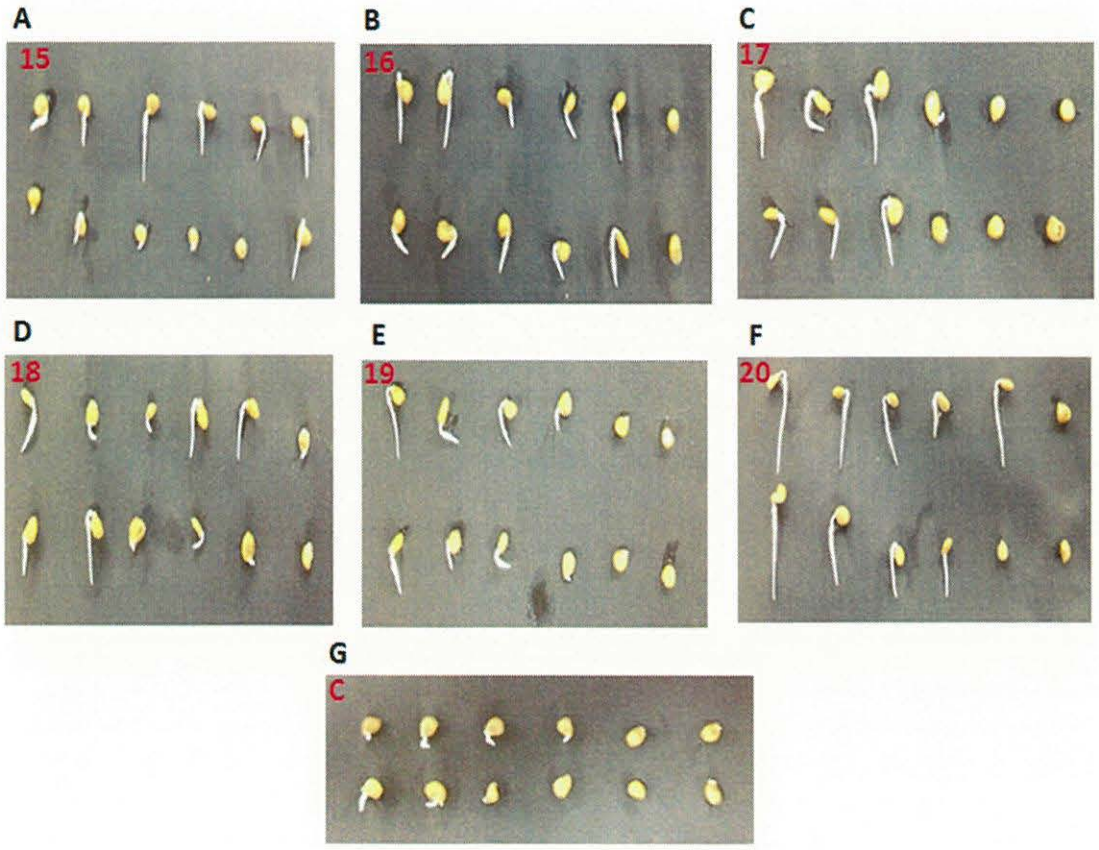

Fig. 2-3a. The activity of *B. thuringiensis* strains on promotion of tomato seed germination was done by seed treatment. C: untreated control.

A: BT15 treament; B: BT16 treament; C: BT17 treament; D: BT18 treament; E: BT19 treatment; F: BT20 treatment; G: control.

Fig. 2-3b.

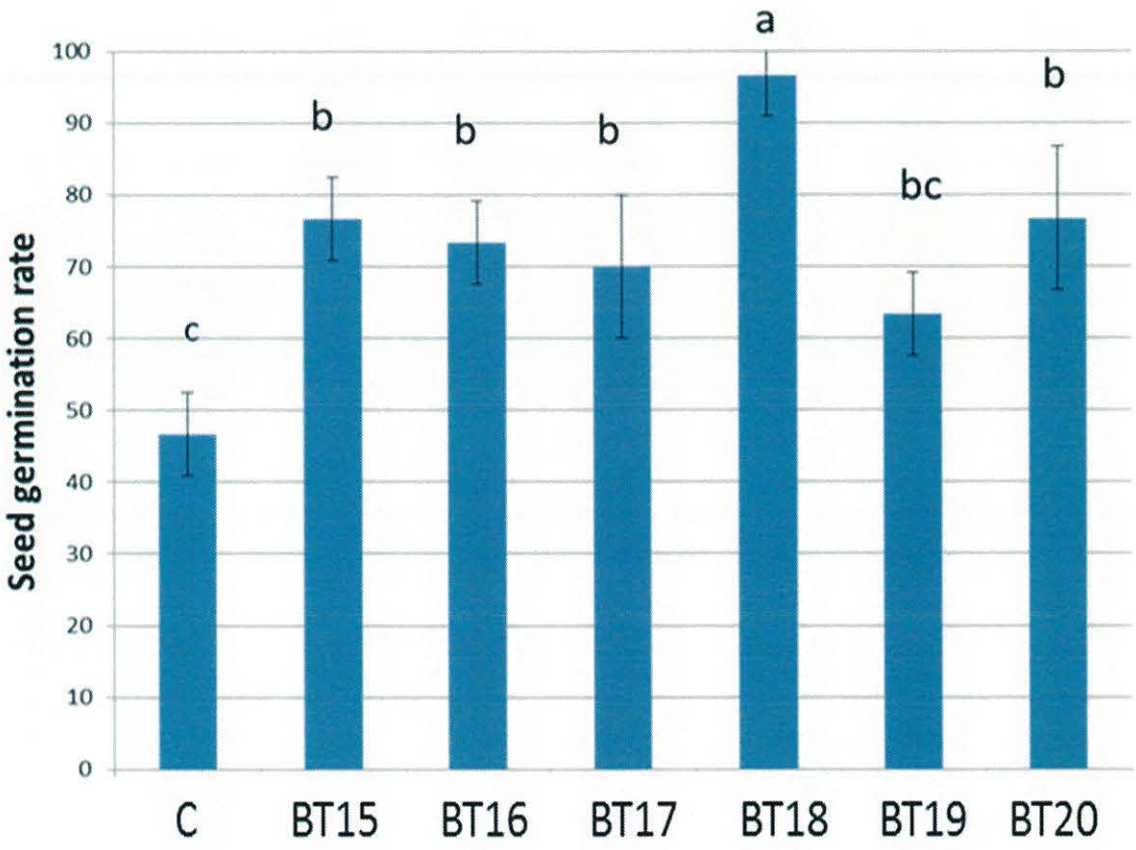

Fig. 2-3b. The activity of *B. thuringiensis* strains on promotion of tomato seed germination was done by seed treatment. C: untreated control. Mean and standard deviation of three replicates per experiment are presented. Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

Fig. 2-4a.

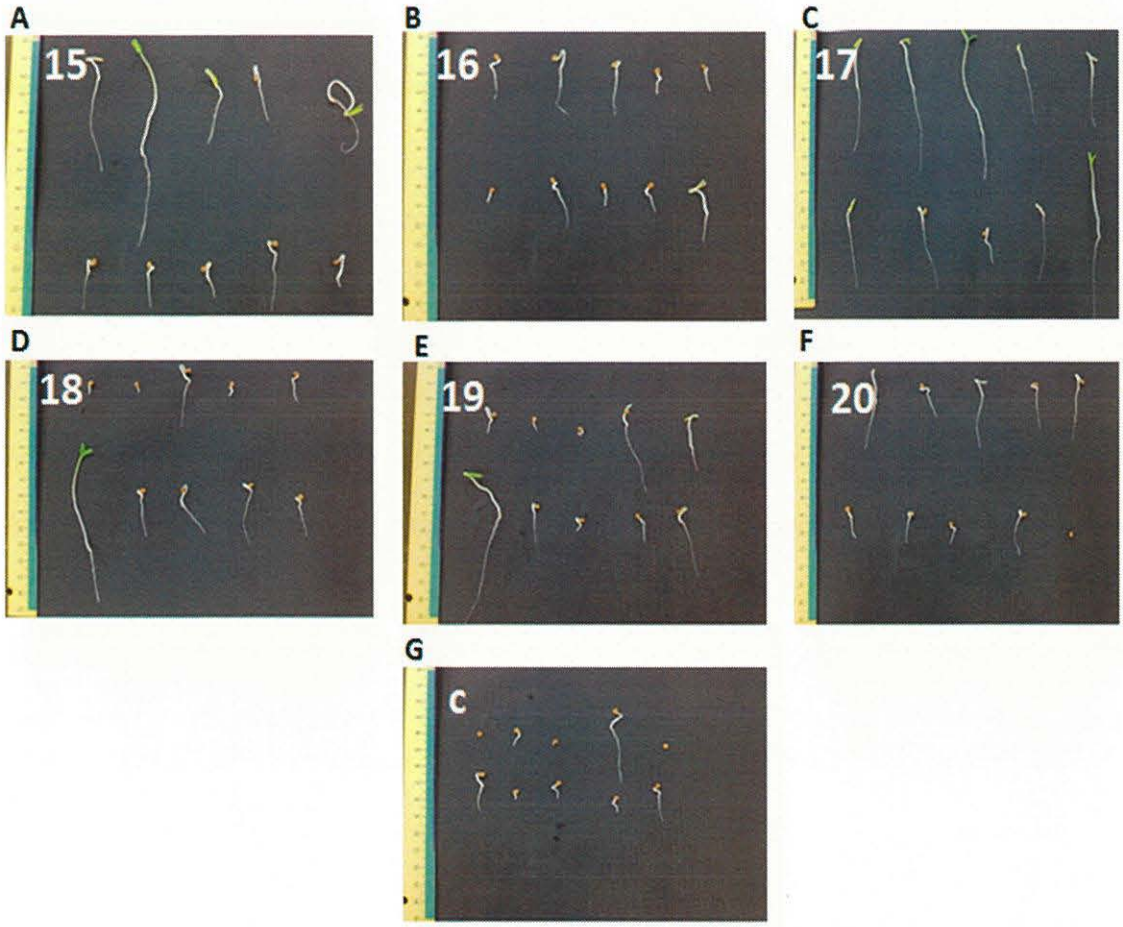

Fig. 2-4a. Shoot and root elongation by roll towel method.

A: BT15 treament; B: BT16 treament; C: BT17 treament; D: BT18 treament; E: BT19 treatment; F: BT20 treatment; G: control.

Fig. 2-4b.

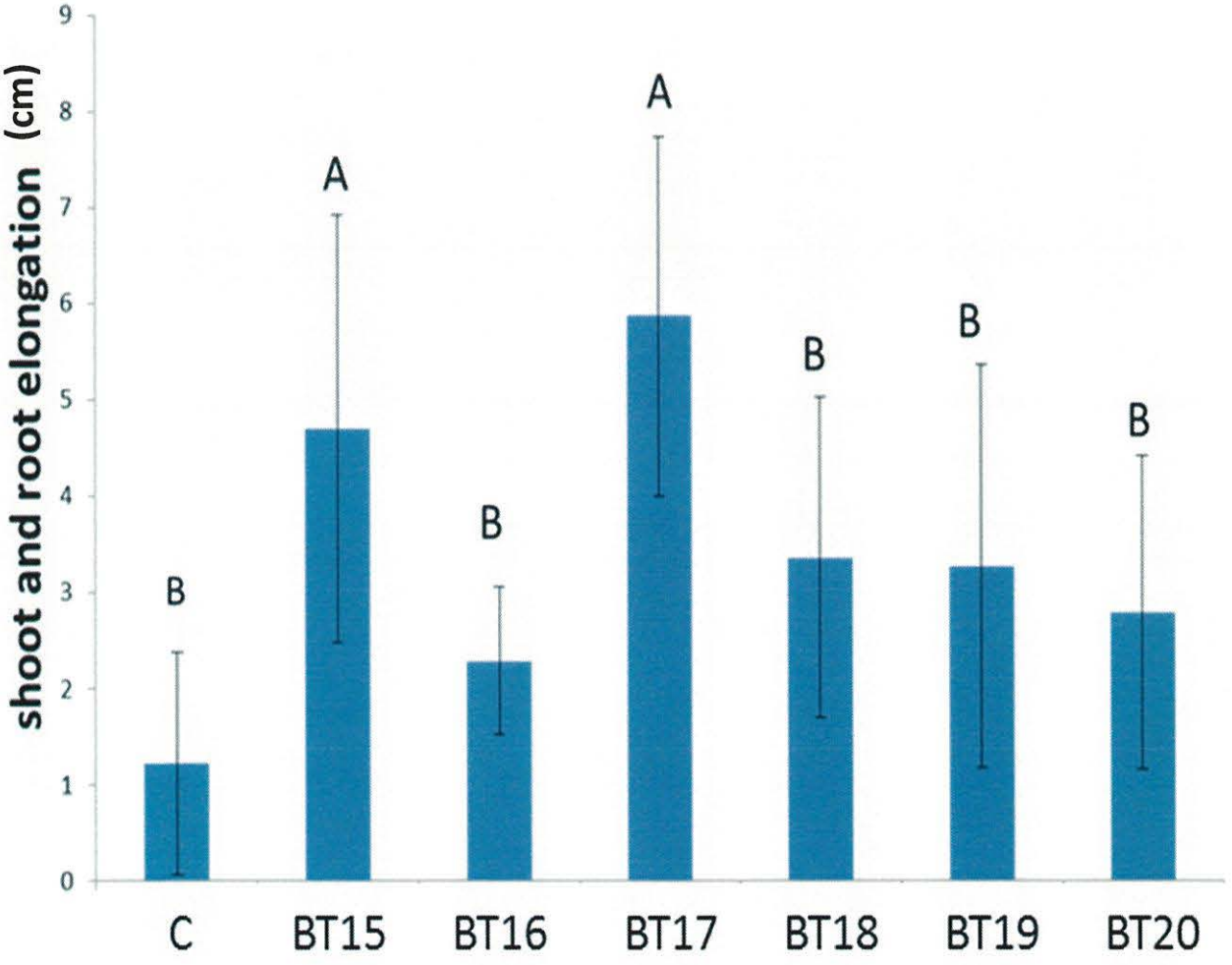

Fig. 2-4b. Shoot and root elongation by roll towel method. C: untreated control. Mean and standard deviation of three replicates per experiment are presented. Values followed by different letters were significantly different according to the Tukey test at a 0.01 level of confidence.

Table 2-1. The shoot, root length and fresh weight of the bacteria and pathogen treated tomato seedlings in pot experiment.

	PC	C	BT15	BT16	BT17	BT18	BT19	BT20
Shoot length, cm	26.00 ± 12.70c	79.00 ± 12.30a	48.40 ± 11.10b	48.40 ± 13.80b	54.10 ± 8.30ab	46.30 ± 13.80b	37.20 ± 21.10b	51.20 ± 17.30b
Root Length, cm	10.90 ± 5.08c	26.00 ± 2.16a	9.80 ± 3.20c	18.10 ± 7.24b	12.40 ± 3.14c	15.80 ± 3.50b	9.70 ± 1.86c	16.60 ± 8.29b
Fresh weight, g	5.78 ± 1.83B	36.55 ± 12.37A	6.44 ± 5.41B	9.47 ± 4.97B	8.73 ± 5.93B	7.98 ± 2.41B	5.68 ± 4.23B	18.50 ± 10.47A

PC: pathogen control; C: untreated control.

Mean and standard deviation of three replicates per experiment are presented. Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence

Table 2-2. Shoot length, fresh weight and dry weight of bacteria treated tomato seeds in PGPR pot experiment.

	C	BT15	BT16	BT17	BT18	BT19	BT20
Length, cm	24.47 ± 4.72a	27.50 ± 4.60a	31.45 ± 2.27a	28.33 ± 3.06a	27.23 ± 3.85a	25.45 ± 3.66a	31.25 ± 2.56a
Fresh weight, g	1.95 ± 1.17b	3.18 ± 1.02b	4.06 ± 1.51a	3.24 ± 1.01b	2.80 ± 1.05b	2.03 ± 0.83b	4.03 ± 0.99a
Dry weight, g	0.05 ± 0.04b	0.13 ± 0.05b	0.15 ± 0.07a	0.11 ± 0.04b	0.11 ± 0.03b	0.07 ± 0.02b	0.15 ± 0.02a

C: untreated control. Mean and standard deviation of three replicates per experiment are presented.

Values followed by different letters were not significantly different according to the Tukey test at a 0.05 level of confidence.

CHAPTER III.

Bacillus thuringiensis biofilm formation on tomato root surface

3.1 **Introduction of chapter III**

B. thuringiensis has been used as an effective bio-insecticide. But, recently *B. thuringiensis* was treated as a biological control agent which can suppress the plant disease. *B. thuringiensis* can suppress the growth of *Ralstonia solanacearum* and the development of wilt symptoms in plants. *B. subtilis*, which is ubiquitous in soil, can promote plant growth, protect against fungal pathogen attack as a biocontrol agent on plants. It is now widely recognized that *B. subtilis* settings persist in association with surfaces by forming biofilm. In this study, we would like to determine if *B. thuringiensis* would colonize and form biofilms on tomato roots. We use 10 under test strains: *B. thuringiensis* strains *B. thuringiensis sotto* (BT15), *B. thuringiensis israelensis* (BT16), *B. thuringiensis japonensis* (BT17), *B. thuringiensis kurstaki* (BT18), *B. thuringiensis roskildiensis* (BT19), *B. thuringiensis CR371-H* (BT20), *B. thuringiensis* DF (BT DF), *B. thuringiensis GC-91* (BT *GC-91*), *B. subtilis MBI600*, *B. simplex CGF2856*. The microtiter plate assay was used to evaluate *B. subtilis* biofilm formation. Biofilm formation was quantified by measuring the OD_{595} for each well using a spectrophotometer. Also the tomato root was soaked in each tested bacterial

strains by co-culture method for 48h, and transplant tomato to soil condition. The result is *B. thuringiensis* has the ability of biofilm formation. Our results also indicate that *B. thuringiensis* formed biofilm can colonize plant root.

3.2 **Materials and methods**

3.2.1 Bacterial strains and tomato seed preparation

The tested *B. thuringiensis* strains (BT 15~20) used for the present investigation were obtained from Research Faculty of Agriculture, Applied Bioscience Applied Molecular Biology Laboratory, Hokkaido University. For inoculum preparation, six strains of *B. thuringiensis* were inoculated in Luria Bertani broth (LB) and grown for 1 week at 30°C.The other tested strains (BT DF, BT *GC-91*, *B.subtilis MBI600*, *B.simplex CGF2856*) were form commercial formulation. For the preparation of bacterial suspension, the ten strains of bacteria were inoculated in liquid LB broth and grown for 24 h with constant shaking (150 rpm) at 28 ± 2°C.Tomato seed (*Lycopersicon esculentum* Mill) was used in this experiment. Tomato seeds were surface-sterilized with 5% antiformin for 30 sec, 70% ethanol for 10 sec, and washed with sterilized water twice.

3.2.2 Formation of biofilms by *B. thuringiensis* in microtiter plates

The ability of the *B. thuringiensis* strains to form biofilms was tested. Pre-cultures in the exponential phase of growth were inoculated at an optical density at 600nm (OD_{600}) of 0.1 into fresh LB medium (10 g/liter bactopeptone, 5 g/liter yeast extract, 5 g/liter NaCl) in 24-well microtiter plates. After 24, 48, 72 h of incubation at 25°C with 3 replicates, the biofilm density was measured as follows: the microtiter plate wells were washed once with phosphate- buffered saline, and bound cells were stained with a 1% (wt/vol) crystal violet solution at room temperature for 20 min. The wells were then washed with phosphate-buffered saline three times, and the dye was solubilized with a 20%/80% acetone/ethanol mixture. The absorbance at 595 nm of the solubilized dye was subsequently determined (Auger et al., 2006).

3.2.3 Formation of biofilms by *B. thuringiensis* with liquid and soil co-culture methods

The ability *B. thuringiensis* biofilm formation on tomato roots was tested by liquid and soil co-culture methods (Bais et al., 2004). The tested bacterial suspensions were prepared as described before. In liquid co-culture, the tomato seeds were placed in

a culture dish for germination in 8 days. Elongated tomato roots were soaked in a glass bottle (4.5cm in height, 2cm in width), which filled with 5ml bacterial suspension for 3 days. In soil co-cultivation, the tomato seeds were placed in a culture dish for germination in 4 or 5 days. After 4 or 5 days, germinated tomato seedlings were plant in 15 ml of tubes added with 2.5g sterilization soil, filled with sterilized water. 1 ml/day tested bacterial suspensions were inoculated to the tomato seedlings, inoculation was taken three days in total. Root of the tomato seedlings treated with liquid and soil co-culture methods were rinsed with a phosphate-buffered saline twice and soaked with 70% methanol for 10 min. The tomato seedlings were dyed with 5% trypan blue for 10 min. An optical microscope was used to observe the tomato root surfaces in a field of vision of 1,000 times. Cut 2cm from the tip of taproot and evaluated the biofilm formation of the tomato root surfaces.

Bacterial concentration in liquid co-culture experiment: BT15: 2.08×10^{8}cfu/ml; BT16: 8.16×10^{7}cfu/ml; BT17: 7.6×10^{7}cfu/ml: BT18: 1.97×10^{8}cfu/ml; BT19: 9.34×10^{7}cfu/ml; BT20: 9.26×10^{7}cfu/ml; BT DF: 1.68×10^{8}cfu/ml; BT *GC-91*: 1.64×10^{8}cfu/ml; *B. subtilis MBI600*: 1.47×10^{7}cfu/ml; *B. simplex CGF2856*: 1.78×10^{8}cfu/ml.

Bacterial concentration in soil co-cultivation experiment: BT15: 1.95×10^8cfu/ml; BT16: 1.04×10^8cfu/ml; BT17: 1.10×10^8cfu/ml; BT18: 2.0×10^8cfu/ml; BT19: 1.13×10^8cfu/ml; BT20: 1.17×10^8cfu/ml; BT DF: 1.81×10^8cfu/ml; BT *GC-91*: 1.79×10^8cfu/ml; *B. subtilis MBI600*: 9.51×10^7cfu/ml; *B. simplex CGF2856*: 1.74×10^8cfu/ml.

3.3 RESULTS

3.3.1 Formation of biofilms by *B. thuringiensis* in microtiter plates

The result of formation of biofilms by *B. thuringiensis* in microtiter plates was all tested strains could form biofilms in microtiter plates (Fig. 3-1). All tested strains showed a rise in absorbance from the first day to the second day. From second day to third day, BT16, BT18, BT20, BT DF, *B. simplex CGF2856* showed a rising trend in absorbance, BT15, BT17, BT19, BT *GC-91*, *B. subtilis MBI600* showed a decrease trend in absorbance (Fig. 3-1). To determine the kinetics of biofilm formation, a microtiter plate was inoculated with the bacterial strain as described above. A measurable amount of biofilm was detected after 24 h of inoculation (Fig. 3-1). The number of viable cells in the biofilm rings was determined as follows. The biofilm was

manually scraped from the sides of the wells using a pipette tip and re-suspended in LB medium. After serial dilutions, cells were plated onto LB medium. The increase in crystal violet staining with time of incubation was proportional to the increase in the number of viable cells in the biofilm (Fig. 3-1).

3.3.2 Formation of biofilms by *B. thuringiensis* with liquid and soil co-culture methods

3-day after liquid and soil co-cultivation, tomato root surfaces was washed, fixed and dyed, then observed the formation of biofilms on tomato root surfaces by an optical microscope. As a result, in the liquid bacteria co-culture experiment, except *B. simplex CGF2856* and control treatment, all of bacterial strains could form biofilms on tomato roots rhizoplane were confirmed (Fig. 3-2). Also, in the soil co-cultivation experiment, except BT 19, BT 20, and *B. simplex CGF2856*, all of bacterial strains could form biofilms on tomato root surfaces were confirmed (Fig. 3-3).

3.4 Discussion

The ability of biofilms formation by bacterial strains was evaluated in 24-well

microtiter plates as described before. As a result, we identified all tested bacterial strains could form biofilm (an annular formed in suspension in the well and the interface with the gas) after 2 days static culture. In addition, because of the interaction between the quantity of CV dyeing and the number of colony forming unit of attached cells, the increase and decrease of absorbance corresponded to quantity of biofilm formation in the well. Furthermore, from the first day to the second day, all tested strains showed an increase in the quantity of biofilms formation, from second day to third day, biofilm quantities showed both increase and decrease for every each strain were confirmed. In biofilm formative process, the biofilm would flow away from direction, which prevent biofilm growth and the structure, composition, and function of biofilm are changing dynamically, during biofilm formation (Brading et al., 1995). So the disintegration of the biofilm or the falling off from a well wall surface may lead to decrease of already developed, thick biofilm.

The results of biofilm formation evaluated by liquid and the soil co-cultivation were that, except some strains colony biofilm were confirmed in all tested strains treated tomato root surfaces under both culture conditions. Under a liquid culture condition, all of the *B. thuringiensis* strains and *B. subtilis MBI600* biofilm formation ability were

confirmed, but there was no biofilm formed by *B. simplex CGF2856*. On the other hand, under soil co-cultivation, except BT19, BT 20, and *B. simplex CGF2856*, all of bacterial strains could form biofilms on tomato root surfaces were confirmed. For *B. simplex* the reason might be the ability for biofilm formation to plant root surfaces was lacked; for *B. thuringiensis* the reason might be a soil particle became the inhibition under soil culture, so there was no biofilm formation in rhizoplane. In this study, we documented *B. thuringiensis* strains have the ability to colonize and form biofilms on plant root surfaces.

Biofilms share an important structural feature: their constituent cells are bound together by an extracellular matrix that mainly consists of macromolecules, including proteins, polysaccharides, and nucleic acids, that are produced by the cells themselves (Branda et al., 2005). Bacterial biofilms are found in most natural and man-made environments where bacteria are associated predominantly with surfaces rather than in a free-floating state (Block et al., 1993). *B. subtilis* has been a model organism for the study of Gram-positive bacterial physiology. Recently, it has been reported that *B. subtilis* forms adhering biofilms on inert surfaces under the control of a variety of transcription factors (Hamon et al., 2001; Stanley et al., 2003). Furthermore, *B. subtilis*

6051 could reduce mortality of Arabidopsis, which root infected by *Pseudomonas syringae*, because of an antimicrobial-producing biofilm formed by *B. subtilis* 6051. So the ability of *B. subtilis* 6051 to control *P. syringae* infectivity of Arabidopsis was directly proportional to its ability to colonize and form biofilms on plant root surfaces was documented (Bais et al., 2004).

B. thuringiensis is a naturally abundant Gram-positive bacterium and a well-known, effective bio-insecticide (Schnepf et al., 1998). *B. thuringiensis* can product crystal proteins, which are highly toxic to insects, but not to mammals, and are not harmful to the environment. It has been widely used as an alternative to chemical pesticides or genetically engineered into crops to provide constant protection (Bravo et al., 2011). Moreover, the activity of *B. thuringiensis* can suppress the growth of *Ralstonia solanacearum* and the development of wilt symptoms has been examined in tomato plants (Hyakumachi et al., 2013).

In this study, although we documented *B. thuringiensis* strains have the ability to colonize and form biofilms on plant root surfaces, in the future, using the fluorescence flagella antibody to observe the three-dimensional biofilm and quantify biofilm formed by *B. thuringiensis* will be necessary. Recently, we proved that *B. thuringiensis* strains

suppressed the development of wilt symptoms caused by FOL in tomato plants. And *B. thuringiensis* strains are plant growth promoting rhizobacteria (PGPR) that can promote plant growth, seed germination and shoot elongation (unpublished). Furthermore, we would like to determine if the biofilm formation and colonization by *B. thuringiensis* strains could provide protection to the plant and control plant disease.

Fig. 3-1.

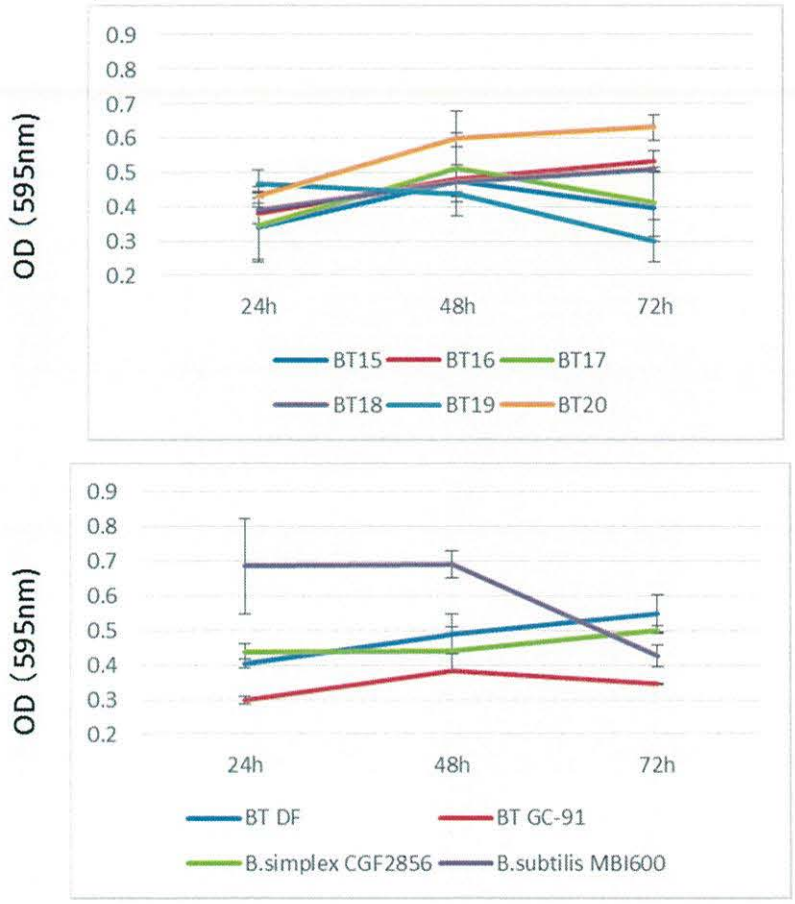

Fig.3-1. OD595 of solubilized crystal violet from microtiter plate assay (filled circles). After 24h, 48h, 72h of incubation, biofilm density was measured as described in the text. The data represent the means of three independent experiments. The error bars represent standard deviations.

Fig. 3-2.

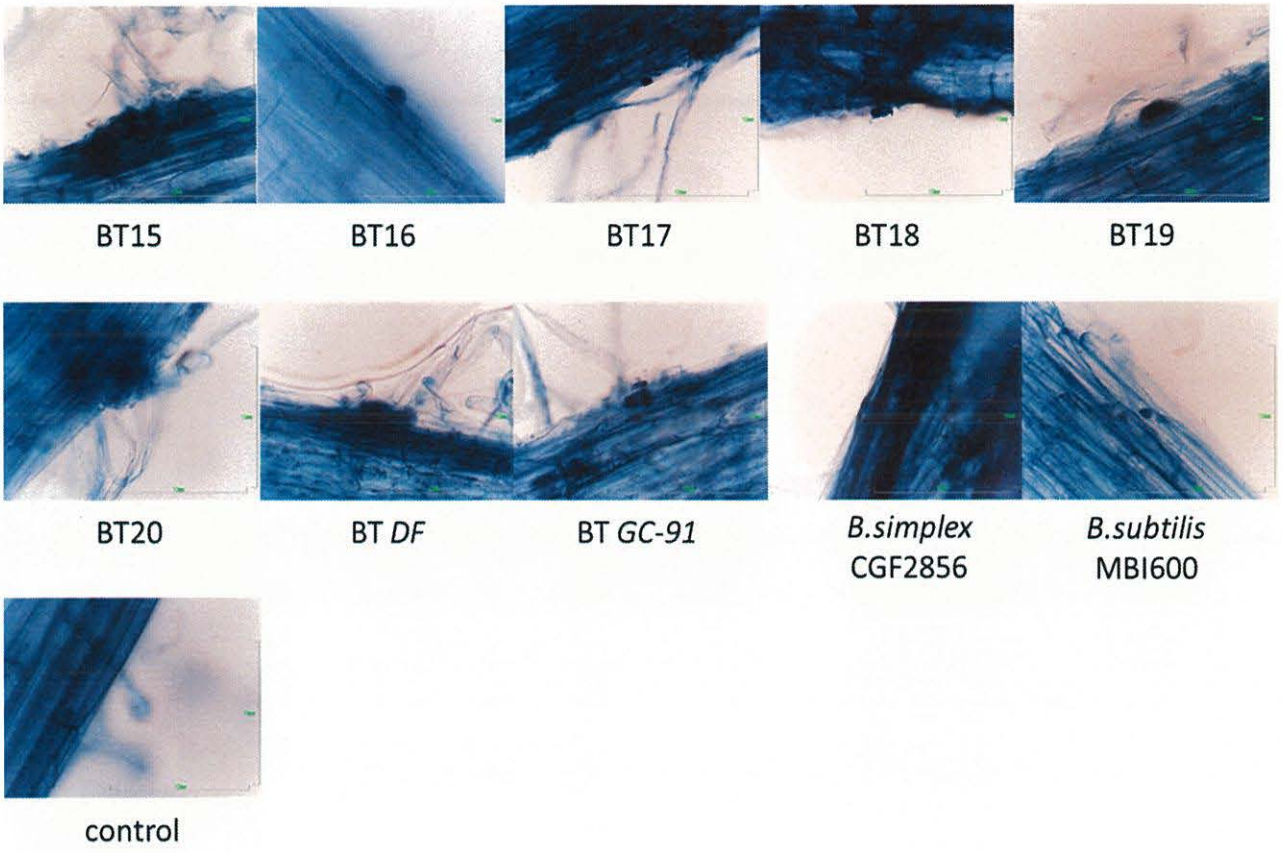

Fig.3-2. In liquid co-culture, the tomato roots cultured with liquid bacterial suspension for 3 days. After 3days culture, the tomato seedlings were dyed with 5% trypan blue. An optical microscope was used to observe the colony formed by bacteria on root surfaces.

Fig. 3-3.

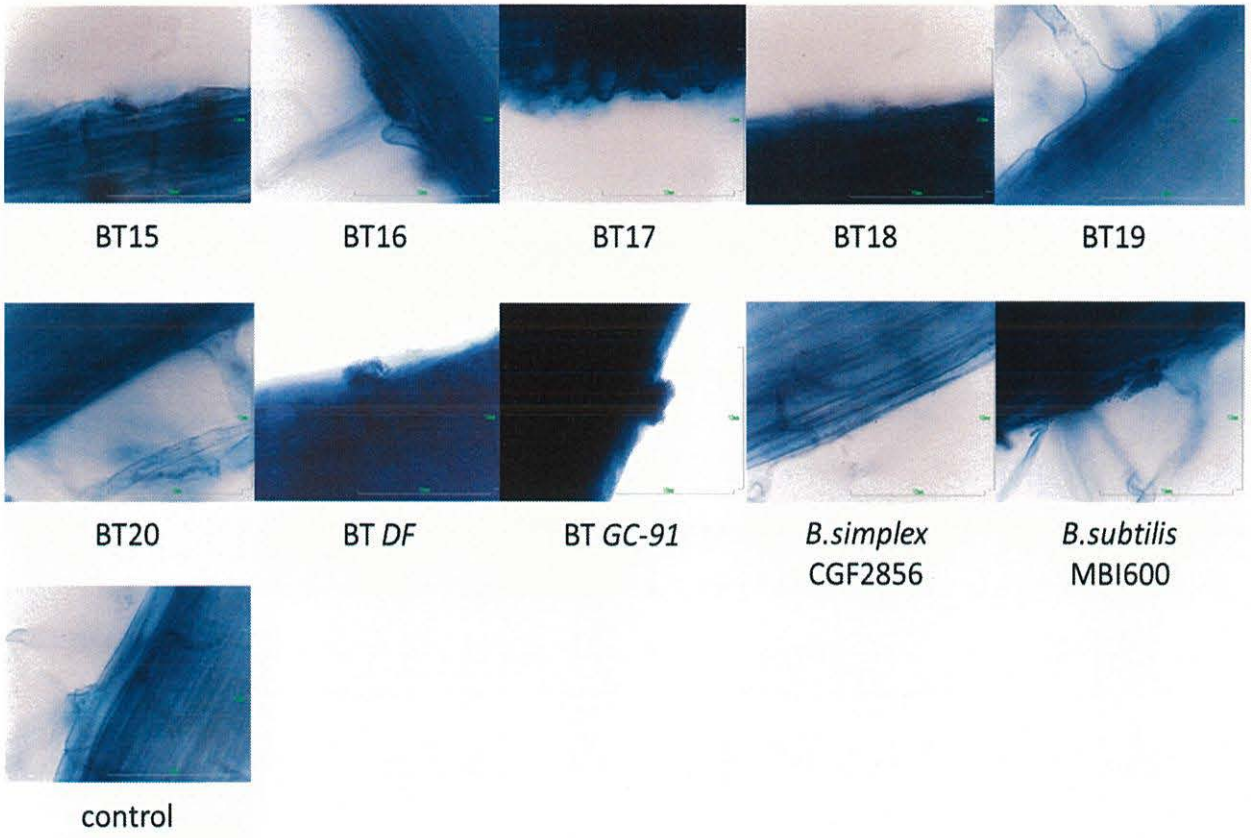

Fig.3-3. In soil co-cultivation, the tomato roots were plant in soil condition and inoculated bacterial suspension. After 5 days culture, the tomato seedlings were dyed with 5% trypan blue. An optical microscope was used to observe the colony formed by bacteria on root surfaces (Bars=10mm).

CHAPTER IV.

Toxicity of *Bacillus thuringiensis* to *Meloidogyne incognita*

4.1 Introduction of chapter IV

B. thuringiensis has been used as an effective bio-insecticide because it produces the proteins Cry and Cyt, which are highly toxic to insects in certain situations. Root-knot nematode *Meloidogyne incognita*, is an important plant parasite, it prefers to attack the root of its host plant and can cause extensive damage to a wide variety of economically important crops. Recently, *B. thuringiensis* strains have the nematicidal activity against root knot nematode was proved. So we focus on using *B. thuringiensis* as a nematicide to control the *M. incognita*.

In this study, the nematicide effect of *B. thuringiensis* was indentified by using bacteria culture filtrate (CF) treated second-stage nematode (J2) and egg sac. J2 suspensions (0.1 ml) were placed into wells of a 24-well tissue culture plate (50J2/well) containing 0.9 ml of bacteria CF. Liquid LB was used as the control. The effect of bacteria CF on the viability of J2 was determined after 12h. Bacteria CF from *B. thuringiensis* strains significantly increased the percentages of J2 motility compare to untreated control.

Also, we inoculated bacteria CF of six strains of *B. thuringiensis* on *M. incognita*

egg sac by co-culture method for 24h, and removed the egg sac into sterilized water. 3 days after inoculation, counted the quantity of hatched J2 in the bacteria CF and un-hatched eggs in the egg sac to calculate the hatchability of egg. *B. thuringiensis* strains reduced the hatchability of egg (from 26.3% to 55.8%) compare to the untreated control.

4.2 Materials and methods

4.2.1 Bacterial strains preparation

Under tested *B. thuringiensis* strains *B. thuringiensis sotto* (BT15), *B. thuringiensis israelensis* (BT16), *B. thuringiensis japonensis* (BT17), *B. thuringiensis kurstaki* (BT18), *B. thuringiensis roskildiensis* (BT19), *B. thuringiensis CR371-H* (BT20) were from Hokkaido University, Research Faculty of Agriculture Applied Bioscience Applied Molecular Biology Laboratory. For inoculum preparation, six strains of *B. thuringiensis* were inoculated in Luria Bertani broth (LB) broth and grown for 48h at 30°C. The bacterial suspension (BS) was adjusted to 1 OD. The preparation of *B. thuringiensis* culture filtrate (CF): inoculum the six strains of bacterial in liquid

LB broth, and grown for 48h with constant shaking (180 rpm) at 28±2°C, the bacterial suspension (BS) was adjusted to 1 OD. The culture obtained at stationery phase was centrifuged at 6000 rev/min for 10 min. The pellet of bacterial cells suspension (CS) was resuspended in phosphate buffer (100mM; pH7.0). The cell concentration was adjusted to 3×10^8 CFU/ml.

4.2.2 Nematode *M. incognita* eggs and second-stage juveniles (J2) preparation

M. incognita was obtained from pure cultures maintained in roots of tomato plants. About 90-day-old tomato seedlings (*Lycopersicon esculentum*) that were heavily infected with root-knot nematodes were used. The roots were removed from the infested soil and washed gently using tape water. Separated the egg masses were from the roots using a sterile dissecting needle and forceps. *M. incognita* egg masses were extracted from severely infected tomato roots, and surface-sterilized using a 1.5 % NaClO solution (Hussey and Barker 1973), and subsequently washed with distilled water three times. The surface-sterilized egg masses were stored in refrigerator at 10°C to prevent hatching before application of treatments.

Active juveniles (J2) of *M. incognita* were obtained by using Baermann funnel

method, incubated surface-sterilized egg masses in sterile water for 5 days at 25°C (Southey, 1986). Emerging J2s were collected daily and stored at 15°C (Oka et al., 2012). The population density of J2s for lethality experiment was determined to 500 J2s/ml.

4.2.3 Effect of Bacterial strains on *M. incognita* J2 lethality

The lethality assay of *B. thuringiensis* strains against J2 of *M. incognita* was set up in a 24-well plate format in five replicates for individual *B. thuringiensis* strains treatments and control (Masler, 2007). A single well consisted of 0.1 ml *M. incognita* J2 suspensions (50J2s/well), 0.9 ml individual *B. thuringiensis* strains suspension (BS, CF, CS) and liquid LB as control. The 24-well culture plates were incubated at 25°C for 4 days and maintain the humidity. J2 of *M. incognita* viability was determined based on movement. A moving J2 was considered as alive. Paralyzed (non-mobile) Juveniles were considered to be dead, if they did not move when probed with a fine needle. The entire assays were carried out in five replicates.

4.2.4 Effect of Bacterial strains on *M. incognita* egg hatching

The effect of *B. thuringiensis* strains on egg hatch of *M. incognita* was determined by co-culture method. Five equal sized egg masses were randomly picked using sterile forceps and were placed in each Petri dish containing the 1ml *B. thuringiensis* strains (BS, CF, CS separately). Egg masses kept in liquid LB were used as a control. The Petri dish containing the suspension and the egg masses were incubated at 25°C. 48 hours after treatment application, the egg masses were washed, then transferred to 24-well plate, 1 egg mass with 1ml sterile water were set up in one well and incubated at 25°C. The numbers of freshly hatched J2s were recorded at 7 days. The experiment was conducted five replications per treatment.

4.2.5 Effect of Bacterial strains on the development of eggs

Five equal sized egg masses were randomly picked using sterile forceps and were placed in each Petri dish containing the 1ml *B. thuringiensis* strains (BS, CF, CS separately). Egg masses kept in liquid LB were used as a control. The Petri dish containing the suspension and the egg masses were incubated at 25°C. 48 hours after treatment application, the egg masses were washed, then transferred to 24-well plate, 1 egg mass with 1ml sterile water were set up in one well and incubated at 25°C for 7

days. Egg masses were placed on slider glass and covered with cover glass, and crushed the egg masses to observe the normal eggs and abnormal eggs using optical microscope. The abnormal eggs were classified into eggs with vacuoles, dark discolored eggs, and floating eggs.

4.3 RESULTS

4.3.1 Effect of Bacterial strains on *M. incognita* J2 lethality

The lethality of *B. thuringiensis* strains against J2 of *M. incognita* ranged from 89.33% to 99.33%. There were significant differences ($P < 0.05$) in mobility of juveniles between the bacterial treatments and control (Fig. 4-1). BS of BT18 caused the highest inhibition in motility of juveniles 24 h after treatment application. Motility of *M. incognita* J2 by *B. thuringiensis* strains increased from 43.3% to 61.3%, compared to untreated control. None of the J2s recovered from the paralysis caused by any *B. thuringiensis* strains, 48h after treatment with water. This result showed that *B. thuringiensis* strains have the lethal activity to J2 of *M. incognita*.

4.3.2 Effect of Bacterial strains on *M. incognita* egg hatching

To evaluate the effect of *B. thuringiensis* strains on egg hatch of *M. incognita*, surface-sterilized egg masses were co-cultured in *B. thuringiensis* strains BS, CF, CS for 48 h, then hatched in sterile water. After 7 days incubation, the numbers of hatched J2s were decreased than control (Fig. 4-2). BS, CF, CS of *B. thuringiensis* strains significantly inhibited egg hatching. BS of *B. thuringiensis* strains decreased egg hatching rate ranged from 95.4% to 78.3%, most effective BS of *B. thuringiensis* strains was BT15 (Fig. 4-2a); CF of *B. thuringiensis* strains decreased egg hatching rate ranged from 66.3% to 95.8%, most effective CF of *B. thuringiensis* strains was BT15 (Fig. 4-2b); CS of *B. thuringiensis* strains decreased egg hatching rate ranged from 72. 7% to 93.1%, most effective CS of *B. thuringiensis* strains was BT18 (Fig. 4-2c). Percent of *B. thuringiensis* strains of egg hatching decreased from 26.3% to 55.8%, compared to control. This result indicated that *B. thuringiensis* strains have the ability of suppression egg hatching of *M. incognita*.

4.3.3 Effect of Bacterial strains on the development of *M. incognita* eggs

The BS, CF, CS of *B. thuringiensis* strains treatment significantly ($P < 0.01$)

increased the abnormal eggs when compared with the untreated control, and demonstrated toxicity against the *M. incognita* eggs (Fig. 4-3). After 7 days treatment, the BS of six *B. thuringiensis* strains killed from 39.0% to 63.0% of the eggs (Fig. 4-3a). The CF of six *B. thuringiensis* strains killed from 19.0% to 42.0% of the eggs (Fig. 4-3b). The CS of six *B. thuringiensis* strains killed from 19.0% to 42.0% of the eggs (Fig. 4-3c). Especially, BT18 showed the most effective activity against eggs.

4.4 DISCUSSION

The most damaging diseases that destroy crops are caused by many plant pathogenic organisms, of these diseases are those caused by plant parasite nematodes. Numerous biological agents have been used to control RKNs. Biocontrol agents reduce nematode populations mainly by several mechanisms: production of toxins; changing nematode physiology behavior; promoting plant growth; interrupting with plant recognition; induction of systemic resistance (Siddiqui et al., 1999; Sikora et al., 1993; El-Nagdi et al., 2004; Oostendorp et al., 1990; Liu et al., 1995). The crystal proteins made by the entomopathogen *B. thuringiensis* are pore-forming toxins that specifically

different target insect orders and nematodes are used worldwide to control insect pests (Wei et al., 2003; Schulenburg and Muller, 2004; Rae et al., 2008). In the last decade, several crystal proteins Cry5, Cry6, Cry12, Cry13, Cry14, Cry21, and Cry55 presented activity against nematode have been observed (Jouzani et al., 2008; Guo et al., 2008). *B. thuringiensis* strains from various locations showed their lethality to J2 and egg of root-knot nematodes (*M. incognita* and *M. javanica*) under laboratory conditions (Al-Banna and Khyami-Horani, 2004).

This study clearly indicated nematicidal activities of *B. thuringiensis* culture filtrate and bacterial suspension against *M. incognita*, including inhibition of egg hatch and J2 lethal activity. This result suggested that *B. thuringiensis* showed a nematicidal activity, and might play a significant role in nematode management, since egg hatching, survival, motility are important behavior for RKN life cycle (Ediz and Dickerson, 1976; Jaubert et al., 2005). Therefore, if any step of the RKN life cycle was interfered, the nematode reproduction would be interrupted effectively. This result indicated that *B. thuringiensis* was a very promising biocontrol agent against RKN.

The utilization of ten different isolates of *B. thuringiensis*, which purified Cry proteins and the supernatant, containing vegetative proteins, was applied to *M.*

incognita, in laboratory and in greenhouse was showed (Mohammed et al., 2008). The results showed that four isolates, *Bt*7, *Bt*7N, *Bt*Soto and *Bt*Den, presented 90 and 100% mortality when utilizing Cry proteins and vegetative protein fractions, respectively. In greenhouse, these isolates were efficient, with *Bt*7N showing the best result in reducing the number of eggs, the egg mass and the number of galls, when compared to the other treatments and control. Our research also indicated Cry proteins and vegetative cell suspension of six *B. thuringiensis* strains showed toxicity to J2 and eggs of *M. incognita.* The results of the current research are in agreement with several numbers of earlier studies (Griffitts et al., 2001; Lopez-Arellano et al., 2002; Mozgovaya et al., 2002; Wei et al., 2003; Griffitts et al., 2003; Hala et al., 2003; Huffman et al., 2004; Kotze et al., 2005).

When *M. incognita* was treated with *B. thuringiensis* culture filtrates, significant difference on mortality, and egg hatching rate than control were observed, which suggests that the nematicidal activity of *B. thuringiensis* is likely attributed to secondary metabolites, not only proteins. *Bacillus* species can product different types of enzymes and antibiotics, which are strongly antagonistic to several pathogens such as RKN (Saxena et al., 2000). Nematode egg shells consists of three main layers namely,

vitelline layer, chitinous layer and lipid layer (Zdarska et al., 2001). It might be possible that *B. thuringiensis* produce some secondary metabolites, which damages the egg shell of root-knot nematode or the juveniles within the eggs. Furthermore, inhibition in J2 emergence and great J2 mortality *in vitro* by culture filtrate application with Bt64 have been recorded (Khan et al., 2010). This may be due to the production of metabolites against root-knot nematodes. Our study also agreed with this opinion, the hatching inhibition and abnormal egg increasing effect of the *B. thuringiensis* culture filtrate might be due to secondary metabolites by *B. thuringiensis* with nematicidal property. However, the nematicidal secondary metabolites produced by *B. thuringiensis* have not been clearly characterized until now, and further search should be carried on.

The eggplant crop is also the target of attack by nematodes, mainly of the genus *M. incognita*. Different microorganisms were used in vitro assays and in a greenhouse (Ashoub and Amara, 2010). Among these microorganisms, three isolates of *B. thuringiensis* identified were Biovar1, Biovar2 and Biovar3. J2s of each isolate were tested separately, with a mortality rate of 70.0%, 85.0% and 90.0%, respectively. However the mortality was higher (92%) when the three isolates were used simultaneously after a 24h exposure. Inspired by these results, we would like use the

mixture of 6 *B. thuringiensis* strains to control the disease in the further study, and the effect was expected.

In conclusion, this study demonstrated that *B. thuringiensis* strains provide effective nematicidal activity against *M. incognita*. This research also suggested that the nematicidal activity of *B. thuringiensis* strains is attributed to both the production of secondary metabolites and vegetative proteins. This indicates that *B. thuringiensis* is an effective agent to be used in the integrated management of RKN in tomato. Further, greenhouse experiment will be in progress to evidence nematicidal activity of *B. thuringiensis* strains.

Fig. 4-1.

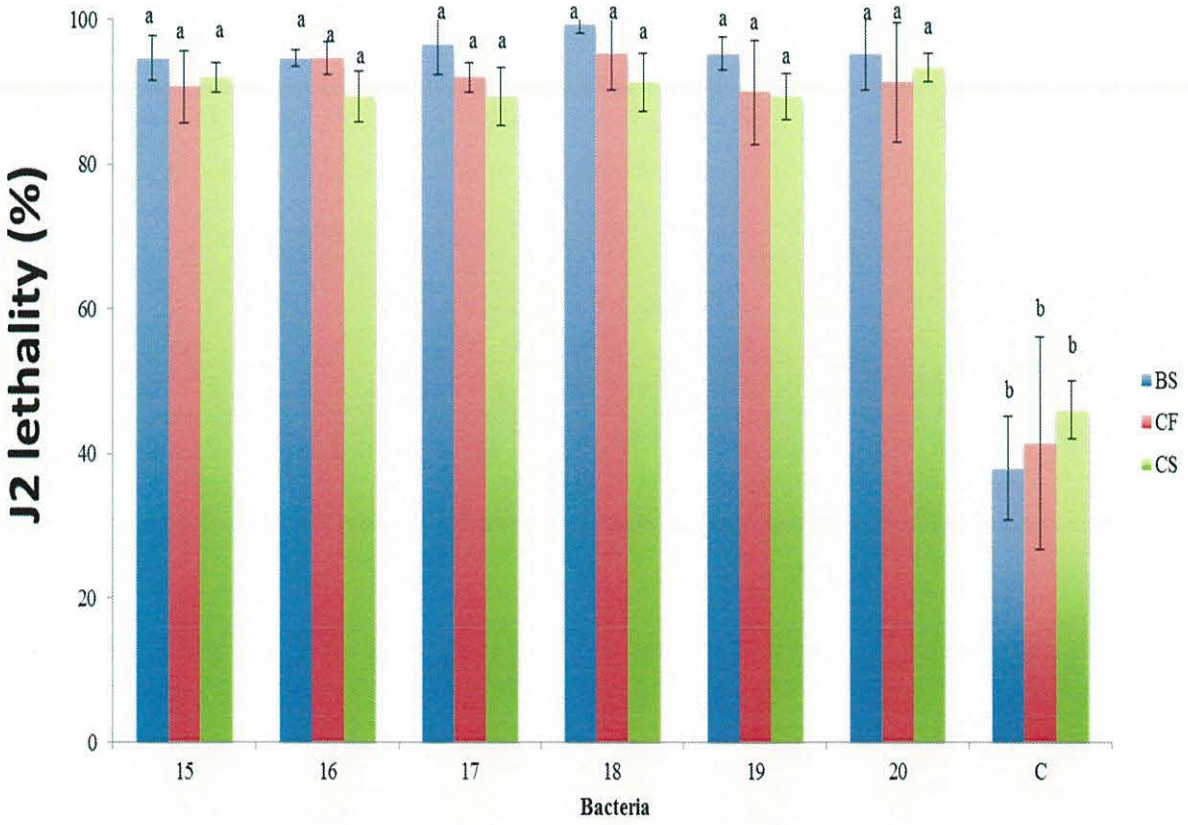

Fig. 4-1. The lethality assay of *B. thuringiensis* strains against J2 of *M. incognita* was done in a 24-well plate format after for 4 days co-culture. Mean and standard deviation of five replicates per experiment are presented. Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

BS: bacterial suspension; CF: culture filtrate; CS: bacterial cells suspension

15-20: *B. thuringiensis* strains; C: LB control

Fig. 4-2.

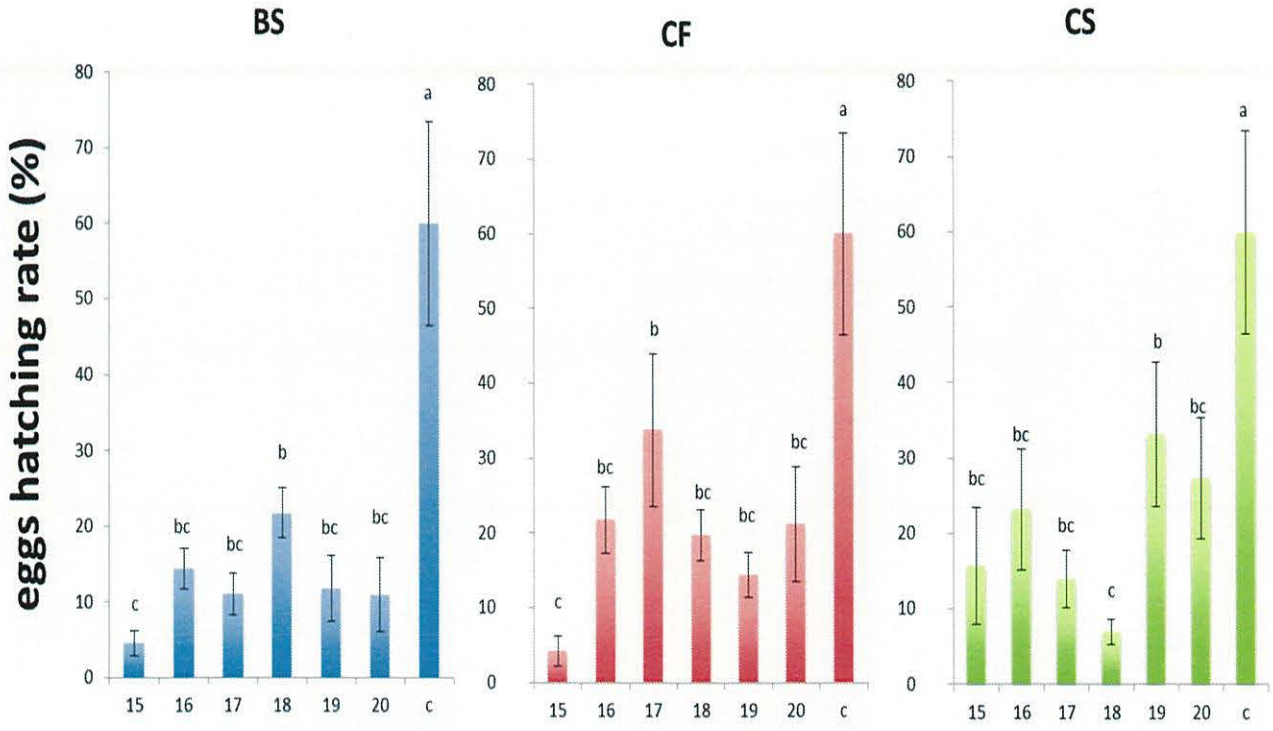

Fig. 4-2. The effect of *B. thuringiensis* strains on egg hatch of *M. incognita* was determined by treating egg mass with *B. thuringiensis* strains for 48h. The numbers of freshly hatched J2s were recorded at 7 days. Mean and standard deviation of five replicates per experiment are presented. Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

BS: bacterial suspension; CF: culture filtrate; CS: bacterial cells suspension

15-20: *B. thuringiensis* strains; C: LB control

Fig. 4-3.

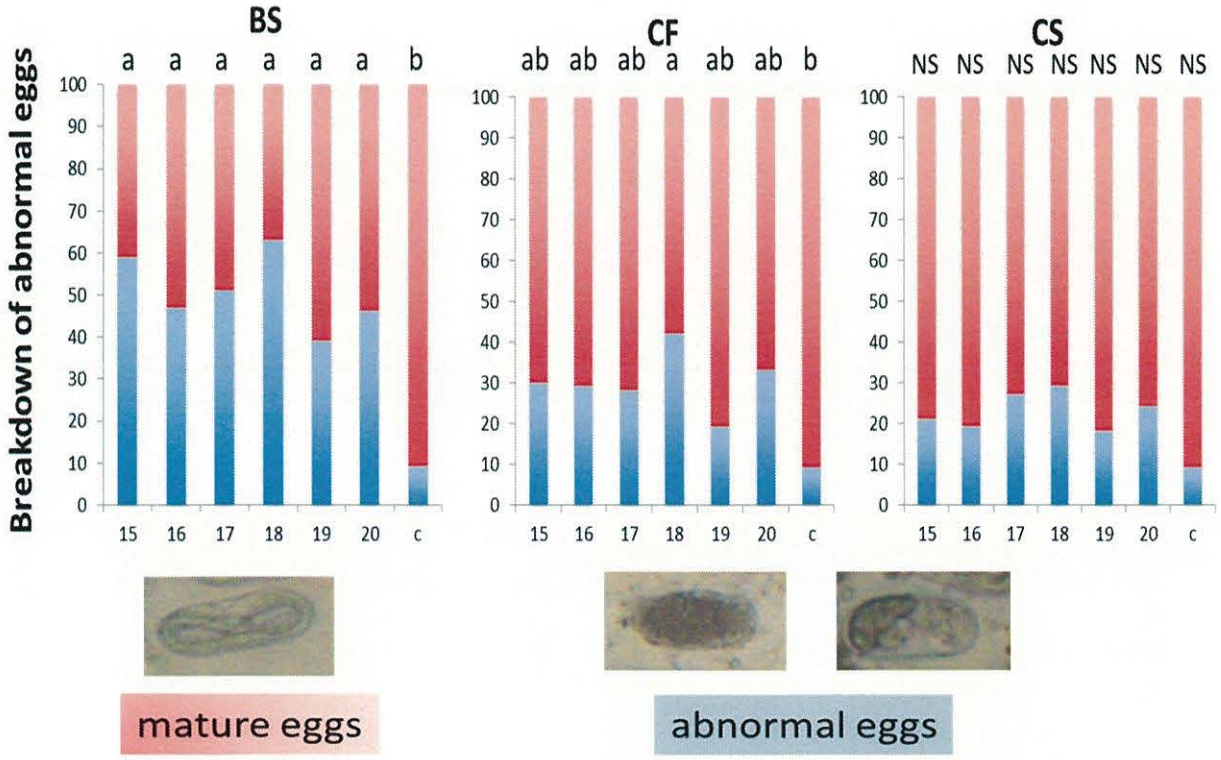

Fig. 4-3. Egg masses treated with *B. thuringiensis* strains for 48h, then transferred sterile water. After 7 days' inoculation, crushed the egg masses, and observed the normal and abnormal eggs using optical microscope. Mean and standard deviation of five replicates per experiment are presented. Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

BS: bacterial suspension; CF: culture filtrate; CS: bacterial cells suspension; 15-20: *B. thuringiensis* strains; C: LB control. Red: normal eggs; Blue: abnormal eggs with vacuoles, dark discolored and floating eggs.

CHAPTER V.

The effect of *Bacillus thuringiensis* to Complex diseases

5.1 Introduction of chapter V

Root-knot nematodes and Fusarium wilt are soil-borne diseases, both of them can cause severely economic losses worldwide to a broad range of plants. Complex diseases in soil caused by soil-borne fungal pathogens, *Fusarium* spp. and root knot nematodes, *Meloidogyne* spp. often damage plants more severely and make more difficultly to control the disease than single pathogens alone. *B. thuringiensis* has been used as an effective bioinsecticide worldwide. Recently, some research reported that *B. thuringiensis* show a potential activity to control plant diseases such as root-knot nematodes and bacterial wilt (Mohammed et al., 2008; Hyakumachi et al., 2013).

Also in our previous study has confirmed that *B. thuringiensis* could suppress the wilt disease caused by *Fusarium oxysporum* f.sp. *lycopersici* (FOL), act as plant growth promoting rhizobacteria (PGPR) and *B.thuringiensis* exhibited a variety of nematicidal properties to *M. incognita* J2, egg mass and egg. So in this study, we would like to use potential *B. thuringiensis* strains which were selected in previous experiments to control complex disease caused by *M. incognita* and FOL in tomato plants.

As a result, *B. thuringiensis* strains could significantly suppress the symptoms of

complex disease; induce root gall; decrease wilting score and internal symptom; promote shoot length and weight.

5.2 Materials and methods

5.2.1 Fungal pathogen and bacterial strains preparation

Fusarium oxysporum f.sp. *lycopersici* (FOL) race 2 was used in the pathogenicity experiments. The isolate (FOL) was grown on potato dextrose agar (PDA) medium at 25°C for 7 days in a culture dish and then stored at 5°C. The isolate, recovered as needed from storage, was grown on PDA at 25°C for 7 days prior to inoculation. FOL-infested soil was prepared. Sterile soil and maize flour were mixed completely at the rate of 2:1 (v/v) to make the culture soil. In a flask filled with 12ml distilled water, 40 ml of culture soil was taken, and sterilized in an autoclave. The FOL was inoculated in the sterilized culture soil, and stored in a 25°C incubator for 7 days. Sterile soil (1 L) and 3% (v/v) FOL infested soil were mixed completely and put in a sterilized plastic plate to make the FOL infested soil. The tested *B. thuringiensis sotto* (BT15), *B. thuringiensis israelensis* (BT16), *B. thuringiensis japonensis* (BT17), *B. thuringiensis*

kurstaki (BT18), *B. thuringiensis roskildiensis* (BT19), *B. thuringiensis CR371-H* (BT20) used for the present investigation were obtained from Research Faculty of Agriculture, Applied Bioscience Applied Molecular Biology Laboratory, Hokkaido University. For inoculum preparation, six strains of *B. thuringiensis* were inoculated in Luria Bertani broth (LB) and grown for 1 week at 30°C. For the preparation of *B. thuringiensis* suspension, the six strains of bacteria were inoculated in liquid LB broth and grown for 24 h with constant shaking (150 rpm) at 28 ± 2°C. The bacterial concentration was adjusted to 3×10^8 cfu.

5.2.2 Nematode *M. incognita* second-stage juveniles (J2) preparation

M. incognita was obtained from pure cultures maintained in roots of tomato plants. About 90-day-old tomato seedlings (*Lycopersicon esculentum*) that were heavily infected with root-knot nematodes were used. The roots were removed from the infested soil and washed gently using tape water. Separated the egg masses were from the roots using a sterile dissecting needle and forceps. *M. incognita* egg masses were extracted from severely infected tomato roots, and surface-sterilized using a 1.5 % NaClO solution (Hussey and Barker, 1973), and subsequently washed with distilled water three

times. The surface-sterilized egg masses were stored in refrigerator at 10°C to prevent hatching before application of treatments.

Active juveniles (J2) of *M. incognita* were obtained by using Baermann funnel method, incubated surface-sterilized egg masses in sterile water for 5 days at 25°C (Southey, 1986). Emerging J2s were collected daily and stored at 15°C (Oka et al., 2012). The population density of J2s for lethality experiment was determined to 100 J2s/ml.

5.2.3 Pot experiment

Seeds of tomato were surface sterilized with 1% sodium hypochlorite for 1 min and washed with sterilized water 3 times. Tomato seedlings were grown at 25°C. After 4-week cultivate, tomato seedlings were treated with bacterial culture at 10 ml/pot (3×10^8 cfu/ml). After 7 days, 5ml J2 suspensions were inoculated around plant roots (500 J2s/pot). After 7 days, the bacteria-nematode treated tomato seedlings were placed in the FOL-infested soil (15 pots/plate). 11 experiment groups were set: 3 BT strains (BT17, BT18, BT20) + FOL; 3 BT strains (BT17, BT18, BT20) + RKN; 3 BT strains (BT17, BT18, BT20) + FOL + RKN; RKN control; FOL control; FOL + RKN control; distilled

water untreated control. 4 weeks after pathogen inoculation, the fresh weight, shoot length, root length were measured and the wilting score, gall score, etiolation score, internal symptom were evaluated. Wilting score was evaluated based on leaf symptoms of wilting as follows: 0 = no wilt symptoms; 1 = <25% of wilting leaves; 2 = 26–50% of wilting leaves; 3 = 51–75% of wilting leaves; 4 = 76–100% of wilting leaves (Bora et al., 2004). Severity of root galling in *M. incognita* infested tomato seedlings was assessed on a 0-4 rating scale according to the percentage of galled tissue (Barker, 1985), where 0 = no galled roots; 1 = 1-25% galled roots; 2 = 26-50% galled roots; 3 = 51-75% galled roots; 4 = 76-100% galled roots. Etiolation score was evaluated based on leaf symptoms of yellow as follows: 0 = no yellow symptoms; 1 = <25% of yellow leaves; 2 = 26–50% of yellow leaves; 3 = 51–75% of yellow leaves; 4 = 76–100% of yellow leaves. Internal symptom score was determined by browning degree of plant vessel as follows: 0 = no browning symptoms; 1 = <33% of vessel browning; 2 = 34–67% of vessel browning; 3 = 68–100% of vessel browning.

5.3 RESULTS

5.3.1 Pot experiment

Wilt severity of Fusarium wilt- root knot nematode disease complex in the pot experiment was significantly reduced by the treatments of *B. thuringiensis* (Fig. 5-1). The control effects were estimated by wilting score, gall score, etiolation score, internal symptom, respectively. There was no significant difference between FOL control and FOL+ RKN control of wilting score and etiolation score, but FOL+ RKN control showed higher disease index synthetically. And *B. thuringiensis* treatments reduced wilting score (from 61.7% to 88.2%) and etiolation score (from 43.2% to 69.8%) than pathogen control (Fig. 5-2; Fig. 5-3). *B. thuringiensis* treatments decreased vascular damage (from 37.8% to 82.2%) than FOL control and FOL+ RKN control (Fig. 5-4). These treatments had also reduced gall formation compared to the pathogen control (from 45.8% to 60.0%). Unfortunately, distilled water untreated control was stained with RKN, so there were small gall formatted in water control (Fig. 5-5). Shoot length, root length and shoot weight of tomato plants were reduced significantly by the infection of both FOL and RKN, and treatments of these *B. thuringiensis* strains enhanced plant growth (increase shoot length from 26.3% to 32.1%; increase root length from 2.6% to 19.4%; increase shoot weight from 50.1% to 51.3%), especially

showing more shoot growth than pathogen control (Fig. 5-6; Fig. 5-7; Fig. 5-8). *B. thuringiensis* treatments presented a decrease of root weight than complex and RKN control, because a large number of root galls were formatted by RKN infection (Fig. 5-9).

5.4 DISCUSSION

Over the past three decades, considerable attention has been given to parasitic nematodes and soilborne fungi (Hirano, 1983). The first nematode-fungus complex disease was found by Atkinson in 1892, who observed that Fusarium wilt of cotton (caused by *Fusarium oxysporum* f. sp. *vasinfectum*) became more severe in the presence of root-knot nematodes (*Meloidogyne* spp.). Synergistic effect between Fusarium wilt pathogens and *Meloidogyne* spp. have been studied and documented in several host crops, including alfalfa (Griffin, 1986); tomatoes (Suleman et al., 1997); chickpeas (Kumar et al., 1998); coffee (Bertrand et al., 2000) and so on. The synergistic effect of *M. incognita* was confirmed in Fusarium wilt of tomato caused by FOL by comparing disease severity between single and combined inoculations of the two pathogens. Nematodes and fungi interact synergistically may cause more damage to a susceptible

host crop than either alone has been reported (France and Abawi, 1994). Combined infection of chickpea plants by *F. oxysporum* f. sp. *ciceri* and *M. javanica* increased the severity of Fusarium wilt (Uma Maheswari et al., 1997). Our results also agreed with these reports, although there was no significantly severity difference between pathogen control alone and complex disease control, complex disease presented a higher damage in appearance.

In the previous study, 6 strains of *B. thuringiensis* were tested for their antifungal and nematicidal activities. Out of those, all of the *B. thuringiensis* strains showed antifungal and nematicidal activities against FOL and *M. incognita*, respectively. The strains had variable effect against both pathogens; 3 strains BT17, BT18, BT20 showed the strongest inhibitory activity and inhibited nematode egg hatch. We used these 3 strains for pot experiment. The pot experiments indicated that addition of *B. thuringiensis* strains into potted soil suppressed the Fusarium wilt severity, reduced root gall formation on tomato and increased plant growth. *B. thuringiensis* controlled the wilt symptom effectively, since wilt score, etiolation score and internal symptom score were significantly reduced than untreated control. Root galls caused by *M. incognita* and FOL were fewer and smaller when treated with *B. thuringiensis* as compared to

untreated control. *B. thuringiensis* showed the plant promoting ability of shoot length, shoot weight and root length than untreated control, but presented a decrease of root weight than complex and RKN control. The unexpected increase in root weight in complex and RKN control was because of the nematodes in the root mass and large root galls.

Although the exact modes of action of *B. thuringiensis* strains are not fully studied in these experiments, they may be supposed that the reduction of the disease complex may be attributed to direct effects of metabolites that inhibit hyphal growth and egg hatch and induce mortality in J2, or enhanced host defence mechanism in roots that reduces invasion and consequent infection by pathogens and inhibition of giant cell formation (Glick et al., 2001). The biocontrol bacteria can affect plant by various mechanisms and the plant uses different abilities for growth promotion at various times. The mechanism involved in *Trichoderma* against *M. incognita* and *F. oxysporum* had been studied intensively in terms of antibiotic and enzyme production as hyphal interactions (Dennis and Webster, 1971; Elad *et al.,* 1982). *P. fluorescens* was found not only effective against *M. incognita* but also against wilt causing fungi. Significant suppression of complex diseases by *P. fluorescens* was due to its capability of altering

root exudates, which could alter nematode behavior and suppress nematode population in root system (Oostendrop and Sikora, 1989). The growth promoting rhizobacteria *Bacillus polymyxa*, may also suppress complex diseases in an indirect way by making the host healthier and stronger to resist pathogens (Khan and Khan, 1998).

There are reports where production of metabolites by rhizosphere bacteria causes lysis of nematode eggs (Westcott and Kluepfel 1993), reduces egg hatching (Oostendrop and Sikora 1989), affects vitality of second stage juveniles (Becker et al. 1988) and degrades specific root exudates resulting in reduced attraction and penetration of nematodes (Oostendrop and Sikora 1990). Siddiqui and Shaukat (2003) also reported that root colonization by rhizosphere bacteria reduced nematode invasion. The present study revealed that the tested *B. thuringiensis* strains had no adverse effect on plants and promoted growth. Thus, the protective properties of *B. thuringiensis* strains make them as environmentally friendly useful agent to reduce deleterious impact of disease complex caused by root-knot nematode and Fusarium wilt on plant growth, especially in organic farming system, where plant nutrition and disease control are the main limiting factor.

Fig. 5-1

Fig. 5-1 4-week tomato seedlings were treated with bacterial culture at 10 ml/pot (3×10^8 cfu/ml). After 7 days, 5 ml J2 suspensions were inoculated around plant roots (500 J2s/pot). After 7 days, the bacteria-nematode treated tomato seedlings were placed in the FOL-infested soil (15 pots/plate). 4 weeks after pathogen inoculation, wilting and etiolation symptoms were presented.

From light to right: distilled water untreated control; FOL control; RKN control; FOL+ RKN control; BT18 strains+ FOL; BT18 strains+ RKN; BT18 strains+ FOL + RKN

Fig. 5-2.

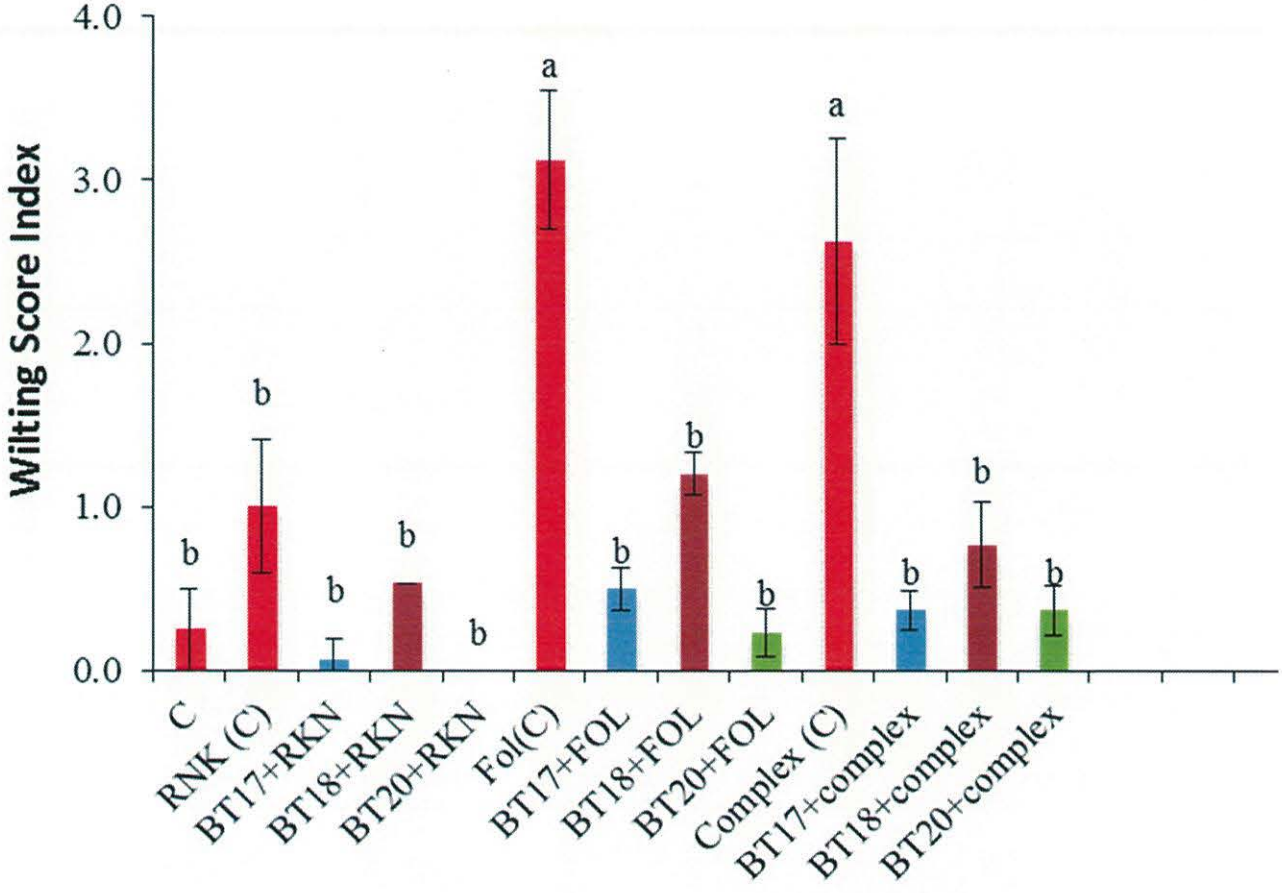

Fig. 5-2. Wilting score was evaluated based on leaf symptoms of wilting as follows: 0 = no wilt symptoms; 1 = <25% of wilting leaves; 2 = 26–50% of wilting leaves; 3 = 51–75% of wilting leaves; 4 = 76–100% of wilting leaves.

Mean and standard deviation of 15 replicates per experiment are presented.

Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

Fig. 5-3.

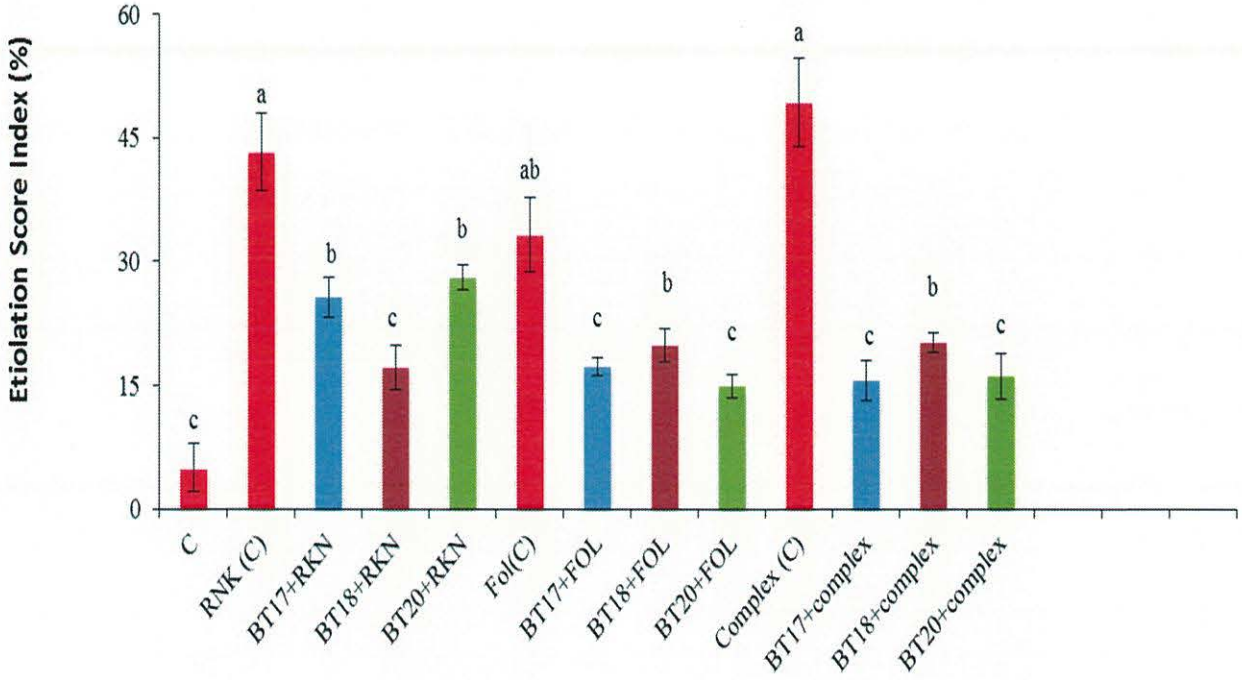

Fig. 5-3. Etiolation score was evaluated based on leaf symptoms of wilting as follows: 0 = no wilt symptoms; 1 = <25% of wilting leaves; 2 = 26–50% of wilting leaves; 3 = 51–75% of wilting leaves; 4 = 76–100% of wilting leaves.

Mean and standard deviation of 15 replicates per experiment are presented.

Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

Fig. 5-4.

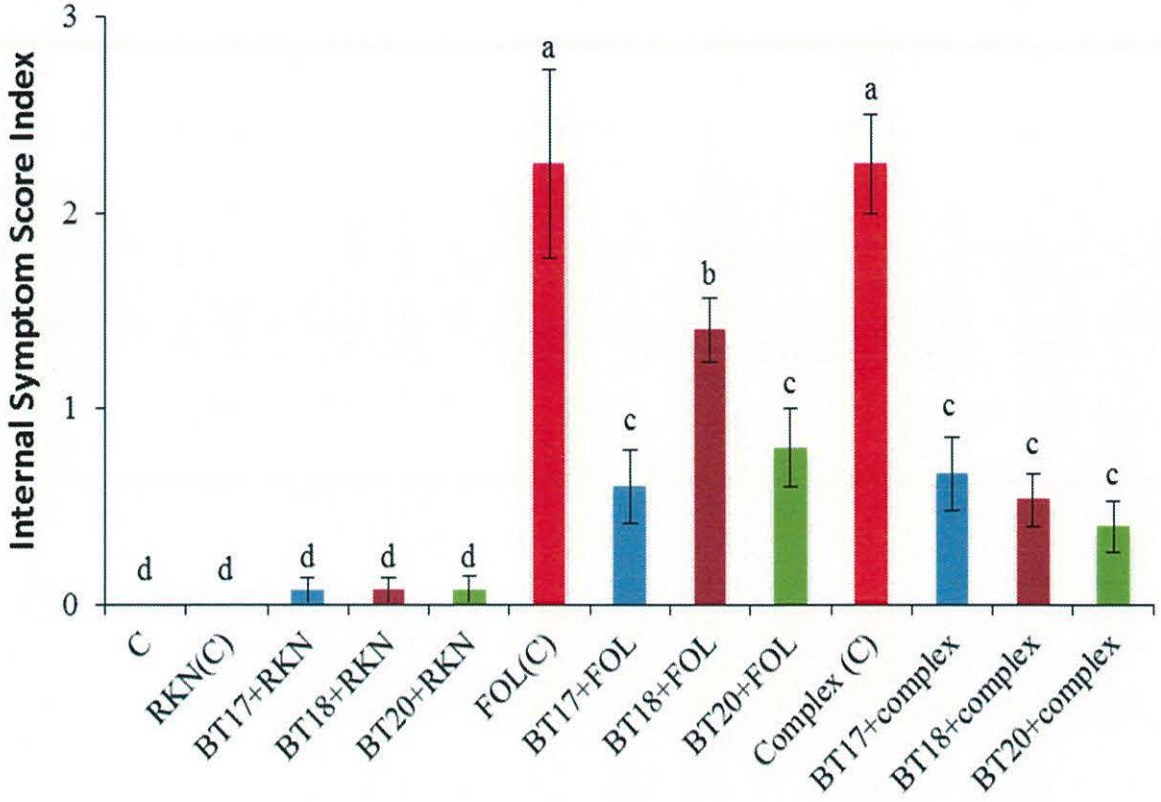

Fig. 5-4. Internal symptom score was determined by browning degree of plant vessel as follows: 0 = no browning symptoms; 1 = <33% of vessel browning; 2 = 34–67% of wilting leaves; 3 = 68–100% of wilting leaves.

Mean and standard deviation of 15 replicates per experiment are presented.

Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

Fig. 5-5.

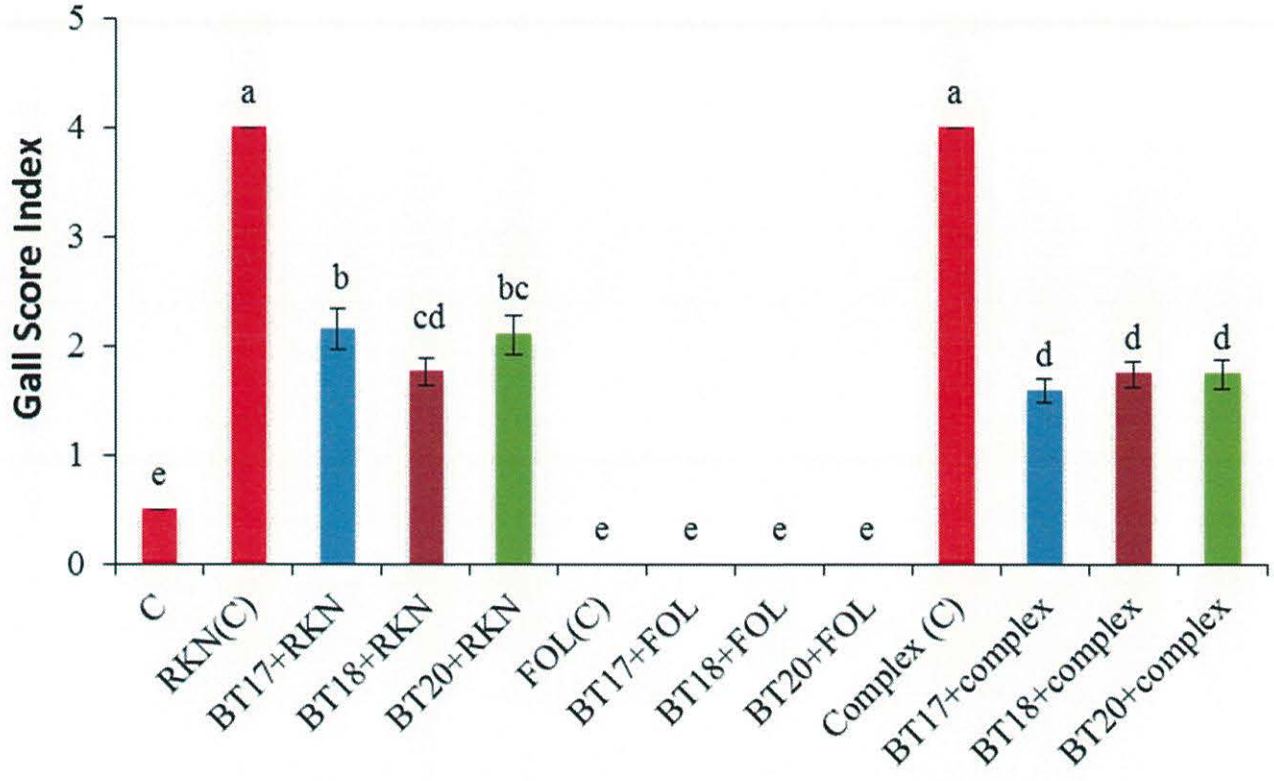

Fig. 5-5. Severity of root galling in *M. incognita* infested tomato seedlings was assessed on a 0–4 rating scale according to the percentage of galled tissue, where 0 = no galled roots; 1 = 1–25% galled roots; 2 = 26–50% galled roots; 3 = 51–75% galled roots; 4 = 76–100% galled roots; 5 = 91–100% galled roots.

Mean and standard deviation of 15 replicates per experiment are presented.

Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

Fig. 5-6.

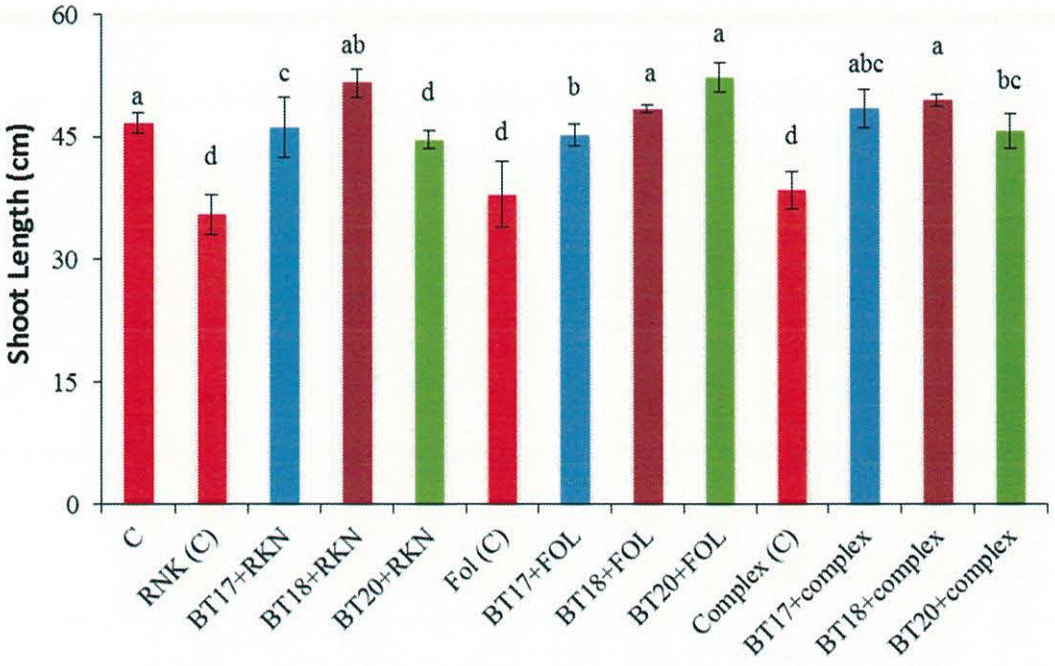

Fig. 5-6. 4-week tomato seedlings were treated with bacterial culture at 10 ml/pot (3×10^8 cfu/ml). After 7 days, 5ml J2 suspensions were inoculated around plant roots (500 J2s/pot). After 7 days, the bacteria-nematode treated tomato seedlings were placed in the FOL-infested soil (15 pots/plate). 4 weeks after pathogen inoculation, shoot length was measured.

Mean and standard deviation of 15 replicates per experiment are presented.

Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

Fig. 5-7.

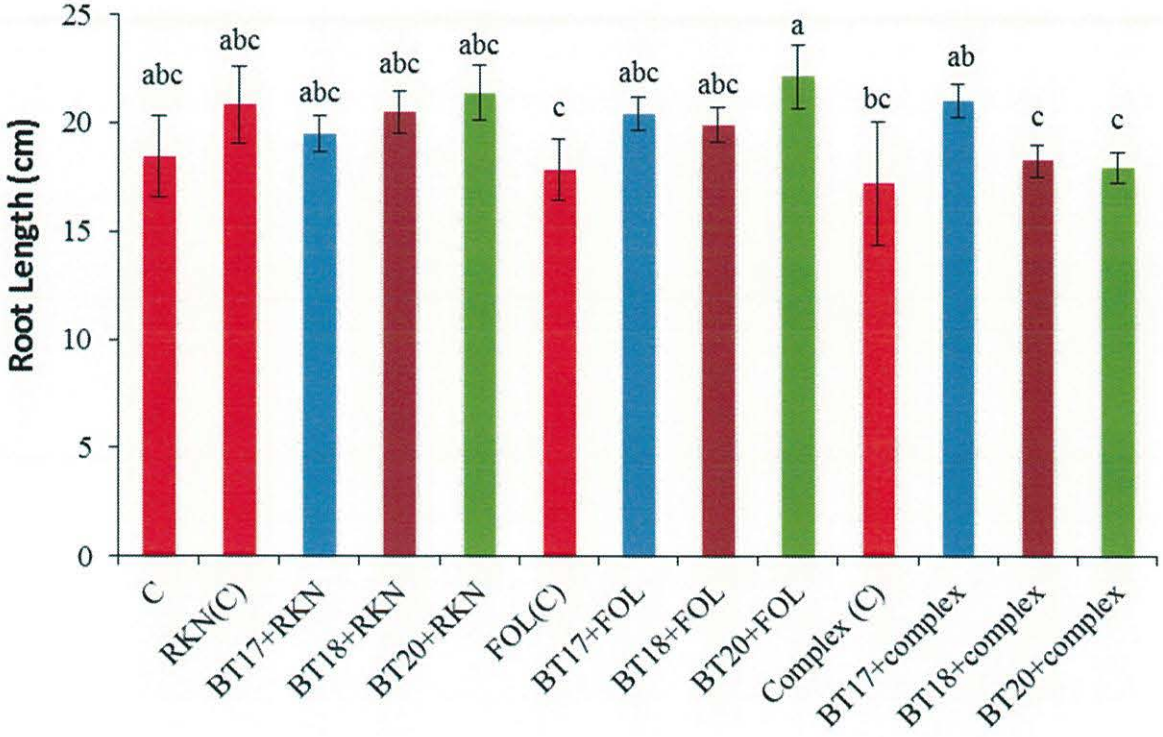

Fig. 5-7. 4-week tomato seedlings were treated with bacterial culture at 10 ml/pot (3×10^8 cfu/ml). After 7 days, 5ml J2 suspensions were inoculated around plant roots (500 J2s/pot). After 7 days, the bacteria-nematode treated tomato seedlings were placed in the FOL-infested soil (15 pots/plate). 4 weeks after pathogen inoculation, root length was measured.

Mean and standard deviation of 15 replicates per experiment are presented.

Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

Fig. 5-8.

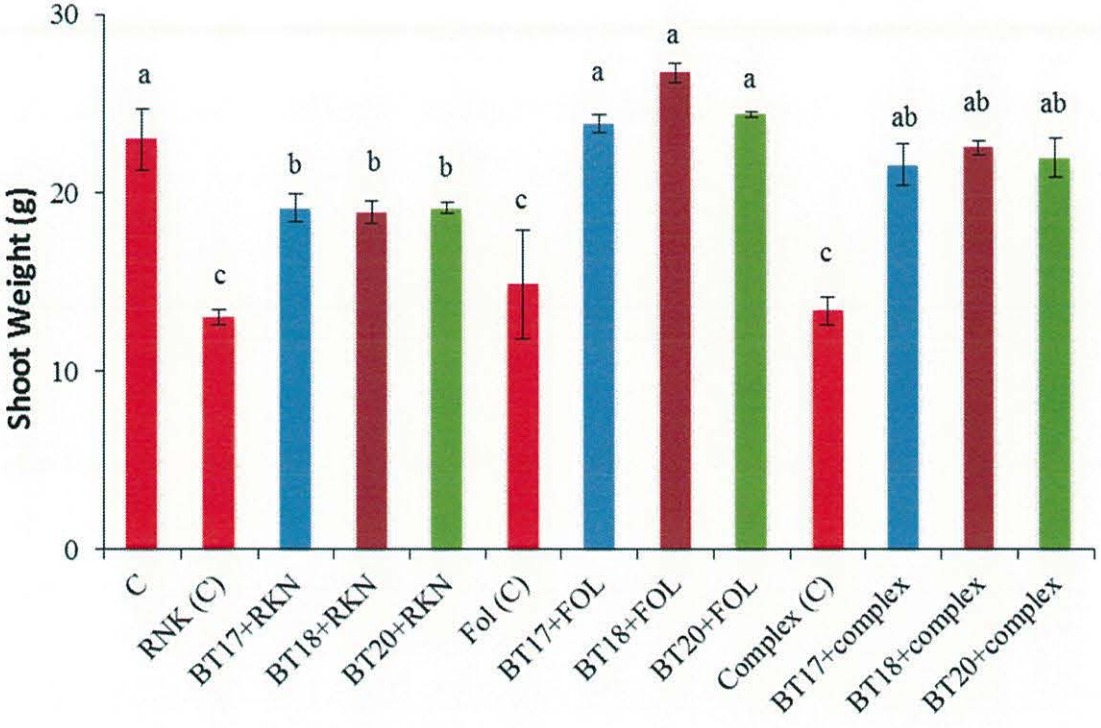

Fig. 5-8. 4-week tomato seedlings were treated with bacterial culture at 10 ml/pot (3×10^8 cfu/ml). After 7 days, 5ml J2 suspensions were inoculated around plant roots (500 J2s/pot). After 7 days, the bacteria-nematode treated tomato seedlings were placed in the FOL-infested soil (15 pots/plate). 4 weeks after pathogen inoculation, fresh shoot weight was measured.

Mean and standard deviation of 15 replicates per experiment are presented.

Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

Fig. 5-9.

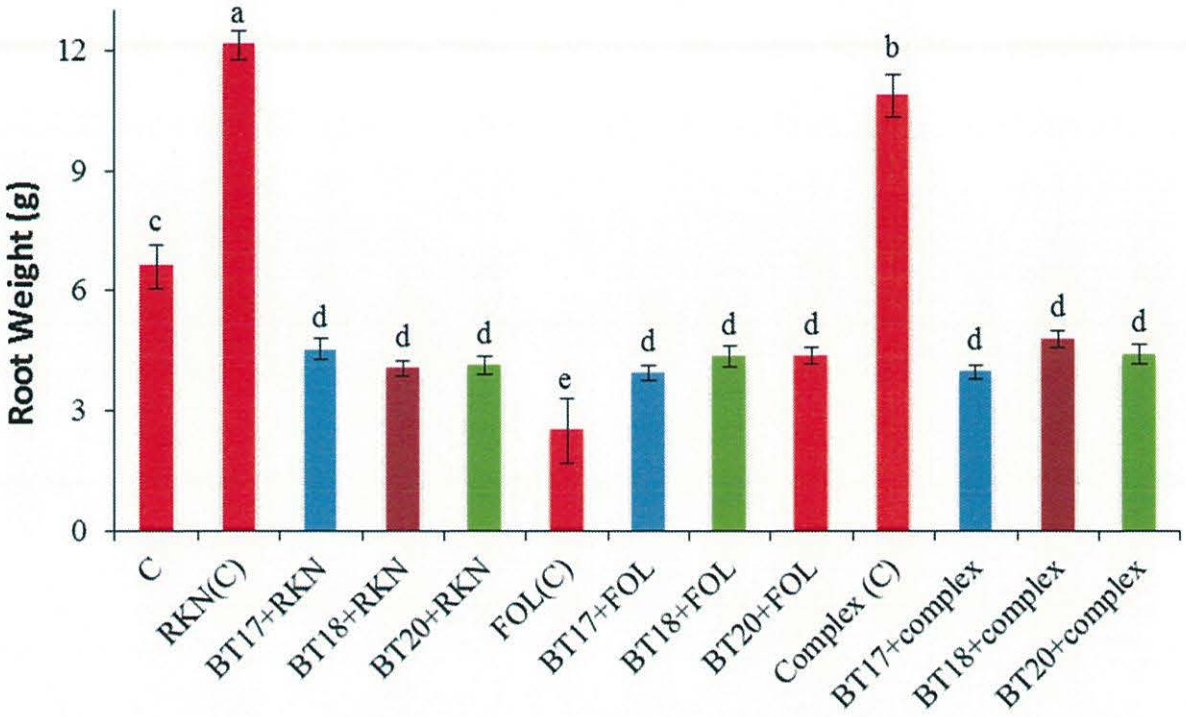

Fig. 5-9. 4-week tomato seedlings were treated with bacterial culture at 10 ml/pot (3×10^8 cfu/ml). After 7 days, 5ml J2 suspensions were inoculated around plant roots (500 J2s/pot). After 7 days, the bacteria-nematode treated tomato seedlings were placed in the FOL-infested soil (15 pots/plate). 4 weeks after pathogen inoculation, fresh root weight was measured.

Mean and standard deviation of 15 replicates per experiment are presented.

Values followed by different letters were significantly different according to the Tukey test at a 0.05 level of confidence.

CHAPTER VI. Overall Discussion

B. thuringiensis is a widespread endospore-forming bacteria, which can produce one or more parasporal crystals with different shapes and insecticidal activity. Since *B. thuringiensis* was first discovered in 1901, it has been used successfully as a biopesticide for many years (Schnepf et al., 1998; Khyami-Horani et al., 2003). However, in recent years, the roles of *B. thuringiensis* to control plant disease have been found out. Except for crystal protein and other insecticidal substances, *B. thuringiensis* also produces other active substances on the control of *Ralstonia solanacearum* (Hyakumachi et al., 2013); root knot nematode (Khalil et al., 2012) and other plant diseases. These findings showed the new functions of *B. thuringiensis* besides insecticidal activity, and help people to take advantage of *B. thuringiensis* more widely in agriculture.

Tomato (*Solanum lycopersicum*) is an important vegetable crop. In tomato cultivate, it is often hampered by vascular wilt caused by FOL and root-knot nematodes. Both of them are soil-borne diseases, and can cause complex disease. Complex disease creates severely economic losses worldwide to a broad range of plants. It is more difficultly to control the disease than single pathogens alone. Since the reports of *B. thuringiensis* on plant diseases control, this study was aimed to investigate the

possibility of *B. thuringiensis* as a potential biological control agent against plant disease and plant parasite nematode. And the plant growth promoting effect, biofilm formation ability, nematicidal effect and complex disease suppression ability of *B. thuringiensis* were confirmed. Overall, results obtained from these serial studies indicate that *B. thuringiensis* could control plant disease and plant parasite nematode effectively, as a potential biological control agent.

B. thuringiensis strains which be biocontrol agent of Fusarium wilt of tomato, were initially screened with the dual culture method. Then the candidates were tested in pot experiment to verify the ability to control disease. Antagonistic activity of six *B. thuringiensis* strains was estimated by the clear inhibition zone to FOL on PDA plates. BT17, BT18 were screened for in vitro inhibition of FOL with the dual culture method, with a clear inhibition zone on PDA plates. And these two strains also showed an effective effect on suppression of Fusarium wilt of tomato in pot experiments. It was found that strain BT20 showed potential biocontrol effect to Fusarium wilt of tomato in pot experiment, while not causing the clear inhibition zone of pathogen on PDA plates. The results showed that some potential biocontrol strains with no antagonistic activity to the pathogen on PDA plates would be ignored by using the dual culture method. Most

of the screening of potential biocontrol *Bacillus* was based on the antifungal activity (Zhang et al., 2008; Chen et al., 2010). *Bacillus* spp. which do not have significant antagonistic effect will not be used for further studies. In experiment 1, we evaluated the biocontrol effects of six *B. thuringiensis* strains by both dual culture method and pot experiments. Our finding showed that BT17, BT18 is a potential biocontrol agent to control Fusarium wilt disease in both PDA plates and pot experiment. No antagonistic activity BT20 showed a potential biocontrol effect in pot experiment. According to this result, we conferred *B. thuringiensis* might be a plant growth-promoting rhizobacteria (PGPR). In experiment 1, we also indicated all of the tested *B. thuringiensis* strains could promote plant growth (seed germination, root and shoot elongation) significantly. It seemed that *B. thuringiensis* control complex disease by two ways, antagonistic activity (or toxicity) and PGPR effect.

PGPR are known for growth promotion and disease reduction in crops. Biocontrol by use of PGPR represents a potentially activity in disease management (Jetiyanon and Kloepper, 2002). Disease reduction by PGPR in colonization of plant roots occur directly and indirectly. PGPR can suppress disease directly, through competition for space, nutrients and ecological niches or production of antimicrobial

substances. Some PGPR do not produce metabolites against the pathogens can control disease indirectly, through induced systemic resistance (ISR) or systemic acquired resistance (SAR) (Kloepper and Beauchamp, 1992; Liu et al., 1995). In experiment 1, BT17 and BT18 showed antagonistic activity against FOL by dual culture assays and inhibited the symptoms of wilt disease in pot. From these results, we conclude that *B. thuringiensis* can produce antimicrobial substances against the growth of FOL, directly. However, in the pot experiment non-antagonistic activity strain BT20 inhibited wilt symptoms with a low wilting score and high plant weight. Tomato roots treated with a cell-free filtrate of *B. thuringiensis* suppressed the development of wilt symptoms caused by bacterial wilt disease *Ralstonia solanacearum*, through the plant defense system. The co-activation of ET-dependent signaling pathway with the SA-dependent signaling pathway and suppression of JA-dependent signaling may play key roles in induced resistance of *B. thuringiensis* to *R. solanacearum* in tomato (Hyakumachi et al., 2014). In experiment 1, it is also possible that some kind of elicitor compounds existed in BT20, and caused induced systemic resistance to control FOL, indirectly.

B. thuringiensis was considered the most used bacteria against plant parasitic nematodes and this agreement with those obtained by Dawar et al., 2008 who recorded

that *B. thuringiensis* significantly reduced eggs hatching of *M. javanica* in vitro whereas mortality of larvae was significantly increased with an increase in exposure time. *B. thuringiensis* Cry6Aa2 toxin exhibits obvious toxicity to J2 of *M. hapla*, and significantly inhibits egg hatching, motility, and penetration to host plant (Yu et al., 2015). Inhibition in J2 emergence and great J2 mortality *in vitro* by culture filtrate application with *Bt64* have been recorded (Khan et al., 2010). This may be due to the production of metabolites against root-knot nematodes. According to these researches, the mechanisms of *B. thuringiensis* control nematode might due to: producing nematicide crystal proteins or metabolites (Jouzani et al., 2008; Khan et al., 2010); changing nematode physiology behavior and interrupting with plant recognition- related to the production of secondary metabolites, which reduce the attraction of the nematodes to the roots or degrade specific exudates of the roots that control the behavior of these species (Sikora et al., 1993). Our research also agreed with these opinions. Experiment 2 showed Cry proteins with vegetative cell suspension and culture filtrate of six *B. thuringiensis* strains showed nematicide effect to J2s and eggs of *M. incognita.* It indicated the toxicity of secondary metabolites and Cry proteins produced by *B. thuringiensis* to *M. incognita.* Many previous studies have demonstrated that the

nematode stylet is too small to pass the proteins of particular size (Bockenhoff and Grundler, 1994). However, the smallest size of nematicide proteins which can enter RKN has been found (Li et al., 2007).

The mechanisms of most biocontrol agents have been elucidated, including antimicrobial substances (Cazorla et al., 2007; Kavroulakis et al., 2010); enzymes (Singh et al., 1999); increasing of natural resistance of the host (Cartieaux et al., 2003); competition for nutrients (Larkin and Fravel, 1999), and formation of biofilm (Haggag and Timmusk, 2007). Since *B. subtilis* is a PGPR, and biofilm formed by *B. subtilis* just like a kind of biobarrier on the roots to protect plants from pathogens being infected was conferred (Bais et al., 2004; Morikawa et al., 2006), we also would like to prove biofilm formation ability by *B. thuringiensis* and biocontrol effect further. As a result, we documented *B. thuringiensis* strains have the ability to colonize and form biofilms on plant root surfaces in experiment 2.

Bacillus species are the most common types of bacteria isolated from soil samples (Hallmann et al., 1998). *B. subtilis* is the best-characterized member of the *Bacillus* genus, and has become an excellent biocontrol agent (Kunst et al., 1997). *B. subtilis* has many characteristics a PGPR, including the production of structurally diverse antibiotics

(Liu et al. 2006), formation of viable spores (Cenci et al., 2006), promotion of plant growth (Ryu et al. 2004), and a ubiquitous presence in soil (Bais et al., 2004). *B. subtilis* strains showed nematicidal and antifungal activities against *M. incognita* (Burkett-Cadena et al., 2008) and *F. oxysporum* (Archana et al., 2009). The reduction of root knot nematodes associated with *B. subtilis* may be attributed to diverse mechanisms which involve phytohormones production, mineral solubilisation, secreting nematicidal products, change of root exudates, inhibition of nematode penetration into the roots, and reduction of the activity of egg hatching factors (Ji et al., 2006; Karanja et al., 2007; Huang et al., 2009). There were also several mechanisms of *B. subtilis* to control soilborne pathogens. *B. subtilis* strains applied to seedlings have been found to be effective in suppressing wilt diseases through producing antifungal compounds; inducing systemic resistance in the treated plants; forming biofilm just like a kind of biobarrier on the roots to protect plants from pathogens infection (Bais et al., 2004; Kloepper et al., 2004; Stein, 2005; Morikawa et al., 2006).

B. thuringiensis is member of *Bacillus* spp. the same with *B. subtilis*, hence the biocontrol effect of plant disease was expected. Our research also indicated *B. thuringiensis* can promote plant growth, form biofilm, suppress wilt disease, inhibit root

knot nematode, and control fungi-nematode complex disease. According to the previous discussion, several mechanisms of *B. thuringiensis* to control complex disease were considered. *B. thuringiensis* showed antagonistic activity and toxicity effect to *Fusarium oxysporum* and *M. incognita*, to suppress the growth of FOL and kill the nematode directly; interrupt the life cycle of the pathogens, such bacterial antibiotics and other compounds present in cultural metabolites might be responsible for J2 mortality and inhibition of egg hatch and fungal growth; stimulate inducing systemic resistance in the plants to inhibit plant diseases indirectly; form biofilm to produce antimicrobial substances and form a biobarrier on the roots to protect plants (Fig. 6). Complex disease inhibition effect of *B. thuringiensis* may be attributed to the comprehensive function of these mechanisms. The current study discovered for the first time that *B. thuringiensis* has activity to control *Fusarium oxysporum*, promote seed germination and root and shoot elongation, inhibit complex disease.

This research indicated that environmentally friendly *B. thuringiensis* is an alternative biocontrol agent, because it can not only kill target insects as a best bio-pesticide, but also control plant disease effectively. Since the cost of biotic pesticide was higher than chemical pesticide, the new way will be considered, such as

dual-control, it means using only one biocontrol agent to control several diseases simultaneously. For instance, we can only use *B. thuringiensis* to control plant disease, nematode damage and pest at the same time to reduce the cost. Furthermore, the results of this study may be capacity to reduce environmental loading, keep food safety and increase production quantity.

Fig. 6.

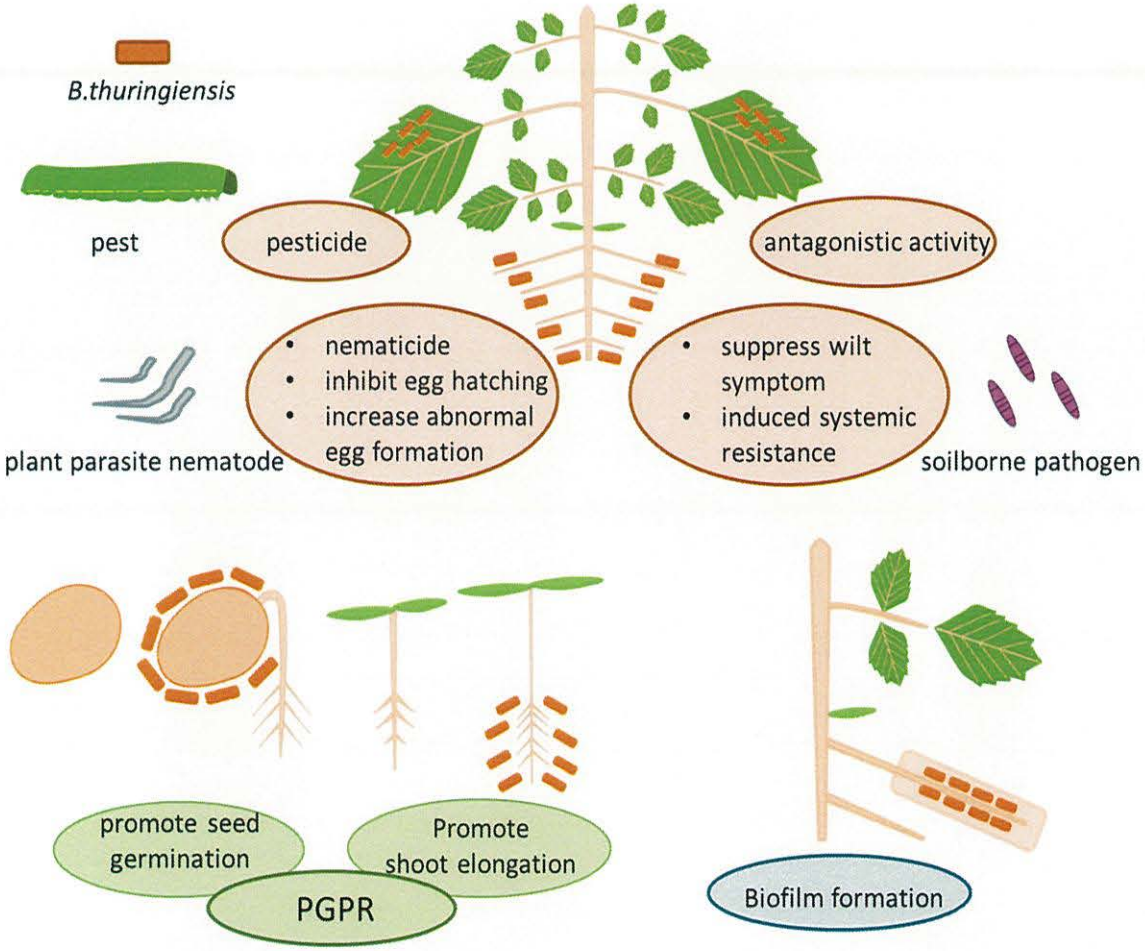

Fig. 6.: Comprehensive interaction of *B. thuringiensis*, plant parasite nematode, pathogen and plant.

REFERENCES

Archana, G., Alok, R.R., Sudhir, U.M., Dongre, A.B., 2010. Isolation, evaluation and characterization of *Bacillus subtilis* from cotton rhizospheric soil with biocontrol activity against *Fusarium oxysporum*. World Journal of Microbiology and Biotechnology 26, 1187–1194.

Arora, N., Ahmad, T., Rajagopal, R., Bhatnagar, R.K., 2003. A constitutively expressed 36 kDa exochitinase from *Bacillus thuringiensis HD1*. Biochemical and Biophysical Research Communications 307, 620–625.

Argôlo-Filho, R., Loguercio, L., 2014. *Bacillus thuringiensis* Is an Environmental Pathogen and Host-Specificity Has Developed as an Adaptation to Human-Generated Ecological Niches. Insects 5, 62-91.

Ana, H., Blanca, L., Rafael, M.J-D., 1997. Influence of chickpea genotype and *Bacillus* spp. on protection from *Fusarium* wilt by seed treatment with nonpathogenic *Fusarium oxysporum*. European Journal of Plant Pathology 103, 631–642.

Ailton, R., Leonardo, S.B., 2007. Outbreak of *Fusarium oxysporum* f.sp. *lycopersici* race 3 in commercial fresh-market tomato fields in Rio de Janeiro State, Brazil. Horticultura Brasileira 25. 3, 451-454.

Auger, S., Krin, E., Aymerich, S., Gohar, M., 2006. Autoinducer 2 affects biofilm formation by *Bacillus* cereus. Applied and Environmental Microbiology 72.1, 937-941.

Atkinson, H.J., Urwin, P.E., Clarke, M.C., McPherson, M.J., 1996. Image analysis of the growth of *Globodera pallida* and *Meloidogyne incognita* on transgenic tomato roots expressing cystatins. Journal of Nematology 28, 209–215.

Ali, N.I., Siddiqui, I.A., Shaukat, S.S., Zaki, M.J., 2002. Nematicidal activity of some strains of *Pseudomonas* spp. Soil Biology and Biochemistry 34, 1051-1058.

Al-Banna, L., Khyami-Horani, H., 2004. Nematicidal activity of two Jordanian strains of *Bacillus thuringiensis* on root-knot nematodes. Nematologia Mediterranea 32, 41–45.

Amara, M., Dahdoh, M., 1997. Effect of inoculation with plant growth-promoting rhizobacteria (PGPR) on yield and uptake of nutrients by wheat grown on sandy soil. Egyptian journal of soil 37, 467–484.

Asghar, H.N., Zahir, Z.A., Arshad, M., Khalig, A., 2002. Plant growth regulating substances in the rhizosphere: microbial production and functions. Advances in Agronomy 62, 146–151.

Ashoub, A.H., Amara, M.T., 2010. Biocontrol activity of some bacterial genera against root-knot nematode, *Meloidogyne incognita*. Journal of American Science 6 (10), 321-328.

Asaka, O., Shoda, M., 1996. Biocontrol of Rhizoctonia solani damping-off of tomato with *Bacillus subtilis* RB14. Applied and Environmental Microbiology 62 (11), 4081-4085.

Bais, H., Fall, R., Vianco, J.M., 2004. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiology 134, 307–319.

Bora, T., Ozaktan, H., Gore, E., Aslan, E., 2004. Biological control of *Fusarium oxysporum* f.sp. *melonis* by wettable powder formulations of the two strains of *Pseudomonas putida*. Phytopathology 152, 471–475.

Bockenhoff, A., Grundler, F.M.W., 1994. Studies on the nutrient uptake by the beet cyst nematode *Heterodera schachtii* by in situ microinjection of fluorescent probes into the feeding structures in Arabidopsis thaliana. Parasitology 109, 249-255.

Betz, F.S., Hammond, B.G., Fuchs, R.L., 2000. Safety and Advantages of *Bacillus thuringiensis*-Protected Plants to Control Insect Pests. Biological Control 32, 156–

173.

Bertrand, B., Nunez, C. and Sarah, J.L., 2000. Disease complex in coffee involving *Meloidogyne arabicida* and *Fusarium oxysporum*. Plant Pathology 49, 383–388.

Benhamou, N., Kloepper, J.W., Tuzun, S., 1998. Induction of resistance against *Fusarium* wilt of tomato by combination of chitosan with an endophytic bacterial strain: ultrastructure and cytochemistry of the host response. Planta 204, 153–168.

Branda, S.S., Vik, S., Friedman, L., Kolter, R., 2005. Biofilms: the matrix revisited. Trends in Microbiology 13, 20–26.

Brading, M.G., Boyle, J., Lappin-Scott, H.M., 1995. Biofilm formation in laminar flow using Pseudomonas fluorescens EX101. Journal of Industrial Microbiology 15, 297-304.

Bravo, A., Likitvivatanavong, S., Gill, S., Soberón, M., 2011. *Bacillus thuringiensis*: a story of a successful bioinsecticide. Insect Biochemistry and Molecular Biology 41, 423-431.

Bird, D.M., Kaloshian, I., 2003. Are roots special? Nematodes have their say. Physiological and Molecular Plant Pathology 62, 115–123.

Biswas, J.C., Ladha, J.K., Dazzo, F.B., 2000. Rhizobial inoculation influences seedling

vigor and yield of rice. Agronomy journal 92, 880–886.

Bin, L., Smith, D.L., Ping, Q.F., 2000. Application and mechanism of silicate bacteria in agriculture and industry. Guizhou Science 18, 43–53.

Bird, L.S., 1981. Pest management systems for cotton diseases. Handbook of Pest Management in Agriculture 3, 203-213.

Block, J.C., Houdidier, K., Paquin, J.L., Miazga, J., Levi, Y., 1993. Biofilm accumulation in drinking water distribution systems. Biofouling 6, 333-343.

Burkett-Cadena, M., Kokalis-Burelle, N., Lawrence, K.S., Santen, E.V., Kloepper, J.W., 2008. Suppressiveness of root-knot nematodes mediated by rhizobacteria. Biological Control 47, 55-59.

Castaagnone, S.P., Bongiovanni, M., Dalmasso, A., 1992. Differential expression of root-knot nematode resistance genes in tomato and pepper: evidence with *Meloidogyne incognita* virulent and avirulent near-isogenic lineages. Annals of Applied Biology 120, 487–492.

Cartieaux, F., Thibaud, M. C., Zimmerli, L., Lessard, P. and Sarrobert, C. 2003. Transscriptome analysis of *Arabidopsis* colonized by a plant growth promoting rhizobacteria reveals a general effect on disease resistance. Plant Journal, 36,

177-188.

Cazorla, F.M., Romero, D., Pé rez-Garcıá, A., Lugtenberg, B.J.J., Vicente, A.D., Bloemberg, G., 2007. Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. Journal of Applied Microbiology 103, 1950-1959.

Cenci, G., Trotta, F., Caldini, G., 2006. Tolerance to challenges miming gastrointestinal transit by spores and vegetative cells of *Bacillus clausii*. Journal of Applied Microbiology 101, 1208–1215.

Cherif, A., Chehimi, S., Limem, F., Hansen, B.M., Hendriksen, N.B., Daffonchio, D., Boudabous, A., 2003. Purification and characterization of the novel bacteriocin entomocine 9, and safety evaluation of its producer, *Bacillus thuringiensis* subsp. *entomocidus* HD9. Journal of Applied Microbiology 95, 990–1000.

Cherif, A., Rezgui, W., Raddadi, N., Daffonchio, D., Boudabous, A., 2008. Characterization and partial purification of entomocin 110, a newly identified bacteriocin from *Bacillus thuringiensis* subsp. *entomocidus* HD110. Microbiological Research 163, 684–692.

Chen, C., Bauske, E.M, Musson, G., Rodriguez-Kaban˜a, R., Kloepper, J.W., 1994.

Biological control of *Fusarium* on cotton by use of endophytic bacteria. Biological control 5, 83–91.

Chen, Z.X., Dickson, D.W., 1998. Review of Pasteuria penetrans: biology, ecology, and biological control potential. Journal of Nematology 30, 313-340.

Chen, F., Wang, M., Zheng, Y., Luo, J., Yang, X., Wang, X., 2010. Quantitative changes of plant defense enzymes and phytohormone in biocontrol of cucumber Fusarium wilt by *Bacillus subtilis* B579. World Journal of Microbiology and Biotechnology 26, 675-684.

Chitwood, D.J., 2003. Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture— Agricultural Research Service. Pest Management Science 59, 748–753.

Chanway, C.P., 1998. Bacterial endophytes: ecological and practical implications. Sydowia 50, 149–170.

Dawar, S., Wahab, S., Tariq, M., Zaki, M.J., 2010. Application of *Bacillus* species in the control of root rot diseases of crop plants. Archives of Phytopathology and Plant Protection 43 (4), 412-418.

Dawar, S. Tariq, M., Zaki, M. J. 2008. Application of *Bacillus* species in control of

Meloidogyne javanica (Treub) Chitwood on cowpea and mash bean. Pakistan Journal of Botany, 40, 439-444.

De Vay, J.E., Gutierrez, A.P., Pullman, G.S., Wakeman, R.J., Garber, R.H., Jeffers, D.P., Smith, S.N., Goodell, P.B., Roberts, P.A. 1997. Inoculum densities of *Fusarium oxysporum* f. sp. *vasinfectum* and *Meloidogyne incognita* in relation to the development of Fusarium wilt and the phenology of cotton plants (*Gossypium hirsutum*). Phytopathology 87, 341-346.

Deviddas, P., Rehberger, L.A., 1992. The effects of exotoxin (*Thuringiensin*) from *Bacillus thuringiensis* on *Meloidogyne incognita* and *Caenorhabditis elegans.* Plant and Soil 145, 115- 120.

Dennis, C., Webster, J., 1971. Antagonistic properties of species- groups of *Trichoderma* 111. Hyphal interaction. Transactions of the British Mycological Society 57, 363-369.

Dong, Y., Zhang, Q., Xu, J.L., 2002. Identification of quorum-quenching N-acylhomoserine lactonases from *Bacillus* species. Applied and Environmental Microbiology 68, 1754–1759.

Emmert, E., Handelsman, J., 1999. Biocontrol of plant disease: a (Gram) positive

perspective. FEMS Microbiology Letters 171, 1–9.

El-Alfy, A.T., Schlenk, D., 2002. Effect of 17 beta-estradiol and testosteroneon the expression of flavin-containing monooxygenase and the toxicity of aldicarb to Japanese Medaka, Oryzias latipes, Toxicological Sciences 68, 381–388.

El-Nagdi, W.M.A., Youssef, M.M.A., 2004. Soaking faba bean seed in some bio-agent as prophylactic treatment for controlling *Meloidogyne incognita* root-knot nematode infection. Journal of Pesticide Science 77, 75-78.

Elad, Y., Chet, I., Hens, Y., 1982. Degradation of plant pathogenic fungi by *Trichoderma hamianum.* Canadian Journal of Microbiology 28, 719-725.

Ediz, S.A., Dickerson, O.J., 1976. Life cycle, pathogenicity, histopathology, and host range of race 5 of the barley root-knot nematode. Journal of Nematology 8, 228–232.

Figueiredo, M.V.B., Burity, H.A., Martinez, C.R., Chanway, C.P., 2008. Alleviation of water stress effects in common bean (*Phaseolus vulgaris L.*) by co-inoculation *Paenibacillus* and *Rhizobium tropici*. Applied Soil Ecology 40, 182–188.

Fagerlund, A., Dubois, T., Økstad, O., Verplaetse, E., Gilois, N., Bennaceur, I., Perchat, S., Gominet, M., Aymerich, S., Kolstøl, A., Lereclus, D., Gohar, M., 2014. SinR

controls enterotoxin expression in *Bacillus thuringiensis* biofilms. Plos one 9.1, e87532.

Fravel, D.R., 2005. Commercialization and implementation of biocontrol1. The Annual Review of Phytopathology 43, 337-359.

Francl, L.J., Wheeler, T.A., 1993. Interaction of plant-parasitic nematodes with wilt-inducing fungi. Nematode Interactions, 79-103.

France, R.A., Abawi, G.S., 1994. Interaction between *Meloidogyne incognita* and *Fusarium oxysporum* f. sp. *phaseoli* on selected bean genotypes. Journal of Nematolog 26, 467–474.

Gupta, A., Gopal, M., Tilak, K.V., 2000. Mechanism of plant growth promotion by rhizobacteria. Indian journal of experimental biology 38, 856–862.

Guo, S., Liu, M., Peng, D., Ji, S., Wang. P., Yu. Z., Sun, M., 2008. New strategy for isolating novel nematicidal crystal protein genes from *Bacillus thuringiensis* strain YBT-518. Applied and Environmental Microbiology 74, 6997–7001.

Glick, B.R., 1995. The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology 41, 109–117.

Glick, B.R., Penrose, D.M., Ma, W., 2001. Bacterial promotion of plant growth.

Biotechnology Advances 19, 135–138.

Griffitts, J.S., Huffman, D.L., Whitacre, J.L., Barrows, B.D., Marroquin, L.D., Muller, R., Brown, J.R., Hannet, T., Esko, J.D., 2003. Resistance to a bacterial toxin is mediated by removal of a conserved glycosylation pathway required for toxin-host interactions. The Journal of Biological Chemistry 278, 45594-45602.

Griffitts, J.S., Whitacre, J.L., Stevens, D.E., Aroian, R.V., 2001. *Bacillus thuringiensis* toxin resistance from loss of a putative carbohydrate-modifying enzyme. Science 293, 860-864.

Griffin, G.D., 1986. The importance of nematode resistance on the interaction of *Meloidogyne hapla* and *Fusarium oxysporum* on alfalfa. Phytopathology 76, 843.

Gray, E.J., Smith, D.L., 2005. Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. Soil Biology and Biochemistry 37, 395–412.

Harris, A.R., Ferris, H., 1991. Interactions between *Fusarium oxysporum* f. sp. *tracheiphilum* and *Meloidogyne* spp. in *Vigna unguiculata*. 1. Effects of different inoculum densities on Fusarium wilt. Plant Pathology 40, 445-456.

Harris, A.R., Ferris, H., 1991. Interactions between *Fusarium oxysporum* f. sp.

tracheiphilum and *Meloidogyne* spp. in *Vigna unguiculata.* 3. Pathogenesis by *F. o. trancheiphilum* as affected by *M. javanica* and host cultivar. Plant Pathology 40, 465-475.

Hamon, M.A., Lazazzera, B.A., 2001. The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. Molecular Microbiology 42, 1119–1209.

Hala, K.H., Hajai,j M., Charles, J.F., 2003. Characterization of *Bacillus thuringiensis* ser. *jordanica* (serotype H71), a novel serovariety isolated in Jordan. Current Microbiology 47, 26-31.

Haggag, W.M., Timmusk, S., 2007. Colonization of peanut roots by biofilm-forming *Paenibacillus polymyxa* initiates biocontrol against crown rot disease. Journal of Applied Microbiology 104, 961-969.

Hallmann, J., Rodrı`guez-Ka ´ bana, R., Kloepper, J.W., 1998. Chitin mediated changes in bacterial communities of the soil, rhizosphere, and internal roots of cotton in relation to nematode control. Soil Biology & Biochemistry 31, 551-60.

Huffman, D.L., Bischof, L.J., Griffitts, J.S., Aroian, R.V., Sebo, P., 2004. Pore worms: using *Caenorhabditis elegans* to study how bacterial toxins interact with their target

host. International Journal of Medical Microbiology 293, 599-607.

Huang, Y., Xu, C., Ma, L., Zhang, K., Duan, C., Mo, M., 2009. Characterisation of volatiles produced from *Bacillus megaterium* YFM3.25 and their nematicidal activity against *Meloidogyne incognita*. European Journal of Plant Pathology 26(3), 417-422.

Hyakumachi, M., Nishimura, M., Arakawa, T., Asano, S., Yoshida, S, Tsushima, S., Takahashi, H., 2013. *Bacillus thuringiensis* suppresses bacterial wilt disease caused by *Ralstonia solanacearum* with systemic induction of defense-related gene expression in tomato. Microbes and Environments 28 (1), 128–134.

ISTA, 1993. Proceedings of international seed test association, international rules for seed testing. Seed Science and Technology 21, 1–152.

Jaubert, S., Milac, A.L., Petrescu, A.J., Almeida-Engler, J., Abad, P., Rosso, M.N., 2005. In planta secretion of a calreticulin by migratory and sedentary stages of root-knot nematode. Molecular Plant-Microbe Interactions 18, 1277–1284.

Jetiyanon, K., Kloepper, J.W., 2002. Mixtures of plant growth promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. Biocontrol 24, 285–291.

Jetiyanon, K., Fowler, W.D., Kloepper, J.W., 2003. Broad spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. Plant Disease 87, 1390–1394.

Ji, P., Campbell, H., Kloepper, J., Jones, J., Suslow, T., Wilson, M., 2006. Integrated biological control of bacterial speck and spot of tomato under field conditions using foliar biological control agents and plant growth-promoting rhizobacteria. Biologial Control 36, 358–367.

Jung, C., Wyss, U., 1999. New approaches to control plant parasitic nematodes. Applied Microbiology and Biotechnology 51, 439-446.

Kumar, R., Ahmad, S. and Saxena, S.K., 1998. Disease complex in chickpea involving *Meloidogyne incognita* and *Fusarium oxysporum*. International Nematology Network Newsletter 5, 12–14.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A., Alloni, G., 1997. The complete genome sequence of the Grampositive bacterium *Bacillus subtilis*. Nature 390 (6657), 249–256.

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

Kloepper, J.W., Hume, D.J., Scher, F.M., 1988. Plant growth promoting rhizobacteria on canola (rapeseed). Plant Disease 72, 42–46.

Kloepper, J.W., Ryu, C.M., 2006. Bacterial endophytes as elicitors of induced systemic resistance. Microbial Root Endophytes, 33–51.

Kloepper J.W., Schroth, M.N., Miller, T.D., 1980. Effects of rhizosphere colonization by plant growth promoting rhizobacteria on potato plant development and yield. Phytopathology 70, 1078–1082.

Kloepper, J.W., Beauchamp, C.J., 1992. A review of issues related to measuring colonization of plant roots by bacteria. Canadian Journal of Microbiology 38, 1219-1232.

Kerry, B.R., 2000. Rhizosphere interaction and the exploitation of microbial agents for the biological control of plant parasitic nematode. Annual Review of Phytopathology 38, 423-441.

Kotan, R., Sahin, F.B., Demirci, E., Eken, C., 2009. Biological control of the potato dry rot caused by *Fusarium* species using PGPR strains. Biological Control 50, 194–198.

Kotze, A.C., O'grady, J., Gough, J.M., Pearson, R., Bagnall, N.H., Kemp, D.H., Akhurst,

R.J., 2005. Toxicity of *Bacillus thuringiensis* to parasitic and freeliving life-stages of nematode parasites of livestock. International Journal for Parasitology 35, 1013-1022.

Kiewnick, S., Sikora, R.A., 2006. Evaluation of *Paecilomyces lilacinus* strain 251 for the biological control of the norther root-knot nematode *Meloidogyne hapla* chitwood. Nematology 8, 69–78.

Kappelman, A.J., Jr., Sappenfield, W.P., 1973. Development of a set of upland cotton lines for comparisons of Fusarium-root-knot nematode disease complex of cotton in California. Crop Science 13, 280-281.

Kavroulakis, N., Ntougias, S., Besi, M.I., Katsou, P., Damaskinou, A., Ehaliotis, C., Zervakis, G.I., Papadopoulou, K.K., 2010. Antagonistic bacteria of composted agro-industrial residues exhibit antibiosis against soil-borne fungal plant pathogens and protection of tomato plants from *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Plant Soil 333, 233-247.

Karanja, N.K., Mutua, G.K., Kimenju, J.W., 2007. Evaluating the effect of *Bacillus* and Rhizobium bio-inoculant on nodulation and nematode control in *Phaseolus vulgaris* L. Advances in Integrated Soil Fertility Research, 863–868.

Khan, M.R., Khan, N., Khan, S.M., 2001. Evaluation of agricultural materials as substrate for mass culture of fungal biocontrol agents of fusarial wilt and rootknot nematode diseases. Annals of Applied Biology 22, 50-51.

Khan, M.R., 2008. Plant Nematodes-Methodology, Morphology, Systematics, Biology and Ecology. Science Publishers.

Khan, M.R., 2007. Prospects of microbial control of root-knot nematodes infecting vegetable crops. Biotechnology: Plant Heatlh Management, 643-665.

Khan, M.R., Altaf, S., Mohiddin, F.A., Khan, U., Anwer, A., 2009. Biological control of plant nematodes with phosphate solubilizing microorganisms. Phosphate Solubilizing Microbes for Crop Improvement, 395-426.

Khalil, M.S., Kenawy, A., Ghorab, M.A., 2012. Impact of microbial agents on *Meloidogyne incognita* management and morphogenesis of tomato. Journal of Biopesticides 5 (1), 28-35.

Khyami-Horani, H., Hajaij, M., Charles, J.F., 2003. Characterization of *Bacillus thuringiensis* ser. *jordanica* (serotype H71), a novel serovariety isolated in Jordan. Current Microbiology 47, 26–31.

Larkin, R.P., Fravel, D.R., 1998. Efficacy of various fungal and bacterial biocontrol

organisms for control of Fusarium wilt of tomato. Plant Disease 82, 1022-1028.

Larkin, R.P., Fravel, D.R., 1999. Mechanisms of action and dose response relationships governing biological control of Fusarium wilt of tomato by nonpathogenic Fusarium spp. Phytopathology 89, 1152-1161.

Lopez-Arellano, M.E., Crespo, J.F., Gives, P.M., Parra, A.B., Rodríguez, D.H., Hernandez, E.L., Prat, V.M.V., Uriostegui, P.V., De-la-Parra, A.B., 2002. *In vitro* lethal activity of *Bacillus thuringiensis* toxins against *Haemonchus contortus* eggs and infective larvae. International Journal of Nematology 12, 66-72.

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

Martin, W. J., Newsom, L. D., Jones, J. E., 1956. Relationship of nematodes to the development of Fusarium wilt in cotton. Phytopathology 46, 285-289.

Morton, C.O., Hirsch, P.R., Kerry, B.R., 2004. Infection of plant-parasitic nematodes by nematophagous fungi—a review of the application of molecular biology to understand infection processes and to improve biological control. Nematology 6, 161–170.

Mohammed, S. H., Saedy, M.A.E., Enan, M.R., Ibrahim, N.E., Ghareeb, A., Moustafa, S.A., 2008. Biocontrol efficiency of *Bacillus thuringiensis* toxins against root-knot nematode, *Meloidogyne incognita.* Journal of Cell and Molecular Biology 7(1), 57-66.

Morikawa, M., Kagihiro, S., Haruki, M., Takano, K., Branda, S., Kolter, R., Kanaya, S., 2006. Biofilm formation by a *Bacillus subtilis* strain that produces g-polyglutamate. Microbiology 152, 2801-2807.

Mozgovaya, I.N., Byzov, B.A., Ryabchenko, N.F., Romanenko, N.D., Zvyagintsev, D.G., 2002. Nematicidal effects of the entomopathogenic bacteria *Bacillus thuringiensis* in soil. Pedobiologia 46, 558-572.

Meyer, S.L., 2003. United States Department of Agriculture— Agricultural Research Service research programs on microbes for management of plant-parasitic nematodes. Pest Management Science 59, 665–670.

Mukhopadhyay, A.N., Shrestha, S.M., Mukherjee, P.K., 1992. Biological seed treatment for control of soil-borne plant pathogens. FAO Plant Protection Bulletin 40, 21-30.

Munees, A., Mulugeta, K., 2014. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. Journal of King Saud University–

Science 26, 1–20.

Mishra, P.K., Mishra, S., Selvakumar, G., Bisht, J.K., Kundu, S., Gupta, H.S., 2009. Coinoculation of *Bacillus thuringiensis*-KR1 with *Rhizobium. leguminosarum* enhances plant growth and nodulation of pea (*Pisum. sativum* L.) and lentil (*Lens culinaris* L.). World Journal of Microbiology and Biotechnology 25, 753–761.

Milligan, S.B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., Williamson, V.M., 1998. The root knot nematode resistance gene *Mi* from tomato is a member of the leucinezipper, nucleotide binding, leucine-rich repeat family of plant genes. Plant Cell10, 1307–1320.

Nagorska, K., Bikowski, M., Obuchowski, M., 2007. Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. Acta biochimica Polonica 54, 3, 495–508.

Oostendorp, M., Sikora, R.A., 1990. In-vitro inter relationships between rhizosphere bacteria and *Heterodera schachtil*. Review of Nematology 13, 269-274.

Oostendrop, M., Sikora, R.A., 1989. Utilization of antagonistic rhizobacteria as a seed treatment for the biological control of *Heterodera schachtii* in sugarbeet. Revue de Nematologie, Journal of Nematology 12, 77-83.

Papavizas, G.C., 1985. Trichoderma and Gliocladium: biology, ecology and potential for biocontrol. Annual Review of Phytopathology 23, 23-54.

Pan, B., Bai, Y.M., Leibovitch, S., Smith, D.L., 1999. Plant growth promoting rhizobacteria and kinetic as ways to promote corn growth and yield in short season areas. European Journal of Agronomy 11, 179–186.

Pal, K.K., Tilak, K.V.B.R., Saxena, A.K., Dey, R., Singh, C.S., 2000. Antifungal characteristics of a *fluorescent pseudomonas* strain involved in the biological control of *Rhizoctonia solani*. Microbiological Research 155, 3, 233 - 242.

Peighami-Ashnaei, S., Sharifi-Tehrani, A., Ahmadzadeh, M., Behboudi, K., 2009. Interaction of different media on production and biocontrol efficacy of *Pseudomonas fluorescens*. Journal of Plant Pathology 91, 1, 65 - 70.

Pinton, R., Varanini, Z., Nannipieri, P., 2001. The rhizosphere as a site of biochemical interactions among soil components, plants and microorganisms. Biochemistry and Organic Substances in the Soil-Plant Interface. 1–17.

Randhawa, N., Sakhuja, P.K., Singh, I., 2001. Management of root-knot nematode *Meloidogyne incognita* in tomato with organic amendments. Research in Plant Disease 16, 274-276.

Racke, J., Sikora, R.A., 1992a. Influence of plant healthpromoting rhizobacteria *Agrobacterium radiobacter* and *Bacillus sphaericus* on *Globodera pallida* root infection of potato and subsequent plant growth. Journal of Phytopathology/Phytopathologische Zeitschrift 134, 198-208.

Racke, J., Sikora, R.A., 1992b. Isolation, formulation and antagonistic activity of rhizobacteria toward the potato cyst nematode *Globodera pallida.* Soil Biology and Biochemistry 24, 521-526.

Raupach, G.S., Kloepper, J.W., 1998. Mixtures of plant growth promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88, 1158–1164.

Ramamoorthy, V., Viswanathan, R., Raguchander, T., Prakasam, V., Samiyappan, R., 2001. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Protection 20, 1–11.

Raddadi, N., Belaouis, A., Tamagnini, I., Hansen, B.M., Hendriksen, N.B., Boudabous A., Cherif, A., Daffonchio, D., 2009. Characterization of polyvalent and safe *Bacillus thuringiensis* strains with potential use for biocontrol. Journal of Basic Microbiology 49, 293–303.

Rae, R., Riebesell, M., Dinkelacker, I., Wang, Q., Herrmann, M., Weller, A.M., Dieterich, C., Sommer, R.J., 2008. Isolation of naturally associated bacteria of necromenic *Pristionchus nematodes* and fitness consequences. Journal of Experimental Biology 211, 1927–1936.

Roh, J.Y., Choi, J., Li, M.S., Jin, B.R., Je, Y.H., 2007. *Bacillus thuringiensis* as a specific, safe, and effective tool for insert pest control. Journal of Microbiology and Biotechnology 17, 547–559.

Roberts, P., Lohrke, S., Meyerb, L.F., Buyer, S., Bowers, H., Backed, C., Jorge, T. Lewis, C.S., 2005. Biocontrol agents applied individually and in combination for suppression for of soilborne disease of cucumber. Crop Protection 24, 141–155.

Ryu, C.M., Murphy, J.F., Mysore, K.S., Kloepper, J.W., 2004. Plant growth-promoting rhizobacteria systemically protect *Arabidopsis thaliana* against *Cucumber mosaic virus* by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway. The Plant Journal 39, 381–392.

Sakhuja, P.K., Jain, R.K., 2001. Nematode diseases of vegetable crops and their management. Diseases of Fruits and Vegetables and their Management.

Sassser, J.N., Carter, C.C., Taylor, A.L., 1982. A Guide to the Development of a Plant

Nematology Program. Plant Pathology, 21.

Salehi Jouzani, G., Seifinejad, A., Saeedizadeh, A., Nazarian, A., Yousefloo, M., Soheilivand, S., Mousivand, M., Jahangiri, R., Yazdani, M., Amiri, R.M., Akbari, S., 2008. Molecular detection of nematicidal crystalliferous *Bacillus thuringiensis* strains of Iran and evaluation of their toxicity on free-living and plant-parasitic nematodes. Canadian Journal of Microbiology 54(10), 812-822.

Saxena, A.K., Pal, K.K., Tilak, K.V.B.R., 2000. Bacterial biocontrol agents and their role in plant disease management. Biocontrol Potential and its Exploitation in Sustainable Agriculture: Crop Diseases, Weeds, and Nematodes 1.

Somasegran, P., Hoben, H.J., 1994. Handbook for Rhizobia: Method in Legume Rhizobium Technology. Springer.

Sikora, R.A., 1988. Interrelationship between plant health promoting rhizobacteria plant parasitic nematodes and soil microorganisms. Mededelingen Faculteit Landbouwwetenschappen Rijksuniversiteit Gent 53, 867-878.

Singh, R., Parameswaran, T.N., Rao, P.E.V.S., Puttanna, K., Kalra, A., Srinivas, K.V.N.S., Bagyaraj, D.J., Divya, S., 2009. Effect of arbuscular mycorrhizal fungi and *Pseudomonas fluorescens* on root-rot and wilt, growth and yield of *Coleus*

forskohlii. Biocontrol Science and Technology 19 (8), 835-841.

Stirling, G.R., 1991. Biological Control of Plant Parasitic Nematodes. Progress, Problems and Prospects. Wallingford: CAB International.

Seldin, L., Van Elsas, J.D., Penido, E.G.C., 1984. *Bacillus azotofixans* sp. nov. a nitrogen fixing species from Brazilian soils and grass roots. International journal of systematic bacteriology 34, 451–456.

Siddiqui, Z.A., Mahmood, I., 1995. Management of *Meloidogyne incognita* race 3 and *Macrophomina phaseolina* by fungus culture filtrates and *Bacillus subtilis* in chickpea. Fundamental and Applied Nematology 18, 71-76.

Siddiqui, Z.A., Mahmood, I., 1999. Role of bacteria in the management of plant parasitic nematodes: a review. Bioresource Technology 69, 167-179.

Sikora, R.A., Fernandez, E., 2005. Nematode parasites of vegetables. Plant-Parasitic Nematodes in Subtropical and Tropical Agriculture, 319-392.

Sikora, R.A., Hoffmann-Hergarter, S., 1993. Biological control of plant parasitic nematodes with plant-health promoting rhizobacteria. Biologically Based Technology, 166-172.

Singh, P.P., Shin, Y.C., Park, C.S., Chung, Y.R., 1999. Biological control of Fusarium

wilt of cucumber by chitinolytic bacteria. Phytopathology 89, 92-99.

Schnepf, E., Crickmore, N., van Rie L.D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiology and Molecular Biology Reviews 62, 775–806.

Schulenburg, H., Muller, S., 2004. Natural variation in the response of *Caenorhabditis elegans* towards *Bacillus thuringiensis*. Parasitology 128, 433–443.

Stanley, N.R., Britton, R.A., Grossman, A.D., Lazazzera, B.A., 2003. Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. Journal of Bacteriology 185, 1951–1957.

Stabb, E.V., Jacobson, L.M., Handelsman, J., 1994. Zwittermycin A producing strains of *Bacillus cereus* from diverse soils. Applied and Environmental Microbiology 60, 4404–4412.

Sumeet, S., Mukerji, K.G., 2000. Exploitation of protoplast fusion technology in improving biocontrol potential. Biocontrol Potential and its Exploitation in Sustainable Agriculture: Crop。Diseases Weeds and Nematodes 1, 39-48.

Shanmugam, V., Kanoujia, N., 2011. Biological management of vascular wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycopersici* by plant growth-promoting

rhizobacterial mixture. Biological Control 57, 85–93.

Sudhamoy, M., Nirupama, M., Adinpunya, M., 2009. Salicylic acid-induced resistance to *Fusarium oxysporum* f. sp. *lycopersici* in tomato. Plant Physiology and Biochemistry 4, 642–649.

Suleman, P., Sardanelli, S., Krusberg, L.R. and Straney, D.C., 1997. Variability among *Fusarium oxysporum* f. sp. *Lycopersici* isolates in their ability to interact with *Meloidogyne incognita* race 1. Kuwait Journal of Science 24, 299–307.

Taylor, E.C., Brown, F.J.D., 1976. The geographical distribution of *Xiphinema* and *Londgidorus* nematodes in the British Isles and Ireland. Annals of Applied Biology 84, 383-402.

Trudgill, D.L., Blok, V.C., 2001. Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. Annual Review of Phytopathology 39, 53–77.

Terefe, M., Tefera, T., Sakhuja, P.K., 2009. Effect of a formulation of *Bacillus firmus* on root-knot nematode *Meloidogyne incognita* infestation and the growth of tomato plants. Journal of Invertebrate Pathology 100, 94–99.

Uma Maheshwari, T., Sharma, S.B., Reddy, D.D.R., Haware, M.P., 1995. Co-infection

of wilt-resistant chickpeas by *Fusarium oxysporum* f. sp. *ciceris* and *Meloidogyne javanica.* Journal of Nematology 27, 649-653.

Uma Maheshwari, T., Sharma, S.B., Reddy, D.D.R., Haware, M.P., 1997. Interaction of *Fusarium oxysporum* f. sp. *ciceri* and *Meloidogyne javanica* on *Cicer arietinum.* Journal of Nematology 29, 117-126.

Van Loon, L.C., 2007. Plant responses of plant growth-promoting rhizobacteria. European Journal of Plant Pathology 119, 243–254.

Van Loon, L.C., Bakker, P.A.H.M., Pieterse, C.M.J., 1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology 36, 453–483.

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

Vessey, J.K., 2003. Plant growth-promoting rhizobacteria as biofertilizers. Plant Soil 255, 571–586.

Vurro, M., Gressel, J., 2006. An ecological and societal approach to biological control: an integrated approach to biological control of plant diseases and weeds in Europe. Springer 2, 257–274.

Walia, R.K., Vats, R., 2000. Fungal antagonists of Phytonematodes. Biocontrol Potential and its Exploitation in Sustainable Agriculture: Crop Diseases, Weeds, and Nematodes, vol. 1. Kluwer Academy Plenum.

Watnick, P.I., Kolter, R., 1999. Steps in the development of a Vibrio cholerae El Tor biofilm. Molecular microbiology 34 (3), 586-595.

Wei, G., Kloepper, J.W., Tuzun, S., 1991. Induction of systemic resistance of cucumber to Colletotrichum orbiculare by select strains of plant growth promoting rhizobacteria. Phytopathology 81 (11), 1508-1512.

Wei, J.Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S.C., 2003. *Bacillus thuringiensis* crystal proteins that target nematodes. Proceedings of the National Academy of Sciences 100, 2760-2765.

Weller, D.M., Thomashow, L.S., 1994. Current challenges in introducing beneficial microorganisms into the rhizosphere. Molecular ecology of rhizosphere microorganisms: Biotechnology and the release of GMOs, 1-18.

Westcott, S.W. III and Kluepfel, D.L., 1993. Inhibition of *Criconemella xenoplax* egg hatch by *Pseudomonas aureofaciens*. Phytopathology 83, 1245–1249.

Williamson, V.M., Gleason, C.A., 2003. Plant-nematode interactions. Current Opinion

in Plant Biology 6, 327-333.

Wyss, U., Grundler, F.M.W., Munch, A., 1992. The parasitic behavior of second stage juveniles of *Meloidigyne incognita* in root of *Arabidopsis thaliana*, Nematology38, 98–111.

Yu, Z.Q., Xiong, J., Zhou, Q.N., Luo, H.Y., Hua, S.B., Xia, L.Q., Sun, M., Li, L., Yu, Z.N., 2015. The diverse nematicidal properties and biocontrol efficacy of *Bacillus thuringiensis* Cry6A against the root-knot nematode *Meloidogyne hapla*. Journal of Invertebrate Pathology 125, 73-80.

Zdarska, Z., Huffman, D.G., Moravec, F., Nebesarova, J., 2001. Egg shell ultrastructure of the fish nematode, *Huffmanela huffmani* (Trichosomoididae). Folia Parasitologica 48, 231–234.

Zhang, F., Dashti, N., Hynes, R.K., Smith, D.L., 1996. Plant growth-promoting rhizobacteria and soybean [*Glycine max* (L.) Merr.]. Nodulation and fixation at suboptimal root zone temperatures. Annals of Botany 7, 453–459.

Zhou, Y., Choi, Y.L., Sun, M., Yu, Z., 2008. Novel roles of *Bacillus thuringiensis* to control plant diseases. Applied Microbiology and Biotechnology 80, 563–572.

Zhang, S., Raza, W., Yang, X., Hu, J., Huang, Q., Xu, Y., Liu, X., Ran, W., Shen, Q.,

2008. Control of Fusarium wilt disease of cucumber plants with the application of a bioorganic fertilizer. Biology and Fertility of Soils 44, 1073-1080.