Thesis for the Degree of Master of Agriculture

Study on The Mechanism of Suppression Mediated Rhizobacterial Strains against Citrus Canker Caused by *Xanthomonas citri* subsp. *citri*

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ABSTRACT

Citrus canker caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) is one of the important diseases which have been strictly quarantined in many countries. By increasing side effect of the chemicals due to the mostly controlling by chemical applications, the alternative method of disease control has been extremely required. Four rhizobacterial strains such as *Burkholderia gladioli* MRL408-3 and TRH423-3 and *Pseudomonas fluorescens* THJ609-3 and TRH415-2 showing anti-bacterial activity against *Xcc* on the artificial medium, were selected in the previous study. When these rhizobacterial strains were pre-treated on the citrus leaves following inoculation with *Xcc*, disease severity was significantly suppressed. In this study, in order to illustrate the mechanism mediated by these selected rhizobacterial strains, the number of *Xcc* on the citrus leaves pre-treated with the selected strains was counted and the infected leaves were observed using a scanning electron microscope (SEM). The number of *Xcc* colonies from the rhizobacterial strains pre-treated leaves was effectively reduced compared to that of non-treated control one on the semi-selective medium, indicating the direct suppression by the selected rhizobacterial strains against *Xcc* on the citrus leaf surface. The counted colonies were identified as *Xcc* and the rhizobacteria, respectively, by the gel electrophoresis of polymerase chain reaction (PCR) amplified and analysis of bacterial rDNA sequence using the specific primer sets. Based on the SEM image, it revealed that the number of total bacterial cells on the leaves pre-treated with the rhizobacterial strains after

inoculating *Xcc* was reduced compared to those on the leaves inoculated with *Xcc* only. On the other hand, to determine whether a resistance induced by the treatment with the rhizobacterial strains, pathogenesis-related protein -1 (PR-1 protein) gene was extracted from the citrus leaves. As expected, PR-1 protein gene from the leaves pre-treated with THJ609-3 was amplified. Based on these results, it is suggested that these rhizobacteria could express any anti-bacterial activity on the citrus leaves against *Xcc* and reduce the population of *Xcc* resulting in suppression of disease severity. Therefore, it is expected that rhizobacterial strains could be used as the biological control agents in a green house and field where chemical application is prohibited or limited.

Ⅰ. INTRODUCTION

In Jeju island, citrus industry was increased rapidly since 1964 resulting in cultivating area exceeded from 10,000 ha in 1975 to 25,000 ha in 1996. However since 2009, it has been kept about 20,000 ha by reducing the citrus orchards because overproduction was almost brought to collapse of the citrus prices (Ko, 2009). Nevertheless, citrus has been yet important fruits in Jeju as well as the country, of which amounts of production is mostly at 700,000 ton per year and its cultivating area was occupied by 21,400 ha in this country in 2012 (Lim, 2013).

Some diseases such as scab, canker and melanose has been important issue in the citrus recently (Song, 2014). Especially, *Xanthomonas citri* subsp. *citri* (*Xcc*) causing citrus canker is the most serious pathogenic bacterium which has typical rod-shaped cells 1.0 μm in length, flagella and numerous fimbriae (Kim et al., 2010) on the exported citrus to foreign countries. Five forms of *Xcc* have been described canker ‘A’, ‘B’, ‘C’, ‘D’ and ‘E’. Canker ‘A’ which is the Asiatic form of citrus canker is the most widespread and severe form of the disease (Gottwald et al., 2002). The causal agent of canker ‘A’ was first described by Hasse in 1915 as *Xanthomonas* (*Pseudomonas*) *citri*. It was reclassified as *Xanthomonas compestris* pv. *citri*, *X. axonopodis* pv. *citri* or *X. smithii* subsp. *citri* (Dye et al., 1980; Vauterin et al., 1995; Schaad et al., 2005). Recently this pathogen emended classification as *X. citri* subsp. *citri* (Schaad et al., 2006).

Xcc surviving on infected citrus act as an inoculum next year for the spread of

the disease (Kang et al., 2014). This pathogen propagates in lesions of leaves, stems and fruit. When there is free moisture on the lesions, bacteria transude out and could be dispersed to new growth part. Rainwater collected from leaf with lesions contains between 10^5-10^8 cfu/ml of the bacterium (Goto, 1962; Stall et al., 1980). Rainstorms with wind are the majority of natural dispersal source. Wind helps the invention of pathogen through the stomatal chambers or wounds made by insects such as the citrus leafminer (*Phyllocnistis citrella*), thorns and blowing sand (Gottwald and Graham, 1992; Graham et al., 1992). The serpentine mines under the leaf cuticle caused by the larvae of the citrus leafminer provide sufficient wound on new leaves to increase citrus canker infection (Cook, 1988; Rodrigues et al., 1998). *Xcc* is capable of naturally infection with green citrus tissues most readily while the tissues are in the last half of expansion phases of growth. Once leaves, twigs and fruit approach mature size, a thickened cuticle forms, these begin to harden off physiologically, and become more resistant to infection (Stall and Seymour, 1983).

Occurrence of citrus canker has been less 1 % in Jeju (Hyun, 2012) because Satsuma mandarin cultivars mostly planted in the island have moderate resistance against *Xcc* (Myung et al., 2003). Moreover using windbreaks and spraying antibiotics such as streptomycin or copper sulfate have successfully controlled the disease (Kang et al., 2014). However, some varieties such as Sweet orange, Grapefruit and Mexican lime are susceptible against *Xcc*. When the disease is severe at the high susceptible cultivar, defoliation, dieback and fruit drop could occur and infected fruit are less valuable or entirely unmarketable (Graham and Gottwald 1991; Koizumi, 1985). Because *Xcc* could act as an inoculum agent to the disease epidemic

in citrus canker-free countries, strict quarantine has been acquired in a lot of countries including the USA. Also only citrus canker free fruits might be exported to the countries such as England or Russia. Therefore, controlling citrus canker is necessary in Jeju for export to these countries.

Controlling the citrus canker has been removal source of infection, managing windbreaks and thinning out. Also, chemical applications such as streptomycin, bordeaux mixture, iminoctadine tris, cooper hydroxide, copper sulfate and mixture cupper hydroxide with streptomycin have been used. In general, the chemicals were applied at the citrus orchards in mid-May, late in June to early in July and at the beginning of August. Certainly, these chemicals have been applied before or/and after rainstorm with wind (Hyun, 2012).

However, intensive chemical control has caused negative effects such as destruction of global environment as well as agriculture ecosystem, residual chemical applications, mammalian toxicity and emergence of antibiotic-resistant strains (Weller, 1988). Streptomycin, an aminoglycoside antibiotic, has been mostly used to control citrus canker disease, but the streptomycin resistant strains have emerged since 1962 (Stall and Thayer, 1962). The resistance in bacteria could occur as results of chromosomal mutation or through gene acquisition or both (McManus et al., 2002). Furthermore, most of the streptomycin resistant strains of *Xcc* in Jeju expressed the resistance by both chromosomal mutation and gene acquisition. The streptomycin-sensitive strains easily get the resistance by mixed culture with resistant strains (Hyun et al., 2012). In addition, market scale of environment-friendly agricultural product has been increase continuously about 20 % in developed

countries such as the USA and EU since 2000 as a result from the spread of Well-being trend and consumer demands for environment-friendly product. In 2009, organic agricultural product has reached approximately 79,200,000 ha regions in 160 countries. Also, the environment-friendly product has been steadily increased in the domestic market since early 2000s (Kim et al., 2012). Thus, these reasons have led to research with the aim of replacing chemicals with biological control such as using plant growth-promoting rhizobacteria (PGPR).

PGPR are commonly known as a heterogeneous bacterial group living in plant rhizophere, contributing to plant growth and improving stand under stress conditions (van Loon et al., 1998; Lugtenberg and Kamilova, 2009). These rhizobacteria have been introduced to soil, seeds or roots and improve plant growth and health through diverse mechanisms to synthesize phytohormones including indoleacetic acid, gibberellic acid, cytokinins and ethylene and to provide an array of nutrients such as the phosphorus or nitrogen fixation for plant (Kloepper et al., 1980; Bloemberg and Lugtenberg, 2001; Idris et al., 2007; van Loon, 2007; Vessey, 2003). *Pseudomonas putida* and *fluorescens* caused increase of the root and shoot elongation in canola, wheat and potato (de Freitas and Germida, 1992).

Furthermore, rhizobacterial strains act as a biological control agent. In order to suppress the pathogenic disease, these strains have the potential to colonize rapidly the rhizosphere and compete with pathogen at the root surface (Rangarajan et al., 2003) or parasite the pathogen. Also, they produce the antibiotics which are diffusible and volatile organic compounds of low molecular weight including toxins, biosurfactants and cell wall-degrading enzymes (Ryu et al., 2003; Compant et al.,

2005; Haas and Défago, 2005). Induced systematic resistance (ISR) is defined as an enhancement of the plants defensive capacity by rhizobacterial strains (Kabir, 2012). When these strains settle down in the rhizosphere, then the signal transfer material is produced and the resistance is induced at above-ground part of the plant. The ethylene and jasmonic acid, that is the plant hormone, play vital role in this process (van Loon et al., 1998).

Until now, most of the studies on rhizobacteria with gram negative strains have been well reported, mainly on *Pseudomonas* spp. For example, *Pseudomonas aureofaciens* Q2-87 inhibited take-all, a root disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* as producing the antibiotic 2,4-diacetophloroglucinol (Vincent et al., 1991). Besides, several *Pseudomonas* spp. have been revealed as potential biological control agents against *Ralstonia solanacearum* which cause the bacterial wilt in pepper (Guo et al., 2001). Moreover, *Pseudomonas putida* WCS358 suppressed fusarium wilt of radich caused by *Fusarium oxysporum* by competing for iron through the production of its pseudobactin siderophore (de Boer et al., 2003).

However, different *Bacillus* spp. have also gained much attention because of an obvious advantages. The genus Bacillus can produce endospores enduring heat and desiccation-resistance and, consequently, it is able to be more easily stored and transported as stable products (Emmert and Handelsman, 1999; Francis et al., 2010) such as *B. subtilis* strain QST 731 (Miille, 2006) and KB-401 (Nam et al., 2010). *Bacillus ehimensis* YJ-37 showed the suppression of vegetables damping-off disease caused by *Rhizoctonia solani* AG-4 and *Pythium ultimum* (Kim et al., 2002). Also, *B.*

mycoides strain Bac J reduced the severity of Cercospora leaf spot on the sugar beet caused *by cercospora beticol* (Kloepper et al., 2004). Besides these famous 2 bacteria, many anti-fungal or bacterial rhizobacteria have found such as *Burkholderia* spp. In the genus, *Burkholderia solanacearum* reduced disease severity of vascular wilt caused by *Fusarium oxysporum* f. sp. *vasinfectum* on cotton (Chen et al., 1995). Additionally, *Burkholderia cepacia* strain AMMDR1 suppressed pythium damping-off disease caused by *Pytium aphanidermatum* and *Aphanomyces euteiches* lysing the zoospore, preventing the cyst germination and inhibiting germ tube growth of both oomycetes (Heungens and Parke, 2000).

On the other hand, plants have developed a lot of defense mechanisms against biotic and abiotic stress condition such as pathogen invasions, water stress, cold and wounding (Kim et al., 2009). These mechanisms include synthesis of pathogenesis-related (PR) proteins which are induced and accumulate in host plant as a result of pathogen attack by fungi, bacteria, virus, nematodes, insects and herbivores. Since the finding out the PR protein, which was first observed in tobacco infected with tobacco mosaic virus (TMV) in 1970 (van Loon and can Kammen, 1970), 17 proteins have been grouped based on amino acid sequences, serological cross-relationship and/or enzymatic or biological activity (van Loon et al., 2006). Especially, PR-1 protein, which dominates the PR group induced by pathogen or abiotic agent such as salicylic acid (SA) is commonly used as an indicator for systemic acquired resistance (SAR) (van Loon and van Strien, 1999). A basic PR-1 protein gene in pepper, CABPR1 has been reported to be induced after infection of *Tobacco mosaic virus*, ethephon treatment or wounding. Also, over expression of

CABPR1 in tobacco plants promoted tolerance to the oomycete pathogen *phytophthora nicotianae* and the bacterial pathogens *Ralstonia solanacearum* and *Pseudomonas syringae* pv. *tabaci* (Sarowar et al., 2005).

Although many researches about biological control using PGPR have been carried out at the annual plants or leaf vegetables, few research about perennial woody plant such as citrus tree have been conducted (Cho et al., 2007; Kim et al., 2002). Furthermore, it has been not investigated concerning the mechanism of disease reduction by PGPR against citrus canker disease.

Thus, this study was designed to investigate the mechanism of suppression of disease severity on the citrus leave by 4 selected rhizobacterial strains. In order to illustrate the control evidence by the selected rhizobacterial strains, the number of *Xcc* was counted on the Satsuma mandarin leaf pre-treated with the rhizobacterial strains following inoculation with *Xcc*. Also, these leaves were observed under scanning electron microscope (SEM) to confirm the reduction of *Xcc* number on the leaves which may cause the reduction of disease severity. Furthermore, PR-1 protein gene was extracted from the Satsuma mandarin leaf treated with rhizobacterial strain or *Xcc* to determine whether resistance could be induced.

Ⅱ. MATERIALS AND METHODS

1. Plant

The seeding of Satusma mandarin (variety: Gungcheon) grafted with the trifoliate orange from orchard was planted in a plastic pot (Ø 25 cm, 40 cm, high) which was filled with the soil mixing the ratio of the mountain soil and nursery bed soil (Tuksimi®, Nongwoo green Tec.) into 1:3. In order to suppress the moisture evaporation and weed generation, the soil in the plastic pots was covered with the pine bark about 2-3cm high from the ground. The seedings were watered every 2 days and kept in green house maintained at 25-27 ℃ temperature and 50-70 % humidity. Branches which contain 4-5 young leaves were cut from the seedings using a sterile secateurs with a slash because the cut plant absorb the distilled water well. The part of cut of the branch was dipped in 0.5 % sodium hypochlorite solution for 5 sec and washed with sterile water 3 times every for 5 sec. Thereafter, the branch was put in a 100 ml Erlenmeyer flask containing 100 ml sterile distilled water. These branches were used for experience of bacteria inoculation, electron microscopic observation and extracting PR-1 protein gene.

2. Pathogenic bacteria

Citrus canker pathogen *Xanthomoans citri* subsp. *citri* (*Xcc*) was isolated from the leaves of Satsuma mandarin (variety: Gungcheon) with symptoms of typical citrus canker at an orchard in Jeju. The leaves were cut 5x5 mm around the symptom with a blade. The surface of leaf disks was sterilized by 1 % sodium hypochlorite (NaClO) solution for 30 sec and washed by sterile distilled water for 1 min three times. After than these disks were sterilized by 70 % ethanol for 30 sec and washed again by sterile water. The sterilized samples were removed from water with a disinfected filter paper (Ø 90 mm) and quadrisect by a blade. They were put in the 1.5 ml micro tube filled with 1 ml of 5 % peptone solution. The bacterial calls in the samples were eluted at room temparaure for 2 h by using a shaker at 70 rpm (CR300, Finemould Precision Ind. Co., Korea). After spreading 100 µl of the bacterial suspension on the tryptic soy agar (TSA: Becton, Dickson and company, France) medium, the plates were incubated at 28 ℃ for 3 days. The yellow colonies were separated as *Xcc* and incubated at the same condition.

In order to identify the yellow bacteria, the separated colony was determined by sequence analysis of ribosomal DNA. The bacterial isolates, which were separated by the loop from the TSA medium, was grown in the tryptic soy broth (TSB; Becton, Dickson and company, France) at 28 ℃ for 24 h shaking at 120 rpm. The total genomic DNA of the bacteria was extracted by the genomic DNA extraction kit (DNeasy Blood & Tissue Kit 56, QIAGEN™, Germany) according to the protocol of manufacturer and described by Ausubel et al. (1987). The extracted DNA was stored

at -21 ℃. The internal transcribed spacer region (ITS) of 16S/23S ribosome from *Xcc* was amplified using primer 2 (5'-CACGGGTGCAAAAAATCT-3') and primer 3 (5'-TGGTGTCGTCGCTTGTAT-3') (Shiotani et al., 2009; Hartung et al., 1993) for amplifying specific DNA sequencing of *Xcc*. Polymerase chain reaction (PCR) was carried out in a total volume of 40 µl containing 2 µl of total DNA (5-10ng/ml), 1 µl of each 10 pM primer, 1 µl of 2.5 mM dNTP, 4 µl of 10 x Buffer, 1 µl of 5 unit/µl Taq DNA polymerase (iNtRON Biotechnology Inc., Seoul, Korea) and 30 µl of sterile water. After all regents were mixed and heated to 95 ℃ for 10 min, 30 cycles of PCR were run at 95 ℃ for 70 sec, 58 ℃ for 60 sec and 72 ℃ for 60 sec followed by 72 ℃ for 2 min using PCR Thermal Cycler TP600 (TaKaRa, Japan). In order to confirm whether the DNA was amplified or not, 5 µl of PCR products were visualized in 1 % agarose gel containing 0.01 % ethidium bromide using UV transilluminator (I-MAX-H250, coreBiosystem, Seoul, Korea). Nucleotide sequences of the products were analyzed at Macrogen Inc., Seoul, Korea with primer 2 and 3. The sequences were compared with sequences in the GenBank database using the NCBI BLAST program (http://blast.ncbi. nim.nih.gov/Blast.cgi).

The identified pathogen, *Xcc* was grown on TSA medium at 28 ℃ for 2 days. Then, 10 ml sterile distilled water was poured into each agar plate grown *Xcc* and suspended the pathogens with a loop. The concentration of suspension was adjusted to 2.0 x 10^7 colony forming unit (cfu)/ml for inoculation.

3. Rhizobacterial strains

The rhizobacterial strains were isolated from the root of wild annual plants in coastal and mountainous area around Han-la mountain from 2004 to 2010. About 200 strains were separated according to the methods presented by Lee et al. (2003).

In order to isolate the strains from the roots, the soil removed from the roots under running water. After removing water the thin roots were cut off 1 g by scissors sterilized with 70 % ethanol. The roots cut were homogenized with 10 ml of sterile water at a mortar (Ø 120 mm). Then, 1 ml of the filtrate throughout two-fold miracloth (CALBIOCHEM) was diluted by ratio of 10^{-8}. Each 100 µl of 10^{-6}-10^{-8} diluted suspension was spread on TSA medium with a loop. The plates were incubated at 28 ℃ for 48 h. After selecting with its color or form of the colonies, the selected colony was grown another TSA medium in same condition. The collected bacteria with loop were mixed in the 1.5 ml micro tube filled with tryptic soy broth and 20 % glycerin using the vortex and stored at -80 ℃.

In this study, 4 rhizobacteria such as MRL408-3, THJ609-3, TRH415-2 and TRH 423-3 among them showing antifungal activity were selected according to the previous results (Kang et al. 2012). One hundread µl of these strains were spread and grown on tryptic soy agar at 28 ℃ for 48 h. The concentration of each rhizobacterial strain was adjusted to 2.0 x 10^7 cfu/ml for treatments.

4. Pre-treatments with rhizobacteria or Streptomycin®

The branches of Satsuma mandarin with 4-5 young leaves were cut and put in a Erlenmeyer flask which contain 100 ml distilled water, respectively, after sterilization of the cut part with 0.5 % sodium hypochlorite solution and washing it with sterile water, briefly. (Duplication: see '1. Plant')

The selected rhizobacterial strains MRL408-3, THJ609-3, TRH415-2 and TRH423-3 grown in TSA medium 28 ℃ for 48 h, were suspended with 10 ml of distilled water in the petri-dish using loop, respectively. The concentration of each rhizobacterial strain was adjusted to 2.0 x 10^7 cfu/ml by Hemocytometer (Hausser Scientific Inc., PA, USA) followed by adding 0.01 % Tween 20 all rhizobacterial suspension. The leaves of one branch were sprayed with the different rhizobacterial suspensions each other, in order to avoid the error from the different susceptibility of the plants.

In order to compare the efficacy of the selected rhizobacteria, the solution of a commercial antibiotic Streptomycin® (Nongyoungsin®, Kyung Nong Co,. Ltd., Seoul, Korea) was sprayed at the concentration of 1 g/L following the manual as a positive control. Sterile distilled water sprayed instead of the rhizobacterial suspension as an untreated control.

5. Inoculation with *Xcc* on the leaves of branches

After the pre-treated leaves were air-dried at room temperature for 3 h, the suspension of *Xcc* of which concentration was adjusted same as the rhizobacterial suspension was sprayed on both-sides of the leaf of the citrus branches. The inoculated plants were kept in a dew chamber maintained at 100 % humidity in the dark at 28 ℃ for 24 h and then transferred to a growth chamber maintained at 70 - 90 % humidity and at 25-30 ℃ for 15 days.

At 15 days after the inoculation with *Xcc*, the number of lesions per leaf was counted. Three independent experiments were conducted with 3 citrus branches each. The results were analyzed with the Duncan's multiple range test using SAS program for evaluation.

6. Determining the density of *Xcc* on the citrus leaves pre-treated with rhizobacterial strains or antibiotic

In order to investigate the mechanism of citrus canker disease suppression by rhizobacterial strains, the number of *Xcc* on the Satsuma mandarin leaves pre-treated with the selected rhizobacteria was compared with those of untreated control citrus plant. The suspension of selected rhizobacteria and *Xcc* was prepared. (Duplication: see '2. Pathogenic bacteria' and '3. Rhizobacterial strains') The concentration of prepared rhizobacterial strains and *Xcc* were adjusted to 2.0 x 10^7 cfu/ml.

The each of the similar size leaves was cut from the citrus branch, disinfected with 1 % of NaClO for 30 sec and washed with sterile water for 1 min every three times. Each of the rhizobacterial isolates was sprayed onto both sides of the leaves, respectively, following the inoculation with *Xcc*. The leaves were placed in a disinfected culture dish maintaining 100 % humidity and kept at 28 ℃ for 3 days. As positive and negative control, Streptomycin® and distilled water was sprayed, respectively, on behalf of treating rhizobacteria. The concentration of Streptomycin® was 1 g/L.

The incubated leaves was put into a 100 ml Erlenmeyer flask with 50 ml of 0.05 M Sodium phosphate buffer (pH 7.2) (0.05 M Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$), 0.05 M disodium hydrogen phosphate dehydrate ($Na_2HPO_4 \cdot 2H_2O$), 1 L distilled water and 10 M NaOH for adjustment of pH) and strongly suspended using vortex mixer (VM-10, KAIHAN Scientific Co., Ltd., Korea) for 1 min. Then each of the flasks was smoothly suspended in a shaking incubator at 120 rpm, at

20 ℃ overnight. One hundred µl of these suspensions, which were diluted to the ratio of 10^0, 10^{-1} and 10^{-2}, was spread on a semi-selective culture medium (SSM: 2.0 g Peptone, 1.2 g Beef extract, 2.0 g Sucrose, 4.0 g Soluble starch, 7.2 g Agar, 0.1 g $CaCl_2$, 4 ml Tween 80, 400 ml distilled water, 60 µl Crystal violet solution at 1 %, 0.02 g *Cephalexin, 0.004 g *Methyl thyophanate and *Chlorothalonil - *added after culture medium autoclaving, Dezordi et al., 2009) and incubated at 28 ℃. The number of colonies of *Xcc* on the plate was counted at 3 days later. This experiment was conducted with 3 replications and the results were analyzed with the Duncan's test.

7. Identification of *Xcc* or rhizobacteiral strains

To identify the counted colonies formed on the SSM, the genomic DNA of bacteria was extracted using these described protocols of the genomic DNA extraction kit (DNeas y Blood & Tissue Kit 56, QIAGEN™, Germany) and of Ausubel et al. (1987). The selected colony regarded as *Xcc* or each of the rhizobacterial strains on SSM was transferred and spread on tryptic soy agar medium each using a loop. *Xcc* and the rhizobacteria fully grown at the medium at 28 ℃ for 48 h were collected by a loop and incubated again in the tryptic soy broth at 28 ℃ at 120 rpm, for 24 h.

The 1.5 ml bacterial suspension was frozen at -80 ℃ for 10 min. The dissolved cells at room temperature were isolated from the TSB medium using the centrifuge (1730MR, GYROZEN™, Incheon, Korea) at 10,000 rpm for 1 min. After the supernatant was discarded carefully, the pellet was resuspended in 180 µl of tissue lysis buffer. The resuspension added 20 µl of proteinase K was mixed thoroughly by vortex mixer and incubated at 56 ℃ until the tissue was completely lysed. Then, 4 µl RNase A (100 mg/ml) was added to the lysed cell, mixed by vortexing and incubated for 2 min at room temperature. After the incubation, the sample added 200 µl lysis buffer was mixed thoroughly using a vertex. Then 200 µl of 100 % ethanol was added to the mixed sample and mixed again. The mixture was pipetted into the column placed in a 2 ml collection tube and centrifuged at 8,000 rpm 1 min. After the collection tube removed the flow-through was replaced with same column, 500 µl of first washing buffer was added into the column and centrifuged at 8,000 rpm 1

min. The collection tube was empty and replaced again, 500 µl of second washing buffer was added into the column and centrifuged at 14,000 rpm for 3 min. In order to dry the membrane in the column, the column replaced empty collection tube was reused in another centrifugation at 14,000 rpm for 3 min and kept at room temperature overnight. The dried column was placed in a clean 1.5 ml micro tube and 200 µl of elution buffer was added onto the membrane directly. After incubation at room temperature for 1 min, it centrifuge at 8000 rpm for 1 min to elute. All centrifugation was carried out at 4 ℃.

Polymerase chain reaction and gene sequencing were conducted similarly with '2. Pathogenic bacteria'. The ITS of rhizobactiral rDNA was analyzed by the universal primer 38r (5'-CCGGGTTTCCCCATTCGG-3') and 72f (5'-TGCGGCTGGATCTCCTT-3') (Martin-Laurnet et al, 2001). The ITS of rDNA was amplified by heating at 96 ℃ for 10 min, running 30 cycles of PCR at 94 ℃ for 30 sec, 55.9 ℃ for 30 sec and 72 ℃ for 30 sec followed by 72 ℃ for 2 min using PCR Thermal Cycler TP600. 16S/23S ITS from *Xcc* was amplified using primer 2 and primer 3 to analyzed specific DNA sequencing of *Xcc*.

8. Scanning electron microscopic observation of bacteria on the citrus leaves

To illustrate the mechanism of 4 rhizobacterial strains on disease suppression, citrus leaves which were pre-treated with these selected rhizobacteria following the inoculation with *Xcc* were compared with those of untreated or pre-treated with Streptomycin®. The surfaces of citrus leaves pre-treated with the rhizobacterial strains, Streptomycin® and non-treated were observed at 3 days after inoculation with *Xcc* using a Field Emission (FE) scanning electron microscope (JSM-6700F, JEOL Ltd., Japan). The citrus leaves used in this experiment were prepared similarly as those of '4. Pre-treatments with rhizobacteria or Streptomycin®'.

The inoculated leaves were cut into 4 pieces at 0.4 x 0.6 mm^2 using a sterile blade. Fixation, dehydration and embedding of the leaves were performed according to be described by Hayat (1989). The samples of leaf were fixed in 2 % glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.2) on a 6 well cell culture plate at room temperature for 2 h. After moving into 2 ml micro tube containing the phosphate buffer carefully, the samples were washed by the sodium phosphate buffer for 10 min each three times. Post fixation was conducted with 2 % osmium tetroxide in the phosphate buffer at 4 ℃ for 2 h. After washing three times, the samples were dehydrated through an ethanol series (30, 50, 70 % two times, 90 % once and 100 % three times for 30 min each). The ethanol in the samples was exchanged by 30, 50, 70 and 100 % acetic acid for 10 min each.

The samples were gently dried using a critical point drier (CPD 030, BAL-Tec). Then the samples were mounted on metallic stubs, gold-coated (~100 Å) with the

Osmium Plasma Coater (OPC80T, FILGEN, Japan) and viewed under the scanning electron microscope (JSM-6700F, JEOL Ltd., Japan) at 20 kV. The numbers of total bacteria was randomly selected and counted. Four samples of each experiment were carried out and the experimental data were analyzed using the Statistical Analysis System.

9. PR-1 protein in the citrus leaves

To determine whether resistance could be induced by the rhizobacterial strain in the leaves of Satsuma mandarin, pathogenesis-related protein - 1 (PR-1 protein) gene was extracted by easy-spin™ II p Plant RNA Extraction Kit (iNtRON Biotechnology Inc., Seoul, Korea), based on theory of central dogma.

The citrus branches having 4-5 young leaves which were put in a flask containing 100 ml distilled water. The rhizobacterial and *Xcc* suspension were prepared and the concentration of these suspensions was adjusted to 2.0 x 10^7 cfu/ml, each. (It carried out like '2. Pathogenic bacteria' and '3. Rhizobacterial strains') The rhizobacterial and *Xcc* suspension and distilled water were sprayed onto the both side of the leave, respectively. The treated plants were kept in a dew chamber maintaining at 100 % humidity in the dark at 28 ℃ for 6 h.

The leaves were air-dried for about 1 h and cut off. The leaf was homogenized after freezing it by liquid nitrogen. The completely grinded sample was collected by disinfected spatula up to 0.05 g into the 1.5 ml microcentrifuge tube and added by 200 μl of pre-lysis buffer, then incubation for 5 min at room temperature. Additionally, the sample was pipetted into 800 μl of lysis buffer and vigorously mixed by vortex in 20 sec. The suspended sample was poured to the column placed in the 2 ml micro tube and centrifuge at 13,000 rpm for 1 min. After the filtrate was added 200 μl of chloroform, it was mixed by vortex.

After this solution was centrifuged at 13,000 rpm for 10 min, 400 μl of upper aqueous layer containing RNA was transferred to 1.5 ml new micro tube. The fluid

was mixed well with 400 µl binding buffer by pipetting gently and it was loaded to another column combined with collection tube without wetting the rim. Loaded sample on the column was centrifuged at 13,000 rpm for 1 min. Then, the flow-thorough was discarded from the collection tube and the column was placed back in the some collection tube. In order washing the membrane in the column, 700 µl of the first washing buffer was added and centrifuged at 13,000 rpm for 1 min. After removal of filtrates and placement back, 700 µl of the second washing buffer was loaded to same column followed by centrifugation at 13,000 rpm for 1 min. To dry the column membrane, the column which was replaced with the empty collection tube was centrifuged at 13,000 rpm for 1 min and it was kept at room temperature overnight. The column was placed in a new 1.5 ml micro tube and 50 µl of elution buffer was added onto the membrane, directly. After incubation at room temperature for 1 min, it was centrifuged at 13,000 rpm for 1 min to elute total RNA.

To detect the PR-1 protein gene from the total RNA, reverse transcription (RT) PCR was conducted using ONE-STEP RT-PCR PreMix Kit (iNtRON Biotechnology Inc., Seoul, Korea). The solution for RT-PCR was added 8 µl of the kit, 2 µl of RNA template, 1 µl of each 10 pM primer and 8 µl of RNase-free water to a total volume of 20 µl each tube. After all regents were mixed carefully, these mixtures were reverse transcribed reacted to 45 ℃ for 30 min and heated to 94 ℃ for 5 min. Thirty cycles of PCR were run at 94 ℃ for 30 sec, 45 ℃ for 35 sec and 72 ℃ for 60 sec followed by 72 ℃ for 1 min using PCR Thermal Cycler TP600 (TaKaRa, Japan).

The cDNA from PR-1 protein mRNA was formed and amplified by citrus PR-1 forward primer (5’-CCT GTG ACA TGG GAC GA-3’) and reverse primer (5’-TTG

CAA ACT ACT TAA TCA TACA-3'). In order to confirm whether the cDNA hybrids were amplified or not, 5 µl of PCR products were visualized in 1 % agarose gel containing 0.01 % ethidium bromide under the ultraviolet.

10. Statistical analyses

The data of disease severity and *Xcc* density on the citrus leaves pre-treated with rhizobacterial isolates after inoculation with *Xcc* and inhibition efficacy against the pathogen by antibiotics were statistically analyzed using Duncan's multiple range test (DMRT). Statistical analysis of the experimental date were carried out using Statistical Analysis System (SAS Institute, version 9.0) program for the comparison between analysis of the averages and variance of the treatments in each experiment.

The total bacterial number on the citrus leaf surface pre-treated with rhizobacteiral strains following inoculation with *Xcc* on the SEM images were compared using a paired *t* - test.

Ⅲ. RESULTS

1. Suppression of disease severity by the pre-treatment with rhizobacterial strains

In order to investigate suppression of disease severity on Satsuma mandarin leaves by treatment with rhizobacterial strains, the bacterial isolates MRL408-3, THJ609-3, TRH415-2 and TRH423-3, which showed anti-bacterial activity *in-vitro* test (Kang, 2012), were treated on the citrus leaves before inoculation with *Xcc*. As negative and positive control H_2O and Streptomycin® were treated instead of the rhizobacterial strains, respectively.

Disease symptoms on the leaves pre-treated with H_2O began at 5-7 day after the inoculation with *Xcc*. The symptoms formed small pale brown protruded lesions like cork surrounding yellow on the back side of the leaf and turned yellow on the front side. As time went on, the lesions were bigger than before and became irregular as combining with the other lesions. Moreover, some of the young leaves, of which lesions were developed further, were fallen down. Numbers of lesion were found on the untreated control plants (Fig. 1A and Table. 1).

When the rhizobacterial strain MRL408-3, THJ609-3, TRH415-2 and TRH423-3 were pre-treated on the leaves, the lesions were formed as fast as the untreated control one. However, fewer lesions were formed on the pre-treated leaves and lesions were less apparent compared with those of untreated control. Furthermore, the lesion development was limited resulting that no leaves fallen down. Disease severity of

treatments with MRL408-3, TRH609-3 and TRH415-2 were significantly decreased about 55 % compared to those of untreated control (Fig. 1B, C, D and Table 1). On the leaves pre-treated with TRH423-3 the disease severity was largest reduced among the plants pre-treated with the rhozobactrial strains (Fig. 1E and Table 1).

To evaluate the suppression efficacy of rhizobacterial strains against *Xcc*, Streptomycin® was treated instead of the bacterial strains, as a positive control. Streptomycin® remarkably reduced the disease severity resulting in no lesions on the any pre-treated leaves (Fig. 1F and Table 1).

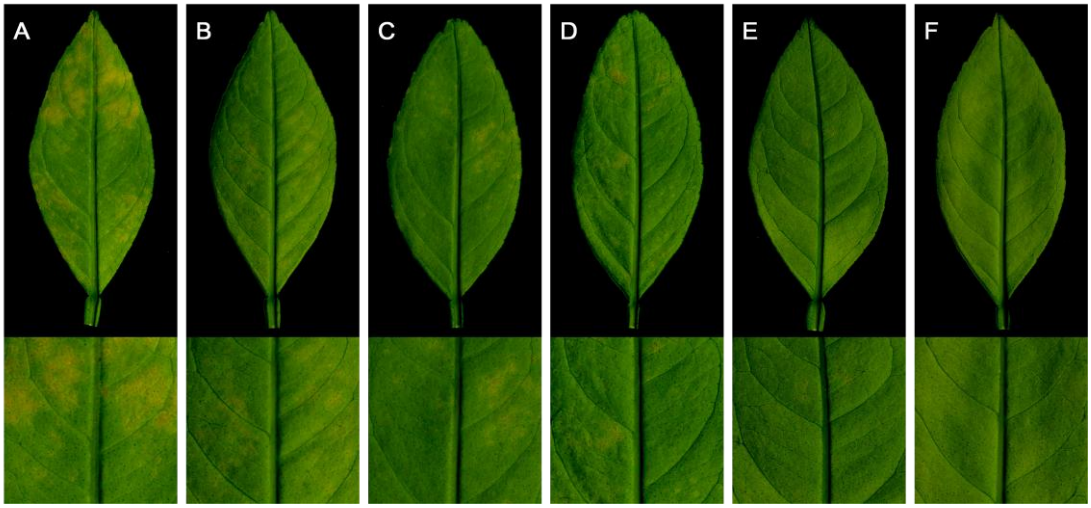

Fig. 1. Satsuma mandarin leaves pre-treated with H_2O (A), rhizobacterial isolate MRL408-3 (B), THJ609-3 (C), TRH415-2 (D), TRH423-3 (E) and commercial antibiotic Streptomycin® (F) at 14 days after inoculation with citrus canker pathogen *Xcc*. The concentration of the bacterial isolates and *Xcc* were 2.0 x 10^7 cfu/ml and the antibiotic was 1 g/L.

Table 1. Number of lesions on Satsuma mandarin leaves pre-treated with H_2O, the selected bacterial isolates and commercial antibiotic Streptomycin® after inoculation with *Xcc*.

Treatment[a]	Number of lesions	Duncan′s-test
Control	643.5 ± 294.0[c]	a[d]
MRL408-3	291.3 ± 149.1	bc
THJ609-3	320.7 ± 186.4	b
TRH415-2	262.3 ± 170.0	bc
TRH423-3	135.0 ± 55.7	b
Streptomycin®[b]	0.0 ± 0.0	c

[a] The concentration of the bacterial isolates suspension and citrus canker pathogen *Xcc* were 2.0 × 10^7 cfu/ml. The control was pre-treated with sterilized distilled water instead of the bacterial strains.

[b] The concentration of the antibiotic was 1 g/L.

[c] Values represent means ± standard error of three separated experiments, each containing three plants of six leaves per treatment.

[d] The different letters are significantly (*P=0.001*) different according to Duncan's multiple test.

2. Reduction of *Xcc* on the leaves pre-treated with rhizobacterial strains

To illustrate the mechanism of disease suppression by rhizobacterial strains, the number of *Xcc* on the citrus leaves pre-treated with the selected bacterial strains was compared with those of untreated control plants. In order to do that, the washing suspension of the citrus leaves pre-treated with H_2O, each of the rhizobacteria and Streptomycin® following inoculation with *Xcc* was incubated on the semi-selective medium and the colonies were counted.

Whereas plates of the washing suspension from the untreated control plants only colonies of *Xcc* were found, on the plates from the leaves pre-treated the bacterial strains not only *Xcc* but also each individual rhiaobacterial strains were observed. The formation of *Xcc* colonies began to show at 2 days after incubation with small transparent round shape. The colonies became bigger and turned yellow at 3 days later (Fig. 2A).

The colonies of the rhizobcterial strains were began to observe at 1 day after incubation which was faster than formation of *Xcc* colony. At that time, MRL 408-3 and TRH423-3 formed small and transparent round shape. THJ609-3 and TRH45-2 was bigger than MRL408-3 and TRH423-3 and had beige round shape. At 3 days after incubation, the colonies of MRL408-3 turned beige and TRH423-3 became beige surrounding pale narrow one (Fig. 2B, E). Also, THJ609-3 turned yellow-beige surrounding wide pale shape and TRH415-2 had 3-fold round beige colonies (Fig. 2C, D).

On the plate of the washing suspension from pre-treated with the 4 rhizobacterial

strains, number of *Xcc* colonies were decreased compared with that from untreated control (Table 2). Among the bacterial strains, TRH415-2 was showed lower anti-bacterial activity presented approximately 80 % reduction on the leave than other pre-treatment with MRL408-3, THJ609-3 and TRH423-3 presented over 85% reduction compared with untreated control (Table 2). Especially, MRL408-3 remarkably suppressed the density of *Xcc* with about 95 % (Table 2). On the other hand, no colony of *Xcc* was observed on the medium incubated with the washing suspension from the leaves pre-treated with Streptomycin® (Fig. 2F and Table 2). The result presented that *Xcc* populations were suppressed by these rhizobacterial strains on the citrus leaf surface.

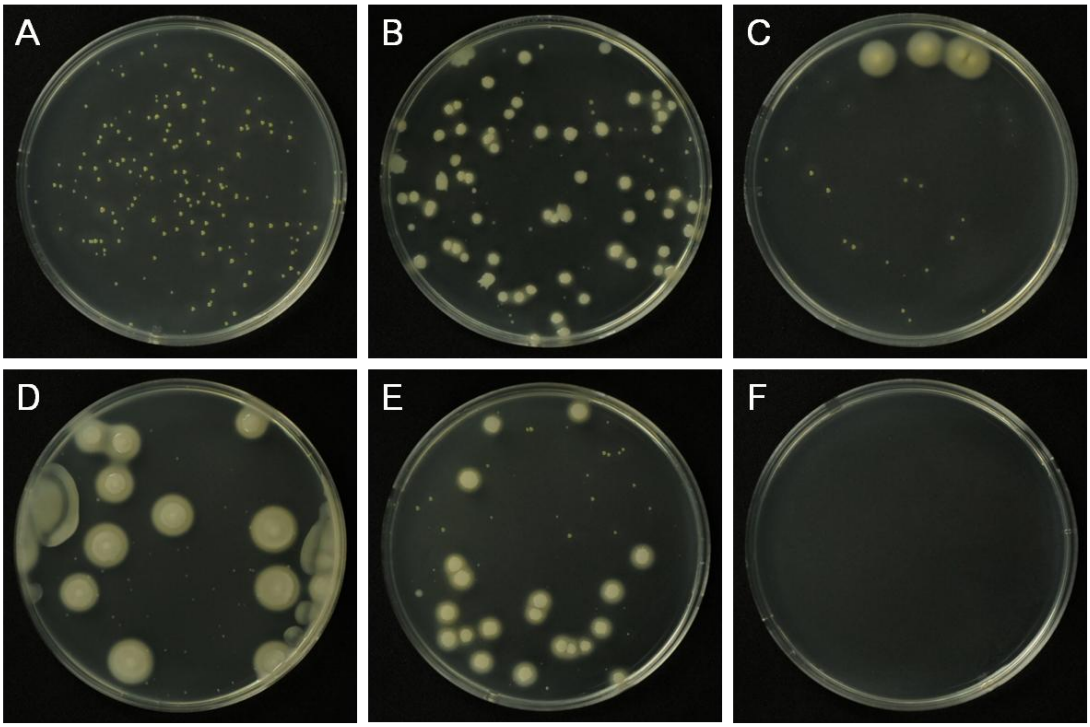

Fig. 2. Photographs of microbe on semi-selective medium incubated with washing suspension from the Satsuma mandarin leaves pre-treated with H_2O (A), the rhizobacterial strain MRL408-3 (B), TRH690-3 (C), TRH415-2 (D), TRH423-3 (E) and the antibiotic Streptomycin® following inoculation with *Xcc* at 28 ℃ for 3 days. The concentration of the bacterial isolates and *Xcc* were 2.0×10^7 cfu/ml and the antibiotic was 1 g/L.

Table 2. The number of bacterial colonies of *Xcc* on the semi-selective medium incubated with washing suspension from the citrus leaves pre-treated with H_2O, the bacterial strains and the commercial antibiotic Streptomycin®

Treatment[a]	Colony number of *Xcc*	Duncan′s-test
Control	169.3 ± 12.9[c]	a[d]
MRL408-3	9.0 ± 4.4	cd
THJ609-3	23.3 ± 17.0	bc
TRH415-2	33.0 ± 11.5	b
TRH423-3	22.7 ± 10.7	bc
Streptomycin®[b]	0.0 ± 0.0	d

[a] The concentration of the bacterial isolates and *Xcc* were 2.0×10^7 cfu/ml. The control was pre-treated with sterilized distilled water instead of the bacterial strains.

[b] The concentration of the antibiotic was 1 g/L.

[c] The letters represented mean ± standard deviation.

[d] The different letters are significantly (*P=0.001*) different according to Duncan's multiple range test.

3. Identification of *Xcc* or rhizobacteria on the semi-selective medium

To identify the counted colonies grown on the semi-selective medium obtained from leaves untreated or pre-treated with the rhizobacterial strains, specific regions of ribosomal DNA (rDNA) from the counted colonies were amplified using each of the corresponding primers. Using the primers 2 and 3 the specific regions of rDNA from the colonies were successfully amplified for identification as *Xcc*, in which the PCR product showed a thick and clear band at about 200 bp on the agarose gel electrophoresis (Fig. 3 lane 1), indicating that the bacteria was identified as *Xcc*.

The universal primers 38R and 72F amplified 16S/23S internal transcribed spacer resions (ITS) of rDNA from the rhizobacteria MRL408-3, THJ609-3, TRH415-2 and TRH423-3. The PCR product from MRL408-3 and TRH423-3 appeared a thick and clear band at 700 bp, whereas those of THJ609-3 and TRH415-2 appeared thin and dim band at 600 bp on the agarose gel electrophoresis (Fig. 3 lane 2, 3, 4, 5).

Furthermore, the sequencing results of PCR fragments from rDNA of the rhizobacterial strains were compared to the sequences in the GenBank database using the NCBI BLAST program. The amplified PCR product of MRL408-3 and TRH423-3 had 99 % nucleotide similarity as *Burkholderia gladioli* (Fig. 4A and D). Also, THJ609-3 and TRH415-2 by amplified by PCR were identified as *Psedomonas fluorescens* having 95 % and 97 % nucleotide similarity each (Fig. 4B and C). On the other hand, sequencing results of PCR product of the citrus canker pathogenic bacteria, *Xcc* was identified *as Xanthomonas axonopodis* pv. *citri* having 100% similarity in the GenBank database (Fig. 4E).

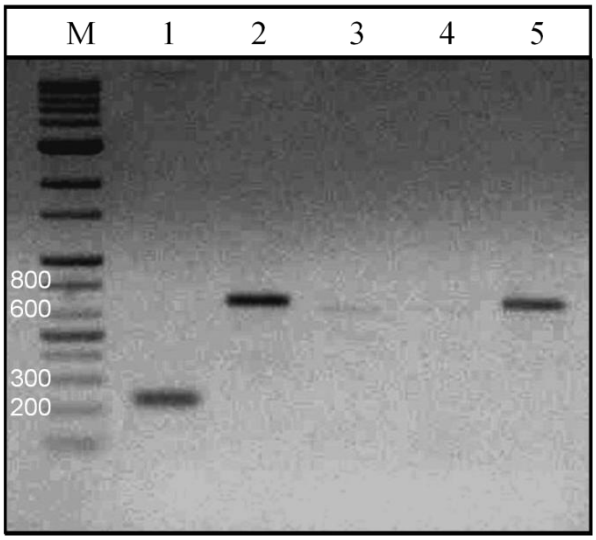

Fig. 3. Gel electrophoresis of PCR amplified specific regions from *Xcc* (lane 1) using the primers 2 and 3 and 16S/23S internal transcribed spacer region (ITS) from the selected bacterial isolates MRL408-3 (lane 2), THJ609-3 (lane 3), TRH415-2 (lane 4) and TRH423-3 (lane 5) using universal primer 38r and 72f M: 1kb molecular size marker (BIOFACT Co. Ltd, Daejeon, Korea).

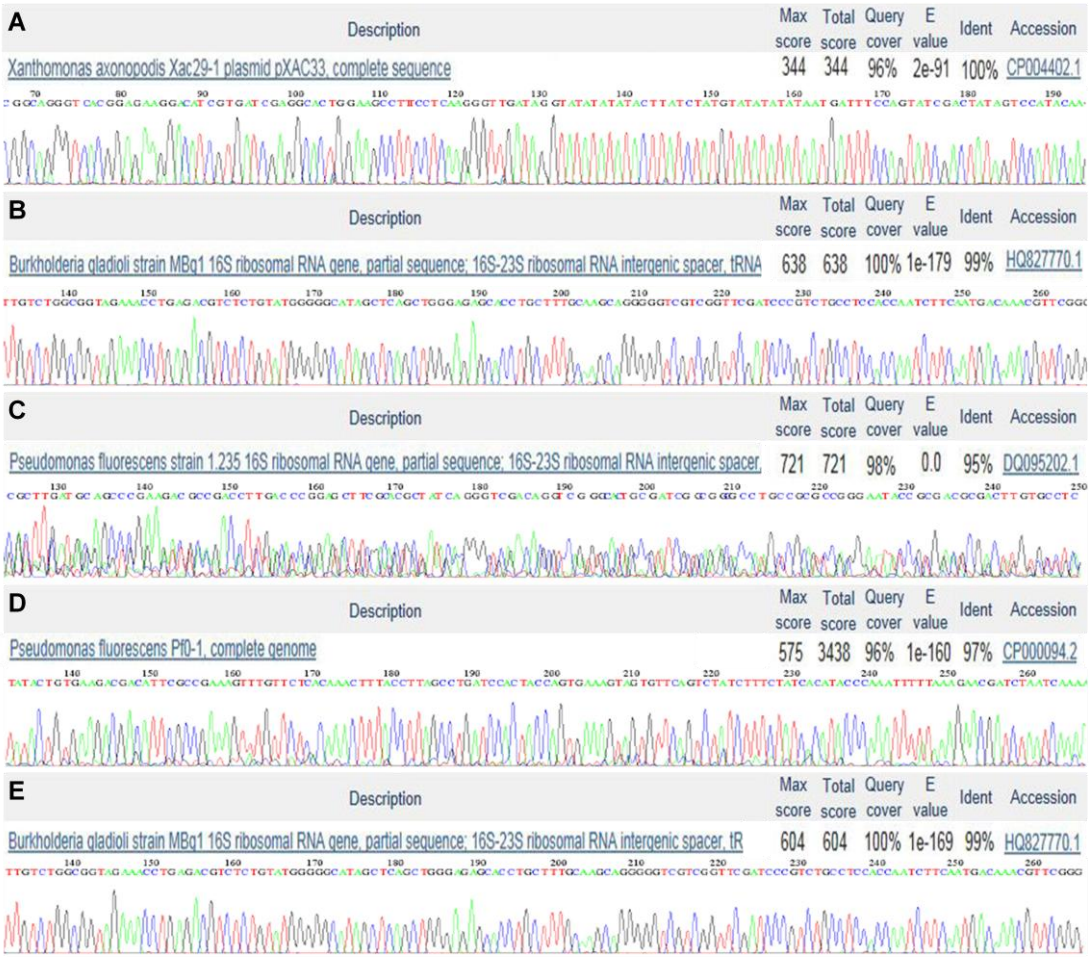

Fig. 4. BLAST results on the NCBI based on the rDNA sequence of citrus cnaker pathogenic bacteria *Xcc* (A) also, of rhizobacterial strains MRL408-3 (B), THJ609-3 (C), TRH415-2 (D) and TRH423-3 (E).

4. Scanning electron microscopy on the citrus leaves pre-treated the rhizobacteria

To illustrate the mechanism of the selected rhizobacterial strains on disease suppression, Satsuma mandarin leaves pre-treated with selected rhizobacterial strains after the inoculation with *Xcc* were compared with those of untreated or pre-treated with Streptomycin® under a scanning electron microscope (SEM).

No bacterial cells were observed on the untreated control citrus leaves whereas numbers of bacterial cells were found on the leaves inoculated with *Xcc* only (Fig. 5) indicating the population of *Xcc* for successful infection.

On the leaves pre-treated with the rhizobacterial strains a lot of bacterial cells were found. However, all of the bacterial cells observed under the SEM were about 1.0 µm long and rod-shaped which could not distinguish between *Xcc* and the rhizobacterial strains. Therefore, although the observed bacterial cells was not identified whether they are *Xcc* or rhizobacteria, total number of cells was reduced compared to those on the only *Xcc* inoculated leaves (Table 3), indicating the number of *Xcc* might be decreased by pre-treatment with the rhizobacterial strains.

Indeed, the number of bacterial cells of the treatment with only *Xcc* counted the most among all the other treatments, although there were not significant each other (Fig. 5F and Table 3). Pre-treatment with THJ609-3 decreased number of bacterial cells a little bit compared to treatment with *Xcc* whereas more number of bacterial cells was reduced by the pre-treatment with TRH415-2 (Fig. 5B, D and Table 3).

As expected, there were only a few bacterial cells in the leaves pre-treated with Streptomycin® following inoculation with *Xcc* (Fig 5I and Table 3), indicating the

antibiotic was effective before *Xcc* invaded into the citrus leaves.

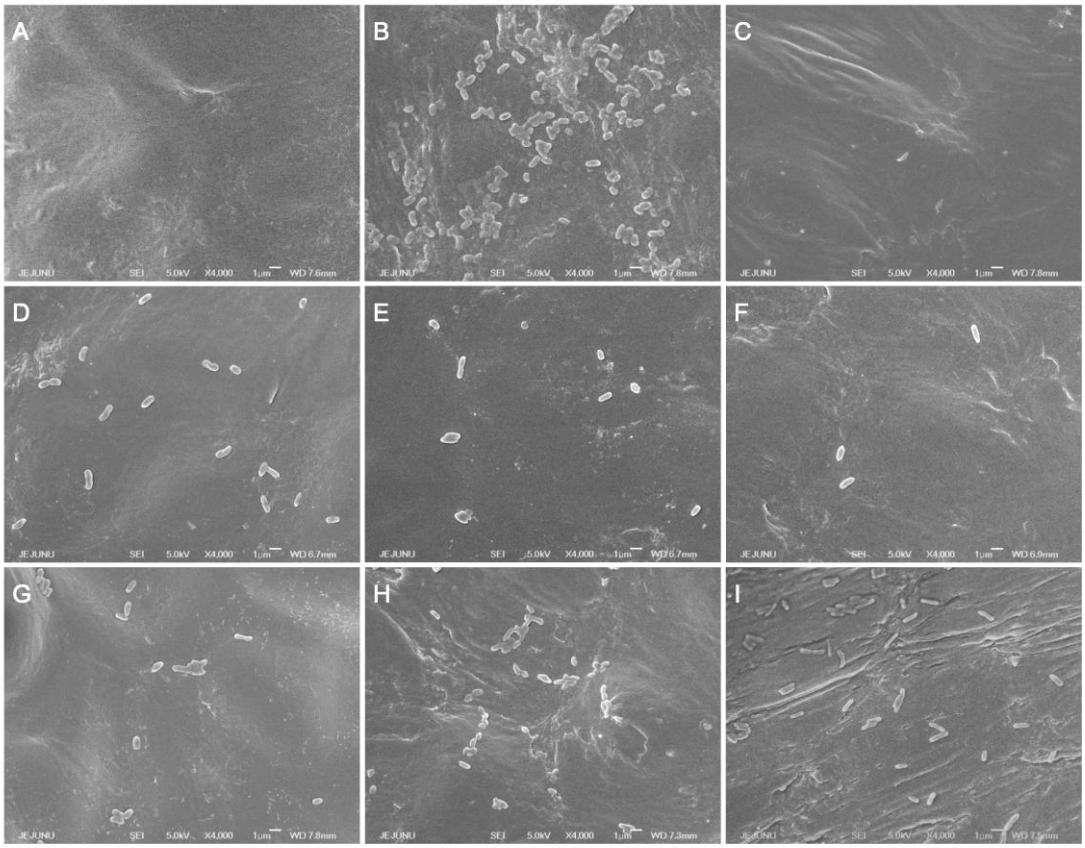

Fig. 5. Scanning electron microscopical observation on Satsuma mandarin leaves untreated (A), inoculated with *Xcc* (B), pre-treated with Streptomycin® (C), following the inoculation with *Xcc* and leaves treated the rhizobacterial strains MRL408-3 (D), THJ609-3 (E) TRH415-2 (F) and TRH423-3 (I). The rest photographs showed the leaves pre-treated with the bacterial strains MRL408-3 (G) and TRH415-2 (H) after the inoculation with *Xcc*. The concentration of the strains and *Xcc* were 2.0×10^7 cfu/ml and the antibiotic was 1g/L, respectively.

Table 3. The number of total bacterial cells observed on the Satsuma mandarin leaves pre-treated with H_2O, the bacterial isolates and Streptomycin® following the inoculation with *Xcc* for 3 days.

Treatment[a]	Number of bacterial cells	t-test[d]	P-value
Xcc	31.3 ± 15.7[c]	-	-
MRL408-3 + *Xcc*	18.5 ± 8.5	*	0.024
THJ609-3 + *Xcc*	30.0 ± 27.0	ns	0.444
TRH415-2 + *Xcc*	14.5 ± 6.1	*	0.021
TRH423-3 + *Xcc*	25.0 ± 6.7	ns	0.139
Streptomycin[b] + *Xcc*	0.7 ± 0.6	*	0.015

[a] The concentration of the bacterial isolates suspension and citrus canker pathogen *Xcc* were 2.0 × 10^7 cfu/ml.

[b] The concentration of the antibiotic was 1 g/L.

[c] Values represent means ± standard deviation.

[d] ns: non-significant

*: significant at 5% probability level

5. PR-1 protein gene in Satsuma mandarin leaves treated with rhizobacterial strain

To determine whether resistance could be induced by treatment with the rhizobacterial strain THJ609-3 in the Satsuma mandarin leaf or not, pathogenesis-related protein -1 (PR-1) gene was amplified by reverse transcription (RT) PCR using citrus PR-1 forward and reverse primer.

The gene was extracted from the leave treated by THJ609-3 or *Xcc*. Agarose gel electrophoresis of the RT-PCR amplified product from PR-1 protein gene observed a band at 400 bp (Fig. 6). The band of PR-1 protein gene from the leaf treated with *Xcc* was apparent compared to another band but that of gene from the leaf treated with H_2O was rarely visible (Fig. 6 lane 1 and 3). This showed that the PR-1 gene is especially synthesized in the citrus leaves by invasion of *Xcc*.

The RT-PCR product from the leaf treated with THJ609-3 showed the band dimly compared with the band of treatment with *Xcc* (Fig 6 lane 2) indicating less amount of PR-1 protein than that inoculated with *Xcc*.

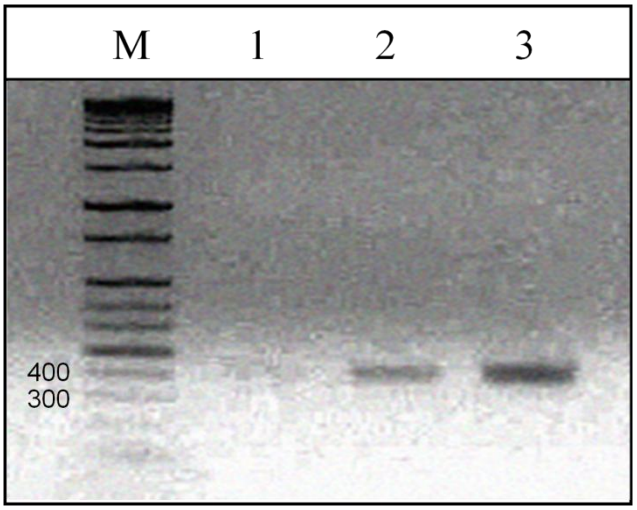

Fig. 6. Gel electrophoresis of RT-PCR amplified PR-1 protein gene from Satsuma mandarin leaves treated by distilled water (lane 1), the rhizobacterial strain THJ 609-3 (lane 2) and *Xcc* (lane 3) after 6 h. The concentration of the rhizobacterial strain THJ 609-3 and *Xcc* were 2.0×10^7 cfu/ml. The presented gel electrophoresis of the bands were used the citrus PR-1 forward and reverse primer. M : 1kb molecular size marker (BIOFACT Co. Ltd, Daejeon, Korea).

Ⅳ. DISCUSSION

Plant growth-promoting rhizobacteria (PGPR) has been known as an eco-friendly control agent in order to reduction of chemical control which caused the problem such as getting chemical resistance of the pathogen, environmental pollution and harmfulness to the human. A lot of studies about PGPR against an array of the pathogen have conducted in all over the world in the last couple of decades (Weller, 1988). Based on the studies about PGPR having anti-fungal or bacterial activity, several PGPR-based products became commercialized since 1990s. Almost of these products contain *Bacillus* spp., especially *Bacillus subtilis* strains (Kroepper et al., 2004). For example, Serenade® product based on *B. subtilis* strain QST 731 has been used in 20 countries including the United States for controlling plant diseases such as downy mildew or powdery mildew (Miille, 2006). Furthermore, Shooting star® product containing *B. subtilis* strain KB-401 was effective against cucumber powdery mildew as well as citrus melanose (Nam et al., 2010). Also, *B. subtilis* strain GB-0365 was commercialized for controlling against pythium blight caused by *Pythium* spp. (Jung et al., 2006). Besides *B. subtilis* strains, *Paenibacillus polymyxa* strain CW could suppress powdery mildews of red pepper and tomato (Kim et al., 2013). Furthermore, *P. polymyxa* strain AC-1 was already commercialized (Ko, 2012).

Bacteria of the genus *Burkholderia* containing over 30 species has been exploited for biological control, bioremediation and plant growth promotion

purposes (Coenye and Vandamme, 2003). The effect of this biological agent appeared to result from antibiosis because the antibiosis-deficient mutant bacteria didn't affect zoospore or cyst germination. Especially, *Burkholderia gladioli* (MRL408-3 and TRH423-3) which is gram-negative bacteria presented anti-fungal activity against *Botrytis cinerea*, *Aspergillus flavus*, *A. niger*, *Penicillium digitatum*, *P. expansum*, *Sclerotinia sclerotiorum*, *Phytophthora cactorum* (Elshafie et al., 2012) and Grey mould on lily caused by *B. elliptica* was controlled by *Bur. gladioli* (Chiou and Wu, 2001). Elements of *Bur. gladioli* produce extracellular hydrolytic enzymes such as protease, cellulase, amylase, chitinase, amylase and glucanase that might directly affect to the pathogen (Elshafie et al., 2012).

Members of the genus *Pseudomonas* comprise a large group of the active biological control strains. There is general ability at these *Pseudomonas* strains is producing a variety of strong anti-fungal metabolite including 2,4-diacetyphloroglucinol, pyrrolnitrin [3-chloro-4-2(2'- nitro-3'-chlorophenyl)-pyrrole] and phenazine-1-carboxylic acid, macrocyclic lactone, 2,3-de-epoxy-2,3-didehydro-rhizoxin (Ligon et al., 2000). There are also antibiotics, hyderogen cyanide, siderophore (O'Sullivan and O'Gara, 1992) and isolation chitinase production (Velazhahan et al., 1999) in the other antifungal metabolites of this genus. Gram-negative bacteria, *Pseudomonas fluorescens* (THJ609-3 and TRH415-2) which was used in this study suppressed stem rot diseases in garlic caused by *Sclerotium cepivorum* plant (Kang and Jeun, 2012) as well as in groundnut cuased by *S. rolfsii*. The anti-fungal activity against stem rot in groundnut was more effective by treatment with combined application of *Pseudomonas. fluorescens* and *Trichoderma*

viride than treatment with only *Pseutomoans. fluorescens* (Manjula et al., 2004).

Also, biological control using PGPR has been required at the citrus industry in Jeju and some of the studies about PGPR have carried out and reported. Rhizobacterial strain MRL 408-3, THJ609-3, TRH415-2 and TRH423-3 which were conducted in this study were already reported that these rhizobacterial strains have anti-fungal activity against citrus scab caused by *Elsinoe fawcettii* (Kim et al., 2011) as well as citrus melanose caused by *Diaporthe citri* (Ko et al., 2012). Additionally, these strains suppressed the growth of citrus canker pathogen *Xanthomonas citri* subsp. *citri* (*Xcc*) *in-vitro* test (Kang, 2012).

In this study it was presented that the lesion development of *Xcc* was effectively suppressed about 70 % by TRH423-3 compared with those of nonbacterized control. Similarly, the disease developments were reduced approximately 55 % by MRL408-3 and TRH415-2 (Table 1). Therefore, it seems that as the other PGPR these rhizobacterial strains has suppression efficacy against *Xcc*, practically, although the efficacy of rhizobacteria was lower than that of Streptomycin®.

Actually, some studies showed that the control efficacy by rhizobacterial strains were lower than that by chemicals. For instance, chemical fungicide Cyazofamid® showed higher suppression against citrus late blight caused by *Phytophthora citrophthora* than rhizobacterial strains *Bur. gladioli*, *B. cereus* and *B. circulans* which showed anti-fungal activity against the pathogen (Kang and Jeun, 2010). Also, effective rhizobacteria *Bur. gladioli* and *Pseudomonas putida* strains had lower disease control efficacy against citrus melanose caused by *D. citri* and citrus scab caused by *E. fawcettii* in citrus than chemical applications such as Dithianon® and

Imibenconazol® (Ko et al., 2012; Kim et al., 2011). It has been shown not only in citrus but also in other plants. Late blight caused by *P. infestance* in potato was suppressed by rhizobacterial strain *Pseudomonas putida*, *Micrococcus luteus* and *Flexibacteraceae bacterium* showing anti-fungal activity against this pathogen lower than chemical fungicide Mancozeb WP® (Kim, 2006). One study reported that inoculation with *B. subtilis* GB-0365 following treatment with chemicals Metalaxyl® at 3 days later successfully controlled pythium blight caused by *Pythium aphanidermatum* compared to treatment with only chemicals. Whereas that, the treatment with only *B. subtilis* GB-0365 showed the lowest suppression among the other treatments (Jung et al., 2006).

To illustrate the mechanism of disease reduction by these rhizobacterial strains, rhizobacterial strains we were counted the number of *Xcc* colonies on the leaves of Satsuma mandarin pre-treated each of the rhizobacterial strains or antibiotic Streptomycin® after inoculation with *Xcc*. All of the rhizobacterial strains significantly suppressed the density of *Xcc* on the medium (Table 2) indicating the number of *Xcc* was reduced on the citrus leaves by pre-treatment with the rhizobacterial strains. This finding suggests that the low concentration of *Xcc* populations which are caused by anti-bacterial activity of rhizobacterial strains make the low disease severity on the Satsuma mandarin leaf, because the antibiotic of these rhizobacteria reduced the invasion of *Xcc* into the citrus leaves. One study reported that concentration of the *Xcc* suspension which was lower than 1.0×10^5 cfu/ml didn't induce canker lesions on the citrus leaves, but general citrus canker symptoms were observed after inoculation with the suspension at the high concentration,

especially 1.0 x 10^7 cfu/ml (Kang, et al., 2014). Thus, it seems that the population of the citrus canker pathogenic cells acts an important for the successful infection to the host tissue such as Satsuma mandarin leaves.

To confirm the counted cells forming colonies on the medium, the bacterial cells were identified by molecular biological detection analyzing bacterial DNA sequence. As expected, the bacterial cells counted visually as *Xcc* were identified as *Xcc* resulting in presence of the band at about 200 bp which was amplified *Xcc* ribosomal DNA (rDNA) using primer 2 and 3 (Fig. 3). Study by Hartung et al. (1993) reported that the primer set allowed the amplification of a 222 bp DNA fragment from *Xcc* pathotype A, which is pathogenicity of the causal agent of Asiatic citrus canker surviving on infected citrus tree in South korea but not other pathotypes of *Xcc* (Yu et al., 2012). In addition, this sequencing result which was compared with the GenBank database had 100 % nucleotide similarity with *Xanthomonas axonopodis* pv. *citri* (Fig. 4). This pathogenic bacterium was reported to be same with *Xcc* (Schaad et al., 2006).

Also, the rhizobacterial strains were identified as ones which were pre-treated on the leaves, respectively. The bands of amplification using universal primers 38R and 72F (Gürtler and Stanisich, 1996) from rDNA of MRL408-3 and TRH423-3 were presented at 700 bp, and of THJ609-3 and TRH415-2 were at about 600 bp (Fig. 3). When these sequencing results were compared to the database, MRL408-3 and TRH423-3 identified as *Burkholderia gladioli* (similarity at 99 %) and THJ609-3 and TRH415-2 were as *Pseudomonas fluorescens* (similarity at 95 % and 97 %, each) (Fig. 4). In previous study also, rDNA of *Burkholderia gladioli* and *Pseudomonas*

fluorescens were similarly amplified at 700 bp and 538 bp, each, on the agarose gel (Kang, 2012). Thus, this result presented that counted bacterial colonies were same with treated 4 rhizobacteria and *Xcc*.

Additionally, to investigate the role of these rhizobacterial strains on disease suppression, citrus leaves pre-treated with these rhizobacterial strains or Streptomycin® following the inoculation with *Xcc* observed under a scanning electron microscope (SEM). Because either *Xcc* or the rhizobacterial strains have about 1.0 μm long in size and rod-shaped, it could not distinguish between *Xcc* and the rhizobacterial strains on the SEM image. Therefore, the total number of bacterial cells which were *Xcc* or the rhizobacterial strains was counted. Interestingly, the most numbers of bacterial cells was counted on the leaves inoculated only *Xcc* (Table 3). Although both *Xcc* and the rhizobacterial strains were sprayed, less number of bacterial cells was observed on the leaves pre-treated with the rhizobacterial strains (Table 3). These observation was coincided with the results in which the decrease number of *Xcc* colonies on the semi-selective medium incubated with the washing suspension from the leaves pre-treated with the rhizobacterial strains (Table 2). Reduction of population of total bacteria on the leaves pre-treated with rhizobacterial strains compared with leaves only inoculated with *Xcc* suggested that the antibiotic from the rhizobacterial strains caused decrease of the population of bacterial pathogen, *Xcc* and then invasion of the pathogen into the citrus leaves.

Mechanisms of biological control with bacterial strains are believed to include competition for nutrient or space bacterial metabolites that adversely affect the pathogen, or induce systemic resistance (Massomo et al., 2004). Agrocin 84 (a di-

substituted nucleotide) which was produced by *Agrobacterium radiobacter*, has controlled the cirulentstrains of *A. tumefaciens* causing crown gall of fruit trees (Cooksey and Moore, 1982). Also, *Erwinia herbicola* could control *E. amylonora* which produced exudates and necrosis on the immature pear and apple fruits (Vanneste et al., 1992) by not only competition with the indigenous plants diverse natural environment but also production of antibiosis such as organic acid, hydroquinones and bacteriocins (Ishimaru et al., 1988). Black rot caused by Xanthomonas campestris pv. campestris, in cabbage was reduced the number and size of lesions on the external leaves and in the absence of internal symptoms by treated Bacillus subtilis strain BB (Wulff et al., 2002). *Pseudomonas fluorescens* and *Serratia marcescens* demonstrated induced systemic resistance (ISR) resulting in suppression of *P. syringae* pv. *lachrymans* (Raupach et al., 1996; Liu et al., 1995).

In this study, it is considered that the reduction of the population of citrus canker pathogen *Xcc* is relative these mechanisms such as production of a variety of siderophores, cyanhdric acid and amino acid from *Pseudomonas* spp. (Dowling and O'Gara, 1994). So, it may be necessary to illustrate the clear metabolites which are potent anti-bacteria of reduction of *Xcc* population before *Xcc* invaded into the citrus leaves in the further research.

In order to investigate whether resistance could be induced by treatment with the rhizobacteiral strain THJ609-3 in the citrus leaf or not, PR-1 protein gene was amplified based on the theory of central dogma. PR-1 protein gene observed a band at 400 bp. The band of PR-1 protein gene from treatment with *Xcc* leaf was most apparent, indicating that PR-1 protein gene is synthesized in the citrus leaves by

infection of *Xcc*. Also, the band from the leaf treated with THJ609-3 was appeared although it was dimly compared to that of inoculation with *Xcc* (Fig. 6). This result showed that perennial plant such as citrus tree could get a Systemic acquired resistance (SAR), like an annual plant.

SAR which have been acted directly anti-fungal activity or catalyst producing other antibiotic materials is associated with accumulation of phenolic compounds (Hunt et al., 1997) and PR (pathogenesis related) proteins. SAR is induced infection of pathogen or signal molecules such as salicylic acid (Malamy et al., 1990; Métraux et al., 1990). It is known that PR-proteins and phytoalexin more and faster present in the acquired resistant plants than not acquired plants (Jeun, 2002).

One research reported that transgenic tobacco plants which expressed high levels of exhibited PR-1a protein significantly reduced disease symptoms caused by infection with 2 oomycete pathogens, *Peronospora tabacina* and *phytophthora parasitica* var *nicotianae* (Niderman et al., 1995). Another research described by Görlach et al. (1996) showed that papillae which prevent invasion of pathogen was more formed on the wheat pre-treated chemical inducer, BRH (Bion®) after inoculation with *Blumeria graminis* f. sp *tritici* and formation of haustoria were reduced. Therefore, in order to confirm whether resistance by treating rhizobacterial strains on the citrus leaf was induce or not, the further research is necessary such as confirmation of phytoalexin, phenolic compound, cell well thickening by lignin and formation of papillae.

Based on these results, it is suggested that 4 rhizobacterial strains could express mainly any antibiotic activity to *Xcc* on the Satsuma mandarin leaf surface, resulting

in suppression of disease severity. Although the reduction of disease severity by the rhizobacterial strains was lower compared to those of commercial antibiotic Streptomycin®, it is expected that the control using the rhizobacterial strains could be helpful in a green house and field where chemical application is prohibited or limited.

Ⅴ. 적　　요

감귤 궤양병은 *Xanthomonas citri* subsp. *citri* (*Xcc*)에 의해 발병하는 세균병으로, 많은 국가에서 검역 대상 병으로 지정한 매우 중요한 식물 병이다. 감귤 궤양병은 주로 화학약제에 의해 방제가 되기 때문에, 화학약제 사용에 따른 부작용으로 인해 화학적 방제를 대신하여 병을 방제할 수 있는 대체 방안이 요구되고 있다. 선행연구에서 인공적인 배지 상에서의 감귤 궤양병균에 항세균 효과를 보이는 네 가지 근권세균 MRL408-3와 TRH423-3은 *Burkholderia gladioli*, THJ609-3과 TRH415-2는 *Pseudomonas fluorescens*이 선발되었다. 이러한 근권세균을 가지고 감귤 잎에 선 처리한 후 감귤 궤양병균을 접종했을 때, 감귤 잎에서 궤양병 발생이 유의적으로 억제된 것을 확인하였다. 본 실험에서는 이러한 근권세균에 의해 감귤 궤양병이 억제된 원인을 알아내기 위해, 근권세균이 처리된 잎 표면에 있는 감귤 궤양병균을 수를 조사하였고. 전사 전자현미경을 이용하여 관찰 하였다. 근권세균을 처리한 잎에서 감귤 궤양병균의 콜로니 수가 무처리 구에 비해 감소되었으며, 이는 근권세균이 잎 표면에서도 감귤 궤양병균의 밀도를 직접적으로 억제시키는 것을 암시하였다. 조사된 세균들은 특이적 primer를 이용한 polymerase chain reaction (PCR)과 rDNA 염기서열 분석을 통해 각각 감귤 궤양병균과 원래의 근권세균들과 동일하게 동정되었다. 주사전자현미경 이미지를 관찰한 결과, 근권세균을 전처리하고 감귤 궤양병균을 접종한 식물체 표면에서의 세균 수가 감귤 궤양병균만 접종한 잎에서의 세균의 수에 비해 감소된 것이 확인되었다. 한편, 근권세균에

의한 식물체의 저항성 유도 여부를 확인하기 위해 병 관련단백질-1 (PR-1 단백질) 유전자를 감귤 잎에서 추출하였다. 예상대로, 근권세균인 THJ 609-3을 처리한 잎에서 PR-1 단백질 유전자가 증폭되었다. 이와 같은 결과들을 기초로 이들 근권세균이 감귤 잎에서 감귤 궤양병균에 대해 항세균 효과를 나타내어 궤양병균의 밀도를 낮춤으로써 감귤 궤양병을 억제된다고 생각된다. 따라서 이러한 근권세균들이 화학 약제의 사용이 금지 또는 제한된 친환경 농가의 하우스 또는 포장에서 생물학적 방제 수단으로 이용이 가능할 것으로 기대된다.

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