Thesis for the Degree of Master of Agriculture

Disease suppression by pre-treatment with bio-sulfur on cucumber leaves inoculated with *Colletotrichum orbiculare*

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ABSTRACT

Bio-sulfur is one of by-product of sulfur that produced as a result in process of desulfurization from a landfill site. In Europe, it is called 'Bio-sulfur' because microorganism such as *Thiobacillus* sp. collected as sulfur element. Many researches have illustrated that some sulfuric materials have an effect on growth of mycelium and disease suppression by fungus. But, they can cause some phytotoxicities on young leaves in eco-friendly orchards because of its high pH. Compare with other sulfuric materials, bio-sulfur has lower pH also the small particle is advantageous to suppress the fungal disease like powdery mildew. So, in this study, in order to investigate the practical use of bio-sulfur as an agent for controlling of plant disease, antifungal activity of bio-sulfur was tested *in vitro* against *Colletotrichum orbiculare*. Efficacy of bio-sulfur for suppressing disease severity was evaluated *in vivo*, against the anthracnose disease too. Mycelial growth of *C. orbiculare* was inhibited on the agar medium containing bio-sulfur. To extent contact area between bio-sulfur and pathogen, *C. orbiculare* was inoculated in the broth medium containing bio-sulfur. As a result, fresh and dry weight of mycelium decreased in broth medium added bio-sulfur. Also, the disease severity on the leaves pre-treated with bio-sulfur was significantly decreased compared to those of untreated one. To understand how the anthracnose disease is suppressed by bio-sulfur, the infection structure of *C. orbiculare* on the cucumber leaves was observed using a fluorescent microscope. Appressorium is a specific mechanism of some fungus including *C. orbiculare* for invasion into plants. There were only a few of appressoria on the cucumber leaves pre-treated with bio-sulfur whereas abundant appressoria were observed on untreated one. Also, similar results of bio-sulfur were observed on the leaves pre-treated with a commercial fungicide Benomyl®. Based on these results, it is suggested

that the inhibition of appressorium formation by bio-sulfur may be the main cause of anthracnose suppression on cucumber leaves. These results may be useful for plant protection especially in eco-friend farms where application with commercial chemicals are limited.

Ⅰ. INTRODUCTION

Searches of new effective changeover methods for the waste into disposable energy have pushed forward under government for offensive action correspond to the expansion of new regeneration energy in Korea (Chun, 2010). Carbon dioxide and methane created from the organic waste are the representative gas that is main cause of global warming. So, it has tried that such greenhouse gas were collected and used as a fuel or electronic energy on the power production facilities (Lim et al., 2012; Seo et al., 2009).

On the other hand, H_2S gas, one of the impurities from landfill site along with moisture, siloxane, and fine dust, caused malodor or environmental pollution because they change to H_2SO_4 resulting in the acidic rain if they combine with H_2O (An et al., 2017; Cha et al., 1994). But, there are some detected H_2S gases from construction waste in the foreign country minimum 0.013ppb to maximum 12,000ppm (Lee et al., 2006). Also, the concentration of H_2S in the emission pipe of domestic waste land fill was 151~358ppm while the Detection Limit (Below Detection Limit, 3 standard deviation) of these gas were 244ppt (Son et al., 2007). Therefore, there were some attempts for remove the H_2S.

The process of the desulfurization is summarized in Fig. 1. First, from the land site, the H_2S-rich gas is injected into the desulfurization equipment, called 'scrubber'. The purified gas is out by adding soda lime and used for making electric power. In bioreactor, during oxidization of gas, sulfur is collected by microorganisms such as *Thiobacillus* spp. which are the effective microorganisms using as sulfur-oxidative agents (Park et al., 2002). These microorganisms are adsorbed to the sulfur aggregation. Finally, soda lime is collected and elemental sulfur is separated from the sulfur handling.

On this desulfurization process practiced at the landfill site, bio-sulfur can be

produced as by-product on the biological process of desulfurization by some kind of microorganisms. These microorganisms remove the H_2S in landfill gas for protection of facilities or prevention of air pollution. In Europe, this elemental sulfur was called 'Bio-sulfur' because the microorganisms can switch over to the biological sulfur. In Korea, also have process of desulfurization system and produce plenty of bio-sulfur. The daily mean and annual average output of bio-sulfur from the landfill site were 15 m^3/day and 5, 475 m^3/year, respectively, from March 2014 to June 2015 in the landfill of metropolitan area in Seoul (Park and Eum, 2016).

Similarly, Shell-Paques/THIOPAQ™ is the biotechnological process of remove H_2S for produce the elemental sulfur which are licensed by Shell and Paques. Bio-sulfur by THIOPAQ™ process has some advantages for agriculture such as a good fertilizer on the canola yield compared with several commercial sulfurs because of its hydrophilic properties and small particle size, while the inorganic elemental sulfurs cannot soluble well in water (Cline et al., 2003; Janssen et al., 2000; 2009).

Likewise, bio-sulfur has advantages on agriculture as fertilizer or growth promoter of plants. The maximum number of silique seeds per plant could be generated by application of bio-sulfur fertilizer. Also, bio sulfur with Nitro Kara, which are a nitrogen bio fertilizer, led to the maximum number of silique and high yield on rape plants (Dahmardeh, 2013). Furthermore, by application of bio-sulfur with salicylic acid, the most significant effect on chlorophyll a and b, which are the main factor of photosynthetic capacity, were shown on the safflower (Jam et al., 2013; Jiang and Huang, 2001).

Recently, importance of environmental friendly pest control has been raised and practiced on many agricultural farms in many countries. Amount of pesticide consumed per unit area on pepper or cucumber plants had the highest level among fruit vegetable in Korea. Indeed, since last 4 years consumed pesticides have been decreased to 10 % whereas usage of the eco-friendly material has been increased for protection of agricultural products by

alternative strategy for controlling the overdose of chemicals. (Ha et al., 2012).

Sulfur compound has been known as one of such eco-friendly materials. Millardet's bordeaux mixture used as a fungicide against common downey mildew and elemental sulfur fungicide was effective on inhibition of fruit and vegetable disease from 1900s (Williams and Cooper, 2004). Nowadays, various sulfur substance have been further used as an inhibition agent of plant diseases. Either spotting or blight disease in Panax ginseng or powdery mildew in sweet pepper were inhibited by treatment Bordeaux mixture or cooking oil -yolk mixture in the environmental friendly agriculture (Lee et al., 2010; 2008). However, some sulfuric compound like Loess-sulfur complex showed toxicity on young crop plants because of its high alkalinity, although they used widely as environmental friendly fungicide on crop cultivation (Paik et al., 2012).

Bio-sulfur is one of the different types of sulfur which has lower pH value than other sulfur compounds. Application of bio-sulfur as soil amendments has caused decrease of the soil pH (Saleh, 2001). Although it has been rarely studied about bio-sulfur in most of the laboratories, some commercial bio-sulfur products were developed in Netherland. Liquid fungicide Cerasulfur® SC containing bio-sulfur has a lower disease index against powdery mildew on tomato plants caused by *Oidium lycopersici*. Also it can be applied to control of apple scab by *Venturia* spp. under rainy conditions with no residue on plants.

Anthracnose disease caused by *Colletotrichum* spp. affects much yield losses and devalues the quality of many crops in Korea. For example, the anthracnose disease by *Colletotrichum acutatum* caused the 10 % annual losses of productivity on pepper (KOSTAT, 2013). Also, *Colletotrichum gloeosporioides* infects the sweet persimmon yearly most of the Asian countries (Kim et al., 2007; Kwon et al., 2013). Symptoms such as sunken necrotic lesions or seedling blight can be observed on the leaves, stems, flowers and fruits which were suffered the anthracnose disease (Cannon et al., 2012).

C. orbiculare, is a hemibiotrophic fungus which causes the anthracnose on

cucurbits including cucumbers, melons and watermelons. Normally, *Colletotrichum* spp. has two stage of infection process in the host tissue. First they take action of biotrophic phase forming primary hyphae. And then they change to the necrotrophic phase when secondary hyphae are developed (Latunde-dada et al., 1996). Also, *C. orbiculare* formed melanized appressoria for invasion through the cell wall in compatible interactions (Hyde et al., 2009; Gan et al., 2013). Some fungus such as *Magnaporthe grisea* caused the blast disease on rice also has dome-shaped appressoria as similar as that of *C. orbiculare* (Lee and Dean, 1994). They produced a penetration peg which helps in invading into the cuticle and cell wall layers of plants and mediated the direct penetration of host epidermal cells (Kubo and Takano, 2013).

There were the some reviews from the Korean famers that used the bio-sulfur on their orchards. Some of farmers were used the bio-sulfur on the soil sterilization or killing insects like insecticide. However they didn't know whether the bio-sulfur has antifungal effect or why bio-sulfur has effect on plant disease exactly.

So, in this study the antifungal activity of bio-sulfur against anthracnose pathogen was tested. Also, suppression of disease severity by bio-sulfur on the cucumber plants was evaluated in the cucumber- *C. orbiculare* interaction. Furthermore, in order to illustrate the suppression mechanism of host-parasite interaction, the infection behavior of the fungus was observed using a fluorescent microscope.

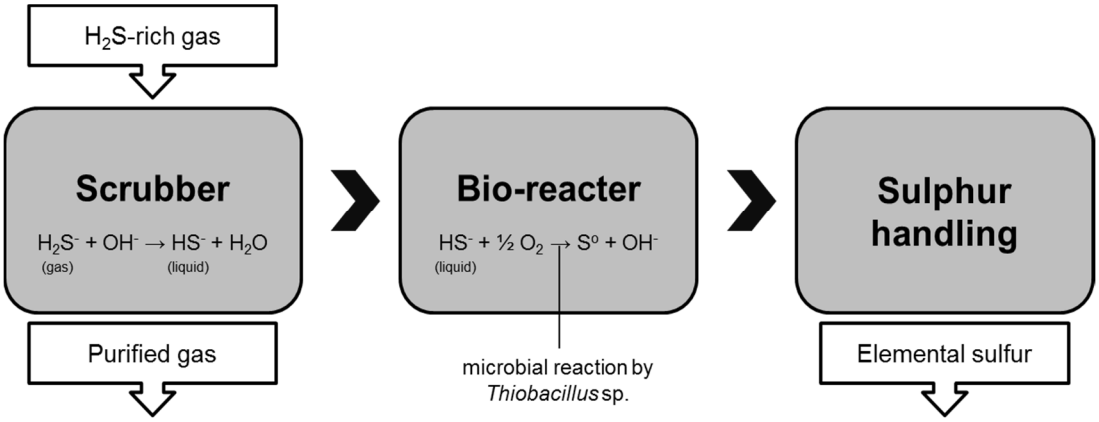

Fig. 1. Summary of the process of manufacturing the bio-sulfur.

Ⅱ. MATERIALS AND METHODS

1. Plant

Cucumber seeds (*Cucumis sativus* L, cv. Jeongseonsamcheok, Dongbu Farm Hannong Co., Ltd, Seoul, Korea), which are susceptible to anthracnose disease, were incubated at 25 ℃ in the dark for 24 h. The sprouted seeds were sown in a pot (Ø 10 cm) filled with mixture with commercial soil (Number-One®, Chungnam-Hongsung, Korea) and Perlite (Parat®, Sam Son, Seoul, Korea) at the rate of 9:1. The seedlings were watered every day and fertilized with a commercial fertilizer (Poly-Feed®, Kyunggi-Pajusi, Korea) once a week after seedling. The plants were grown in a greenhouse maintaining at 25 ± 1 ℃ on daytime, 18 ± 1℃ on night, and photoperiod was 14 h. The plants of first leaf sprouted growth stage were used in this experiment.

2. Bio-sulfur

Bio-sulfur used for this experiment was obtained from Ecobio Holdings Co. Ltd. To investigate the antifungal effect of bio-sulfur, the suspension of bio-sulfur was added to potato dextrose agar (PDA: Becton, Dickinson and company, Claix, France) and potato dextrose broth medium (PDB: Becton, Dickinson and company, Claix, France) each by diluted at 500 times. In detail, after sterilization of medium by autoclave (LAC-5060S,

DAIHAN LABTECH Co., LTD, Korea) at 121 ℃ for 15 min, the suspension of bio-sulfur was added to the medium. In the *in vivo* experiment, in order to illustrate the reduction of anthracnose disease by pre-treatment of bio-sulfur on plants, bio-sulfur was diluted at 500 times with H_2O. And then, the suspension of bio-sulfur was sprayed by a sprayer (Spra-tool®, Crown, U.S.A).The diluted bio-sulfur solution was added 0.01% tween 20 and sprayed on the first leaves of the cucumber plants until the leaves well wet. The treated plants were kept at room temperature until the leaves were dried.

3. Pathogen

Colletotrichum orbiculare KACC40808 which cause anthracnose on cucumber plants was obtained from Korean Agricultural Culture Collection (KACC). The pathogen was inoculated on PDA medium and incubated at 25 ℃ under the 4,000 Lux for a week. For inoculum, 10 ml of sterilized water was added on the plate on which acervuli colored orange or pink the edge of mycelium of the anthracnose pathogen was formed at 7 days after inoculation. Then the conidia were harvested with a loop. The suspension of conidia filtered with a Miracloth® (Calbiochem corporation, Lajolla, U.S.A) and the concentration was adjusted to 1.0 x 10^5 conidia/ml by hemocytometer (Hausser Scientific Inc., PA, USA). To the inoculum 0.01 % tween 20 was added before inoculation.

4. Assay of antifungal effect of bio-sulfur against anthracnose pathogen

To investigate the antifungal effect of bio-sulfur on *C. orbiculare*, the fungus was inoculated on the PDA medium in which the bio-sulfur was diluted to 500 times. After inoculation, the plates were incubated in an incubator maintaining 25 ℃ for a week and the diameter of the mycelia measured by a ruler.

To broaden the area of contact between pathogen and medium, the pathogen was also inoculated into PDB medium added the bio-sulfur diluted 500 times as the case of PDA and incubated at 25 ℃ with a shaking incubator (HB-201SL, Hanbaek Scientific Co., Korea) at 180 rpm for one week. Fresh weight of mycelia was measured using an electronic scale (Entris, Sartonis, Germany) after removing the moisture in the mycelia using filter paper (ADVANTEC, Toyo RoshiKaisha, Japan). Also, some mycelia were dried in a dry oven (Cl-1D-1, J. P. SELECTA s.a., Korea) at 65 ℃ for remove remaining liquid entirely in mycelia. Measure of dry weight was performed at 1 day later.

To evaluate the antifungal effect of bio-sulfur, a commercial fungicide Benomyl® was added instead of bio-sulfur at the concentration with 0.7g/L. Also, H_2O were used as negative control.

5. Suppression of disease severity of anthracnose on cucumber plants by bio-sulfur

Three hours after pre-treatment with bio-sulfur, the inoculum was also sprayed on the cucumber leaves until the leaves wet well. The inoculated plants were kept in a dew

chamber (DA-DC, DONG-A, Siheung-si, Korea) maintaining 100 % relative humidity for 24 h and then transferred to an incubating room keeping 60 % relative humidity under 5,000 Lux for 14 h a day. To assess the disease severity, number of lesions was measured at 7 days after the inoculation. The experiments were separately replicated 3 times and 6 plants were tested on each experiment.

6. Preparation for observation with a fluorescent microscope

The infection structures on the surface of cucumber leaves pre-treated with bio-sulfur were observed by a fluorescent microscope at 1, 3 and 5 days after the fungal inoculation. The inoculated parts of the leaves were cut in size of 1 x 3 mm^2. The leaf segments were fixed with 2 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2 hours and washed 3 times using 0.1 M sodium phosphate buffer (pH 7.2) each for 10 min. The leaf samples were dyed with 0.005 % aniline blue (Sigma-Aldrich®, Steinheim, Germany) for 20 min and washed 3 times with the same buffer. The leaf segments were dyed 0.2 % diethanol (UVtex-2B, Muellheim, Germany) for 20 min and washed 3 times. The leaf tissues were mounted on a slide glass and covered with a cover glass. The leaf surfaces were observed with a fluorescent microscope equipped with a fluorescent filter set (exciter filter, BP 400-440; interference beam splitter, FT 460; barrier filter, LP 470). The rate of germination and appressoria of the fungus and the fluorescent sites of the host tissues were counted.

7. Statistical analyses

The data of antifungal effect, disease severity, the rate of germination and appressoria formation of the conidia and the fluorescent sites on the bio-sulfur pre-treated cucumber leaves were statistically analyzed using Duncan's multiple range test (DMRT) and Statistical Analysis System program (SAS Institute, version 9.0).

The images of the fluorescent microscope equipped with a soft imaging solution (XC10, Olympus, Germany) were edited and saved by an image acquisition software (Get IT, Germany).

Ⅲ. RESULTS AND DISSCUSSION

1. Inhibition of mycelial growth of *C. orbiculare* on an artificial medium added with bio-sulfur

In order to investigate the antifungal effect of bio-sulfur on the mycelia growth of *C. orbiculare* on PDA medium with bio-sulfur was compared with PDA medium without bio-sulfur. Growth of the fungal mycelia on the medium added bio-sulfur was significantly reduced compared with those on the untreated PDA medium at 7 days after inoculation (Fig. 2A, 2B and 2D). However, the inhibition of mycelial growth by bio-sulfur was not comparable to those by a commercial fungicide Benomyl® (Fig. 2C and 2D), one of the MBC (Methyl Benzimidazole Carbamate) fungicides which inhibit the mitosis of fungus (Yang et al., 2011).

Similarly, the inhibition of mycelia growth was observed on the PDB medium with bio-sulfur in which the area of contact between treatments and pathogen was extended. The growth of mycelia of *C. orbiculare* was reduced by bio-sulfur treatment at 7 days after the inoculation compared to the untreated control (Fig. 3A and 3B). Also in the medium containing Benomyl®, the fungal mycelia have hardly grown (Fig. 3C).

Antifungal activity of bio-sulfur was presented clearly on the fresh weight of the mycelium mass which was decreased to almost 90 % than those of untreated one (Fig. 3D). Similar with the fresh weight, the dry weight of the mycelia was also reduced by the treatment with bio-sulfur (Fig. 3E).

Some researches mentioned that sulfur treatments may cause the growth inhibition

of fungal pathogen. For examples, the fumigation with H_2S inhibited significantly the colonial growth of either *Aspergillus niger* or *Penicillium italicum* on the yeast peptone dextrose (YPD) agar medium. Especially in *A. niger* the generation of reactive oxygen species (ROS) was directly induced by the H_2S treatment which caused oxidative damage to molecules vital to mycelia growth and spore germination (Fu et al., 2014). Also, the mechanism of antifungal effect by sulfur has been reported. Inorganic sulfur as fungicide caused the abnormal respiration of mycelia by interrupting the electron transport system of fungi in mitochondria and by producing H_2S that has antifungal effect (Jung et al., 2000).

In our study, the pre-treatment with bio-sulfur showed the direct antifungal effect on *C. orbiculare*. Growth of the fungal mycelia on bio-sulfur medium was reduced both on its length and weight (Fig. 2 and 3). However, the general mechanism of the antifungal activity by bio-sulfur has been not clearly illustrated yet.

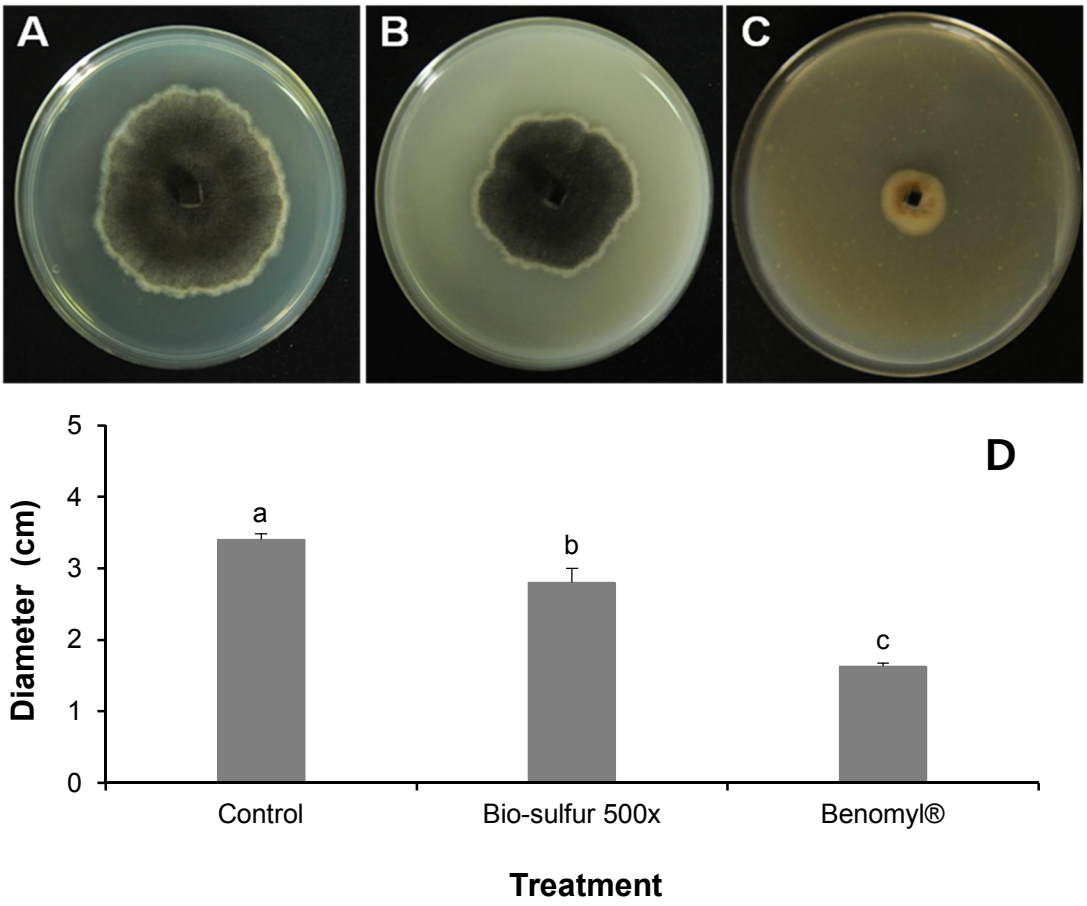

Fig. 2. Suppressed mycelial growth of *Colletotrichum orbiculare* on potato dextrose agar (PDA) medium by bio-sulfur and Benomyl®. Fungal colony formation of *C. orbiculare* on the PDA (A), added bio-sulfur (B) and a commercial fungicide Benomyl® (C). The diameters of colony were photographed at 7 days after cultivation. The suspension of bio-sulfur was diluted with 500 times. The concentration of Benomyl® was 0.7 g/L. The vertical bars indicated the standard deviation of three separating replications of each experiment. Different letters on the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple test.

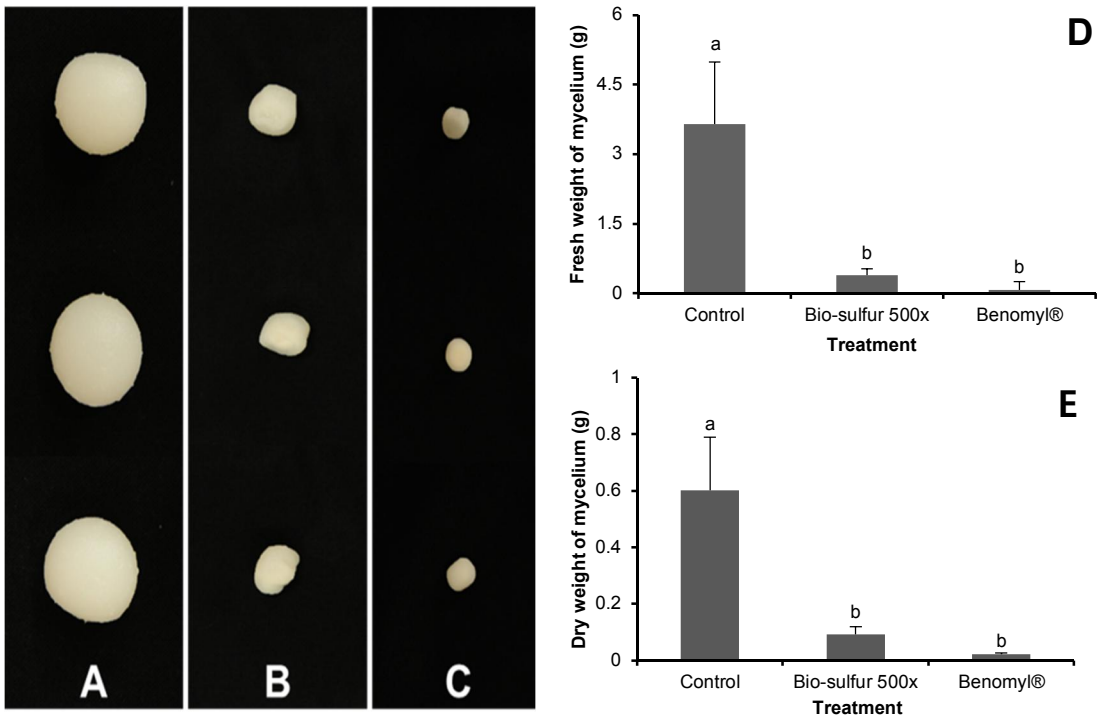

Fig. 3. Suppressed mycelial growth of *Colletotrichum orbiculare* in potato dextrose broth (PDB) medium by bio-sulfur and Benomyl®. Mycelial growth of *C. orbiculare* in the PDB (A), added bio-sulfur (B) or a commercial fungicide Benomyl® (C). The mycelial growth was photographed at 7 days after cultivation. Fresh and dry weight of fungal mycelia from the medium were shown treated with bio-sulfur or Benomyl® (D and E). The suspension of bio-sulfur was diluted with 500 times. The concentration of Benomyl® was 0.7 g/L. The vertical bars indicated the standard deviation of three separating replications of each experiment. Different letters on the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple test.

2. Suppression of disease severity by pre-treatment with bio-sulfur on cucumber plants

To investigate suppression of anthracnose disease by bio-sulfur, the number of lesions on the cucumber leaves pre-treated with bio-sulfur and commercial fungicide Benomyl® were compared with the untreated control leaves.

On the untreated leaves, some of light yellow and irregular lesions appeared at 3 days after inoculation and its color has turned gray. At 6 days after inoculation, the size of lesion on the leaves came to extend and some lesions has united together. Also, the necrosis occurred on the parts of leaves (Fig. 4A).

On the bio-sulfur pre-treated cucumber leaves, some lesions were not developed well compared the typical anthracnose formed on the untreated leaves (Fig. 4B). The number of lesions on the leaves was significantly decreased at 53 % compare to that of untreated one at 7 days after fungal inoculation (Fig. 4D). Reduction of lesions was similar with that on the Benomyl® leaves (Fig. 4C and 4D). These results suggested that the pre-treatment with bio-sulfur caused an inhibition effect to anthracnose disease on cucumber plants.

There were some studies that application of sulfur compound could suppress diseases on plants. Soil applied sulfur fertilization had a significant repressive effect on the infection of powdery mildew on grapes (Klikocka et al., 2005). The occurrence of skin sooty dapple disease on asian pear was decreased by treatment with lime-sulfur (Park et al., 2008). On tomato plants, which were sprayed with loess-sulfur on the leaves, the occurrence of tomato powdery mildew was decreased (Shim et al., 2014). Also, lime- or loess-sulfur was used to prevent the anthracnose caused by *Colletotrichum gleosporioids* or circular leaf disease by *Mycosphaerella nawae* on sweet persimmon, respectively (Kim et al., 2013). Likewise, the pre-treatment with bio-sulfur has triggered the decrease of disease severity on

cucumber plants in this experiment (Fig. 4). Therefore, it was suggested that bio-sulfur may cause the reduction of anthracnose disease on cucumber plants.

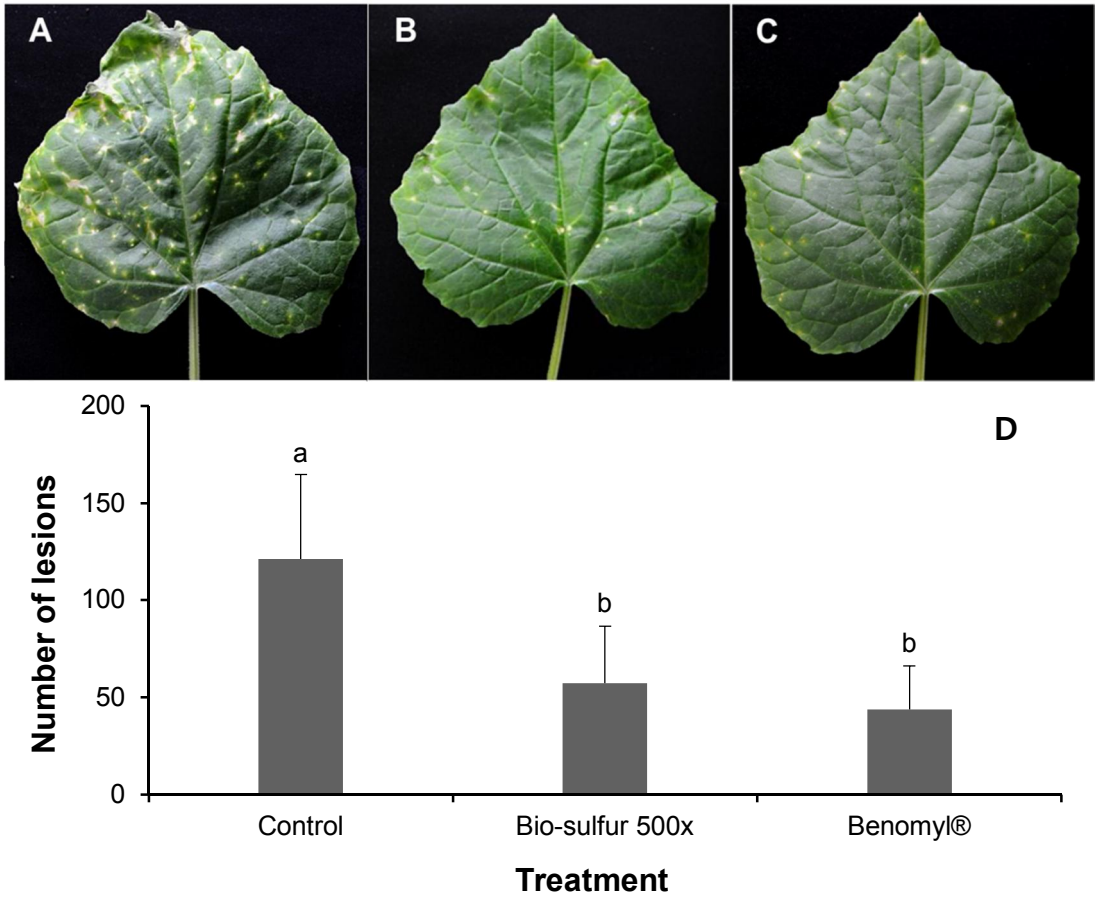

Fig. 4. Number of lesions on cucumber leaves untreated (A), pre-treated with bio-sulfur (B), and a commercial fungicide Benomyl® (C) at 7 days after inoculation with anthracnose pathogen *Colletotrichum orbiculare*. The number of lesions was decreased by pre-treatment with bio-sulfur or Benomyl® (D). The suspension of bio-sulfur was diluted with 500 times. The concentration of Benomyl® was 0.7 g/L. The vertical bars indicate the standard deviation of 3 replications. Different letters on the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple test.

3. Fluorescence microscopic observations of the infection structures on the cucumber leaves pre-treated with bio-sulfur

The infection structures of *C. orbiculare* on the cucumber leaves untreated, pre-treated with bio-sulfur or Benomyl® were observed using a fluorescence microscope at 1, 3 and 5 days after inoculation.

On the surface of untreated leaves most spores were germinated and formed germ tube at 1 day after inoculation. Also, several appressoria were found at that time which have circular or oval shape and dark brown color (Fig. 5A). At 3 days after inoculation, over 40 % of germ tubes formed appressorium (Fig. 5D and 6B). At 5 days, hyphae were grown broadly and most of them were tangled each other. The rate of appressorium was reached at about 60 % (Fig. 5G and 6B).

However, the rate of germination on the bio-sulfur pre-treated leaves was not different with that on untreated leaves at 1 day after inoculation. Most the germ tubes didn't form appressorium unlikely those on untreated leaves (Fig. 6B). At 3 and 5 days after inoculation the rate of conidial germination were weakly reduced than that of untreated leaves (Fig. 6A). Remarkably, much less appressorium were found compared with untreated one. Similarly, at 5 days after inoculation appressorium formation was strongly suppressed on the bio-sulfur pre-treated leaves (Fig. 6B).

Likewise, the rate of appressorium on the surface of tissues pre-treated with a commercial fungicide Benomyl® was similar with that on bio-sulfur pre-treated leaves (Fig. 5 and 6B) indicating bio-sulfur may express antifungal effect as a fungicide on the leaf surfaces. On the other hand, there were no apparently difference on the rate of fluorescent sites, which indicated defense responses of plant, among untreated, pre-treated with bio-

sulfur and Benomyl® (Fig. 6C).

Some sulfur materials were known as fungicide that controlled plant disease by suppressing the spore germination of fungal pathogen. Conidial germination of *Venturia nashicola* causing pear scab was decreased to 93.7 % by treatment with organic sulfur (Song and Seo, 2018). The water soluble sulfur compound BTB® and Hwangstar® was shown the most effective on spore germination of *Botrytis cinerea* (Kwak et al., 2012). In this study the rate of conidia germination on bio-sulfur pre-treated leaves was reduced at 3 and 5 days after inoculation (Fig. 5 and 6A). However, suppression of the conidial germination seemed to not play an important role for the inhibition of anthracnose disease.

Also, there were a few appressorium of *C. orbiculare* on leaves pre-treated with bio-sulfur on observation with a fluorescent microscope whereas lots of appressoria were observed on the untreated leaves at 3, 5 days after the fungal inoculation (Fig. 6B). Some fungi including *Colletotrichum* form appressorium which is a specific invasion structure when they invade host leaves (Bechinger et al., 1999). It has been known that turgor of appressorium is necessary to infect plant cells physically of which structure generate a penetration protrusions into the cuticle (Ryder and Talbot, 2015). Many researchers have proved that suppression of appressorium formation resulted in the decrease of disease severity caused by filamentous fungi. For example, lower formation of appressorium by the pretreatment with an algae *Chlorella fusca* reduced remarkably number of lesions caused by anthracnose pathogen on cucumber leaves (Lee et al., 2016). Formation of appressorium could be reduced by treatment with soluble silicate on which the penetration of powdery mildew was limited (Kanto et al., 2007). Also, inhibition of appressorium formation was observed on pear leaves treated with a commercial sulfur (Song and Seo, 2018). CoHox3 (*Colletotrichum orbiculare* Homeobox transcription factor 3) mutant that shows suppression of lesions on the cucumber cotyledon were not formed the normal appressoria while the mature appressoria formed on the cucumber leaves (Yokoyama et al., 2018). Similarly,

mutants of *C. orbiculare* like *cmk1* mutant cannot form the appressoria or produce swollen structures similar to appressoria and failed the invasion onto the host plants (Kubo and Takano, 2013). So, reduction of appressorium on the bio-sulfur treatments may be the key to disturb the infection of some pathogen.

Similarly, some chemicals could reduce appressorium formation of plant pathogens. Lime-sulfur hindered appressorial formation of *Venturia naequalis* at early stage in infection (Montag et al., 2005). Also, in this study the rate of appressorium formation of *C. orbiculare* was decreased on the leaves pre-treated with a commercial fungicide Benomyl® which may suppress the cell division of plant (Dane and Dalgic, 2005). Therefore, it was suggested that the treatment with bio-sulfur might suppress the appressorium formation which lead to reduction of disease severity of anthracnose on the cucumber plant.

Generally, callose formed in host cells indicates a defense response against fungal invasion (Jeun and Lee, 2005). Callose, called as 'papillae' forming on host secondary cell wall, may play a role as barriers during the early infection stages of pathogen (Luna et al., 2011). Also, callose can be formed at the penetration sites on the resistant expressing plants mediated by ß-Aminobutyric acid (BABA) or jasmonic acid (JA) (Hamiduzzaman et al., 2005), which are chemical agents for systemic acquired resistance in many crop plants (Floryszak-Wieczorek et al., 2015; Truman et al., 2007). As expected, on this study there were any differences on fluorescent cells at penetration sites on the leaves pre-treated with bio-sulfur (Fig. 5) indicating bio-sulfur could not induce systemic resistance in this experiment.

In summary, bio-sulfur has direct antifungal effect against anthracnose pathogen which caused the reduction of disease severity. On the bio-sulfur pre-treated leaves germination of the fungus was not hindered, but appressorium formation was remarkably reduced which may be the main reason of decrease of the disease severity.

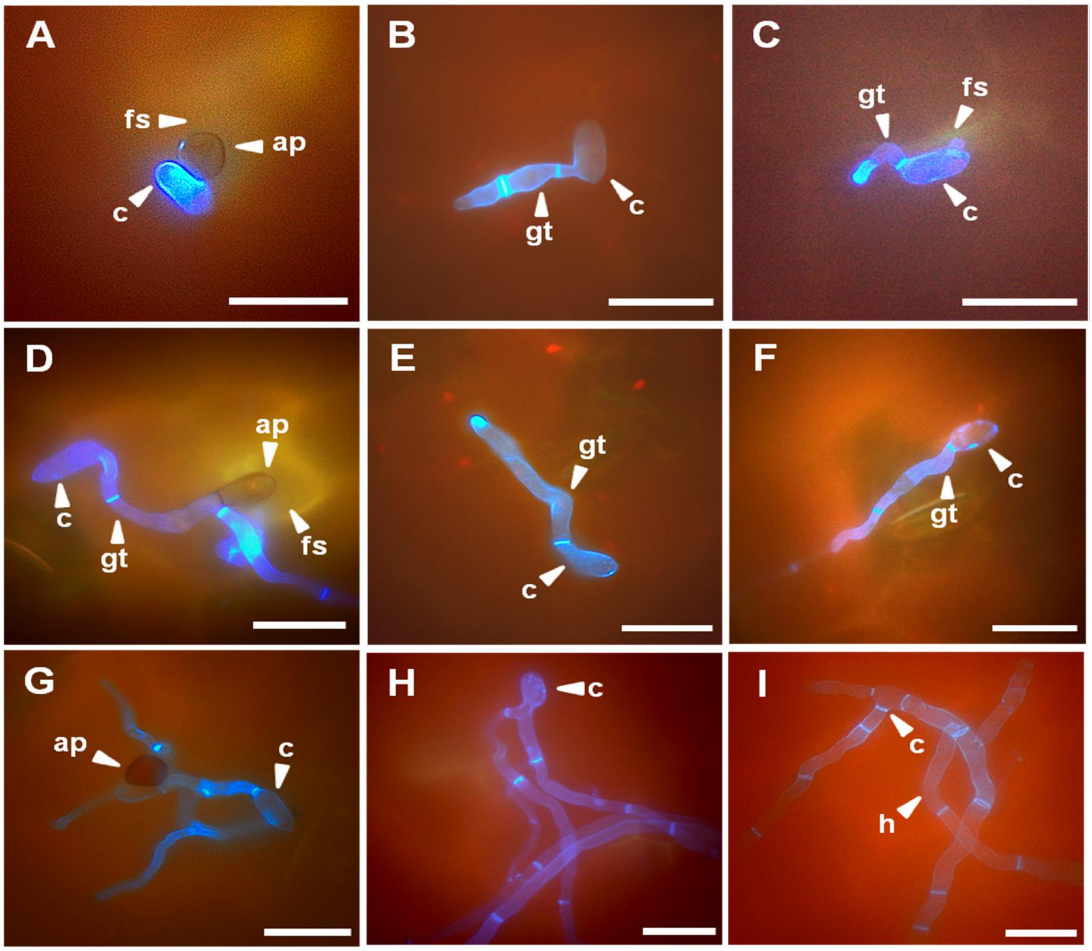

Fig. 5. Fluorescence microscopical observations of infection structures on the leaves of cucumber plants untreated (A, D, G), pre-treated with bio-sulfur (B, E, H) and a commercial fungicide Benomyl® (C, F, I). The suspension of bio-sulfur was diluted with 500 times. The concentration of Benomyl® was 0.7 g/L. The suspension of bio-sulfur was diluted with 500 times. All bars = 20 μm. Abr: ap, appressorium; c, conidium; gt, germ tube; fs, fluorescent site; h, hyphae.

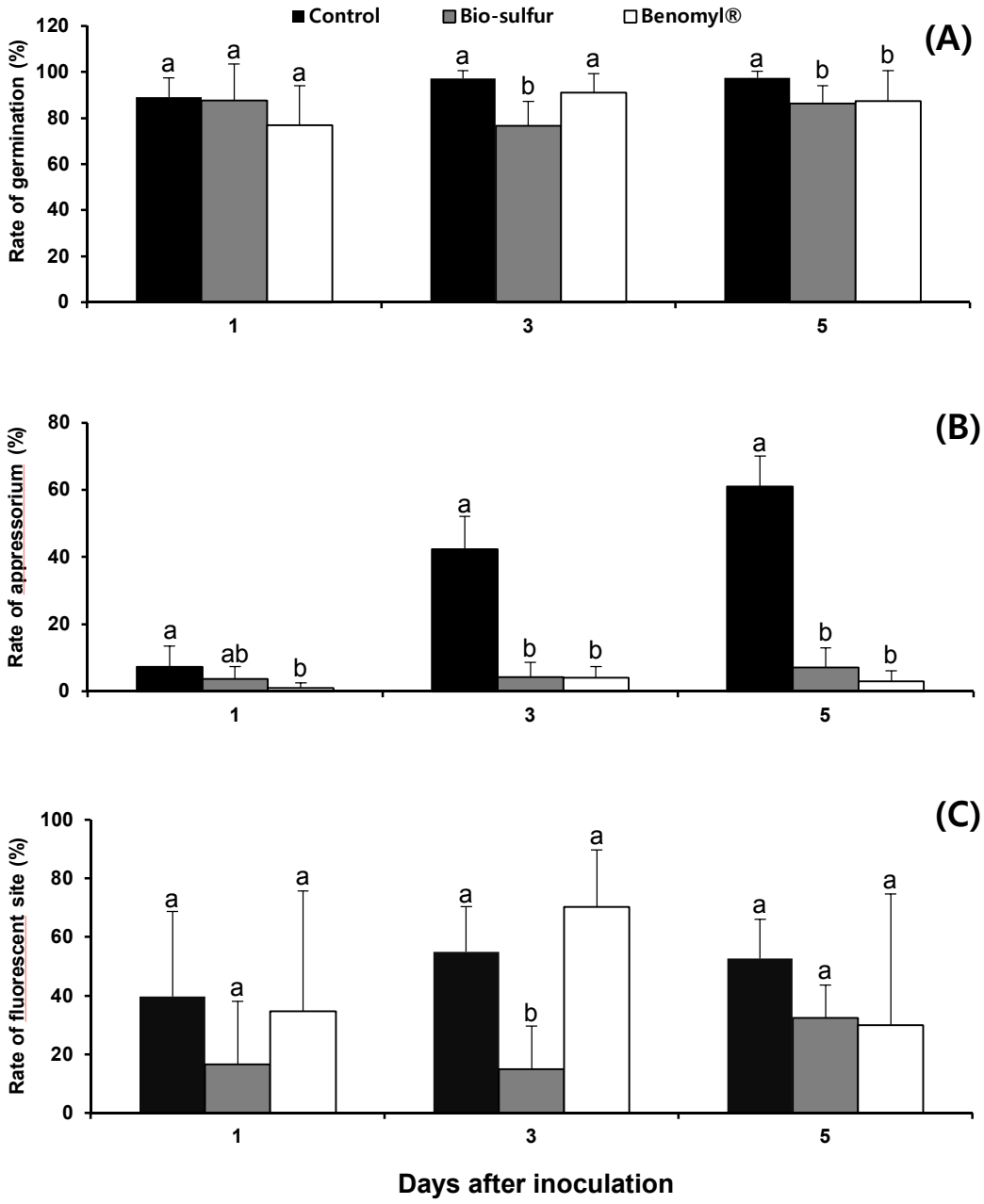

Fig. 6. Rate of germination (A), appressorium formation of fungal conidia (B) and rates of fluorescent sites of host cells (C) on the cucumber leaves untreated, pre-treated with bio-sulfur, and a commercial fungicide Benomyl®. The concentration of Benomyl® was 0.7 g/L. The suspension of bio-sulfur was diluted with 500 times. Different letters on the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple test.

Ⅴ. 적　　요

바이오 황은 수도권 쓰레기 매립지에서 황화수소가스를 포집하고 황을 제거하는 탈황과정에서 만들어지는 황 함량 50%의 부산물이다. *Thiobacillus* sp. 와 같은 미생물들이 이 과정에 관여하기 때문에 유럽에서는 이를 바이오 황 (Bio-sulfur)이라 칭한다. 이미 다양한 연구들에서 황 관련 제재들이 균사의 생장과 곰팡이 병 억제에 효과가 있다는 것이 밝혀졌지만, 높은 pH 로 인해 식물에 해를 입히는 경우가 있어 친환경 농가에서 문제가 되는 경우가 있었다. 바이오 황은 낮은 pH 를 가지고 있기 때문에 식물에 해가 되지 않고 무엇보다 작은 입자 크기 때문에 흰가루병과 같은 곰팡이 병을 방제하는 데 효과가 있다는 사례가 많이 보고되고 있다. 따라서 이 연구에서는 오이탄저병균에 대한 바이오 황의 직접적인 항균활성 및 식물체 내에서의 직접적인 병 억제 효과, 바이오 황을 전 처리한 잎에서 오이탄저병 균의 억제메커니즘을 규명하기 위한 연구를 수행하였다. 바이오 황이 첨가된 고형배지에서 균사 생장 정도를 확인한 결과 무처리 구에 비해 바이오 황을 첨가한 배지에서의 균사 생장이 미세하게 억제되는 것을 확인 할 수 있었다. 좀 더 확실한 결과를 위해 바이오 황 입자와 균사간의 접촉면적을 늘리기 위한 방법을 시도, 바이오 황이 첨가된 액체 배지에 균사를 접종 후 그 생장 정도를 관찰한 결과 바이오 황이 첨가된 배지에서 균사의 생중량과 건중량이 확연하게 줄어드는 것을 확인할 수 있었다. 또한, 위 실험을 바탕으로 바이오 황을 전 처리한 잎에 균사 현탁액을 접종한 결과 바이오 황을 분주한 잎에서의 병반 형성이 무처리 구에 비해서 뚜렷하게

감소하는 것을 확인할 수 있었다. 더 나아가, 바이오 황에 의한 병 억제 기작을 확인하기 위해 바이오 황을 전 처리한 잎에서 오이탄저병균의 침입구조를 형광현미경으로 관찰하였다. 부착기는 오이탄저병균을 포함한 여러 식물병원균들이 식물체 내로 침입하기 위한 필수적인 기작 중 하나로 알려져 있다. 관찰 결과, 무처리구에서 대다수의 균사들이 부착기를 형성한 반면 바이오 황을 전 처리한 잎에서는 부착기 형성이 저지되는 것을 확인할 수 있었다. 이러한 결과들은 시판되는 살균제인 Benomyl® 처리구에서도 유사하게 나타났다. 이를 바탕으로 바이오 황이 항균효과를 가지고 있고 부착기 형성의 감소가 병을 억제하는 핵심적인 원인 중 하나라고 판단된다.

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감사의 글

학부 시절 전공 수업을 통해 가졌던 단순한 호기심과 관심에서 시작했던 실험실 생활을 하다 보니 어느덧 다섯 번째 겨울을 맞으며 대학원 졸업을 바라보고 있습니다. 문득 뒤를 돌아보니 2 년이라는 짧은 시간 동안 수없이 흔들리고 방황하며 제 방향을 찾으려고 버둥거리던 저의 모습과 그런 저를 위해 기꺼이 손을 내밀어주었던 많은 분들의 따뜻함이 떠오릅니다. 소중한 추억을 만들어주었던 분들께 감사의 인사를 전하고자 합니다.

실험실 생활 내내 아낌없이 조언을 해주시고 언제나 따뜻한 마음으로 바라봐주셨던 전용철 교수님께 정말 감사하다는 말씀을 드립니다. 학문적인 지식들 뿐만 아니라 제가 어떤 자세와 마음가짐으로 살아가야 하는지 교수님을 통해서 많이 배우고 느꼈습니다. 교수님께서 배워주셨던 지혜들을 잊지 않고 좋은 제자가 되는 길로 보답하겠습니다.

좋은 마음으로 늘 웃어주시며 칭찬과 격려를 해주셨던 송창길 교수님, 위트 넘치는 말솜씨로 언제나 웃음을 주시고 많은 지식들을 맛있게 전달해주셨던 현해남 교수님, 제자들의 마음을 잘 헤아려 주시고 저의 사소한 질문에도 언제나 열린 마음으로 답해주셨던 김동순 교수님, 학부 시절부터 꾸준한 관심을 가져주시고 응원과 함께 뜻깊은 조언들을 해주셨던 김주성 교수님, 그리고 숫기가 없는 저에게 먼저 적극적으로 다가와주시고 파이팅 넘치게 응원해주셨던 정용석 교수님께 정말 진심으로 감사의 말씀을 올립니다.

한참 부족하고 덜렁대는 막둥이를 과분한 사랑으로 대해주신 식물병리학 실험실 식구들 너무 사랑하고 감사합니다. 먼저 어머니처럼 저를 늘 챙겨주시고 넓은 마음으로 품어주셨던 고평열 박사님께 많이 배웠고 감사한 마음을 전합니다. 실험실에 오실 때 마다 웃는 얼굴로 반겨주시고 격려해주셨던 고순열 선생님, 고효순 선생님, 언제나 제 이야기를 잘 들어주시고 실험에 흥미를 갖도록 도와주셨던 이윤주 선생님, 제가 어려움에 처할 때마다 선뜻 달려와 도와주었던 윤정맘 윤정언니, 늘 저를 배려해주고 물심양면으로 챙겨줬던 경남오빠, 항상 잘하고 있다고 응원해주고 저의 수많은 질문에도 귀찮아하지

않고 실험도 공부도 많이 배워줬던 지순언니, 같이 장난치면서 저를 재미있게 해주고 또한 후배를 위하는 진심 어린 마음으로 아낌없이 챙겨주었던 재신오빠, 멋있는 인생 선배로써 언제나 응원해주면서 좋은 길로 이끌어주고 저를 더욱더 사랑스러운 후배로 만들어주는 민아언니, 많이 투닥거렸지만 동생이라고 뒤에서 언제나 저를 먼저 위해주고 센스있게 챙겨주었던 승학오빠, 대학원 생활 동안 수많은 고민들을 공유하고 실험들을 같이 하면서 제가 든든하게 의지할 수 있었던 친구 용호오빠, 그리고 제가 도움을 주기는커녕 오히려 너무 많은 도움을 받았던 사랑스러운 막다에게 너무 감사하다는 말을 전하고 싶습니다.

그리고 논문이 나오기까지 관심과 도움을 주셨던 많은 분들께 감사합니다. 영원한 저의 회장님 오명협 선생님과 제 논문을 꼼꼼히 봐주시고 여러 조언들을 해주셨던 박원표 선생님께 감사드립니다. 그 외에도 수빈언니, 태옥오빠, 동은오빠, 건이오빠, 희선언니, 성문오빠, 경철오빠, 상희언니, 강해오빠, 명수오빠, 현민오빠를 비롯한 모든 선배님, 후배님들께 감사합니다. 또한 저의 희로애락을 언제나 함께해주는 소중한 평생친구들과 선배로써, 그리고 사랑하는 친구로써 무한히 긍정에너지를 전해주는 성오오빠에게도 감사의 말을 전합니다.

마지막으로 제가 어떤 길을 가더라도 기꺼이 지지해주시고 응원해주시는 사랑하는 엄마, 아빠 그리고 철없는 첫째를 챙겨주고 격려해주느라 늘 고생하는 아끼는 동생 나영이, 한주와 이 기쁨을 함께 나누고 싶습니다.