Bio-Sulfur Pre-Treatment Suppresses Anthracnose on Cucumber Leaves Inoculated with Colletotrichum orbiculare

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ABSTRACT

Bio-sulfur can be produced in the process of desulfurization from a landfill and collected by some microorganism such as Thiobacillus sp. as a sulfur element. In order to investigate practical use of bio-sulfur as an agent for controlling plant disease, in vitro antifungal activity of bio-sulfur was tested against Colletotrichum orbiculare known to cause cucumber anthracnose. Efficacy of bio-sulfur for suppressing anthracnose disease was also evaluated in vivo using cucumber leaves. Mycelial growth of C. orbiculare on medium containing bio-sulfur was inhibited. Disease severity of cucumber leaves pre-treated with bio-sulfur was significantly decreased compared to that of untreated ones. To illustrate how bio-sulfur could suppress anthracnose disease, structures of cucumber leaves infected with C. orbiculare were observed under a fluorescent microscope and a scanning electron microscope (SEM). Cucumber leaves pre-treated with bio-sulfur showed a low rate of appressorium formation whereas untreated ones showed abundant appressoria. Shrunken fungal hyphae were mostly observed on bio-sulfur-pre-treated leaves by SEM. Similar results were observed on leaves pre-treated with a commercial fungicide Benomyl\textsuperscript{R}. These results suggest that inhibition of appressorium formation of C. orbiculare by bio-sulfur may contribute to its suppression of cucumber anthracnose.

1. Introduction

Searches for new effective methods that can change waste into disposable energy have pushed the government to have expanded new regeneration energy in Korea [1]. One of the energy sources that can be created from organic waste is methane [2]. In Korea, bio-sulfur can be produced as a by-product through the biological process of desulfurization by some microorganisms to remove H\textsubscript{2}S in landfill gas for protecting facilities or preventing air pollution. Daily mean and annual average output of bio-sulfur from the landfill site were 15 m\textsuperscript{3}/day and 5475 m\textsuperscript{3}/year, respectively, from March 2014 to June 2015 in the landfill of the metropolitan area in Seoul, Korea [3].

The process of desulfurization is summarized in Figure 1. First, from the land site, H\textsubscript{2}S-rich gas is injected into the desulfurization equipment called “scrubber”. Purified gas released by adding soda lime is used to generate electric power. In a bioreactor, during oxidation of gas, sulfur is collected by microorganisms such as Thiobacillus spp. known to be an effective microorganism that could be used as a sulfur-oxidative agent [4]. These microorganisms are then adsorbed to bio-sulfur aggregation. Finally, soda lime is collected and elemental sulfur is separated from sulfur handling. Bio-sulfur has advantages for agriculture as a fertilizer or growth promoter of plants. With bio-sulfur fertilizer, the maximum number of silique seeds per plant could be generated for canola plants. Also, with a nitrogen biofertilizer, bio-sulfur can lead to the highest yield for grapes [5].

Recently, the importance of environmentally friendly pest management has been raised. Such a pest management strategy has been practiced in many agricultural farms in many countries. Amount of pesticide consumed per unit area on pepper or cucumber plants had the highest level among fruit vegetables in Korea. Indeed, in the last 4 years, the amount of pesticides consumed in many crop cultivation areas has been decreased whereas usage of eco-friendly material has been increased to protect agricultural products by using alternative strategies to avoid an overdose of chemicals [6].

Sulfur compound has been known as one of such eco-friendly materials. Bordeaux mixture has been used as a fungicide against common downy mildews for a long time. Elemental sulfur fungicide can also
effectively inhibit fruit and vegetable diseases [7]. Nowadays, various sulfur substances have been further used as control agents of plant diseases. Spotting or blight disease in ginseng and powdery mildew in sweet pepper could be reduced by environmentally friendly pest management in agriculture [8,9]. However, some sulfuric compounds such as Loess-sulfur complex are phytotoxic to young plants because of their high alkalinity, although they have been used widely as environmentally friendly fungicides for crop cultivation [10].

Bio-sulfur is a type of sulfur that has lower pH value than other sulfur compounds. Application of bio-sulfur as a soil amendment can decrease soil pH [11]. Although bio-sulfur has been rarely studied in the laboratory, some commercial products of bio-sulfur have been developed in the Netherlands. Liquid fungicide Cerasulfur ® SC containing bio-sulfur can decrease tomato powdery mildew caused by Oidium lycopersici [12].

The objective of the present study was to determine antifungal activity of bio-sulfur against C. orbiculare, a hemibiotrophic fungus known to cause anthracnose of various cucurbits including cucumbers, melons, and watermelons. Colletotrichum orbiculare can form melanized appressoria on plant surfaces for invasion through plant cell wall during compatible interactions [13,14]. To illustrate the mechanism involved in the suppression of bio-sulfur on host-parasite interaction, infection behavior of the fungus was observed using an optical microscope for onion peels and a fluorescent microscope and a scanning electron microscope for cucumber leaves.

2. Materials and methods

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

Bio-sulfur used for this experiment was obtained from Ecobio Holdings Co. Ltd. (Seoul, Korea). To investigate the antifungal effect of bio-sulfur on C. orbiculare, the fungus was inoculated onto potato dextrose agar medium (PDA: Becton, Dickinson and company, Clai, France) added with bio-sulfur of which concentration was adjusted as 0.5% (diluted 500 times) of the amount to be applied in the farm. After inoculation, plates were incubated at 25°C for 7 days and colony diameters were then measured. These experiments were three times replicated separately.

The pathogen was also inoculated into potato dextrose broth (PDB: Becton, Dickinson and company, Clai, France) added with bio-sulfur diluted as the case of PDA and incubated at 25°C in shaking (180 rpm) incubator (HB-201SL, Hanbaek Scientific Co., Bucheon, Korea) for 7 days. Fresh weight of mycelia was measured using an electronic scale (Entris, Sartonis, Germany) after removing moisture in the mycelia using filter paper (ADVANTEC, Toyo Roshi Kaisha, Tokyo, Japan). Some mycelia were also dried in an oven (Cl-1D-1, J. P. SELECTA s.a., Barcelona, Spain) at 65°C for 24 h until the remaining liquid in mycelia was removed entirely. Measurement of dry weight was then performed one day later. These experiments were three times replicated separately. To evaluate the antifungal effect of bio-sulfur, a commercial fungicide Benomyl ® was added into PDA or PDB instead of bio-sulfur at a concentration of 0.7 g/L. H₂O was used as a negative control.

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

2.2.1. Plants

Seeds of cucumber (Cucumis sativus L, cv. Jeongseonsamcheok, Dongbu Farm Hannong Co., Ltd, Seoul, Korea) known to be susceptible to anthracnose disease were incubated at 25°C in the dark for 24 h. Sprouted seeds were sown in pots (Ø 10 cm) filled with a mixture of commercial soil (Number-One ®, Hongsung, Korea) and Perlite (Parat ®, Sam Son, Seoul, Korea) at a rate of 9:1. Seedlings were fertilized with a commercial fertilizer (Poly-Feed ®, Paju, Korea) once a week after
seedling. Plants were grown in a greenhouse at 25°C ± 1°C in the daytime and 18°C ± 1°C in the night with a photoperiod of 14 h. Plants at first leaf sprouted growth stage were used in this experiment.

2.2.2. Pathogen

Colletotrichum orbiculare KACC40808 known to cause anthracnose of cucumber plants was obtained from the Korean Agricultural Culture Collection (KACC). The pathogen was inoculated onto PDA medium and incubated at 25°C under the 4000 lux for a week. About 10 ml of sterilized water was added onto the plate on which acervuli of the anthracnose pathogen was formed. Conidia were harvested with a loop. The suspension of conidia was filtered with a Miracloth® (Calbiochem corporation, La Jolla, CA) and its concentration was adjusted to 1 × 10^5 conidia/ml. Before inoculation, 0.01% Tween 20 was added.

2.2.3. Pre-treatment with bio-sulfur and fungicide

Bio-sulfur was diluted 500 times with H_2O and 0.01% Tween 20 was added. The diluted bio-sulfur solution was sprayed onto the cucumber leaves until leaves were well wet. A commercial fungicide Benomyl® was used as a positive control at a concentration of 0.7 g/L. Treated plants were kept at room temperature until the leaves were dried.

2.2.4. Inoculation with pathogen

The conidial suspension was sprayed onto cucumber leaves until the leaves were well wet. Inoculated plants were incubated in a dew chamber (DA-DC, DONG-A, Siheung-si, Korea) with 100% relative humidity under the 4000 lux for 14 h a day. To assess disease severity, the number of lesions was measured at 7 days after the inoculation. Experiments were separately replicated three times and six plants were tested in each experiment.

2.2.5. Observation of onion-peel inoculated with Colletotrichum orbiculare using an optical microscope

Inner peels of onions were used to investigate differences in infection structure after treatment with bio-sulfur. Onion was peeled and cut into size of 2 × 6 cm². Peels were placed in 1.5% of water agar medium and treated with bio-sulfur and Benomyl® at the same concentration as those used to treat plants, respectively. As negative controls, untreated peels were used. The inoculum of C. orbiculare at concentration of 1.0 × 10^5 conidia/ml was dropped on the peel. Infection sites were observed with an optical microscope (BX51, Olympus Ltd., Tokyo, Japan) at 12 and 24 h after the inoculation. Germination rate and hyphae length of C. orbiculare were measured.

2.3. Observation with a fluorescent microscope

Infection structures on the surface of cucumber leaves pre-treated with bio-sulfur were observed under a fluorescent microscope at 1, 3, and 5 days after fungal inoculation. Inoculated parts of the leaves were cut in size of 1 × 3 mm². Leaf segments were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) each for 10 min. These leaf samples were stained with 0.005% aniline blue (Sigma-Aldrich®, Steinheim, Germany) for 20 min and washed 3 times with the same buffer. Leaf segments were then stained with 0.2% diethanol (UVtex-2B, Muelheim, Germany) for 20 min and washed 3 times with the same buffer. Leaf tissues were mounted with 80% glycerol onto glass slides and covered with a cover glass. 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). Rates of germination, appressorium formation of fungus, and fluorescent sites of host tissues were determined.

2.4. Observation with a scanning electron microscope

Fine structure of C. orbiculare on the surface of cucumber leaves pre-treated with bio-sulfur was observed with a scanning electron microscope at 1 and 3 days after inoculation. Leaves were cut with a razor into a size of 1 × 3 mm². Samples were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylic buffer (pH 7.2) for 2 h. After fixation, samples were washed with the same buffer for 10 min three times. To remove moisture from samples, 30, 50, 70, 80, 90, 95, and 100% of ethanol series were used for treatment (10 min for each treatment, three treatments for each ethanol step). Finally, 100% acetone was used for treatment (30 min each time for a total of two times).

Each sample was dried with a critical point dryer (CPD 030; BAL-Tec, Los Angeles, CA) and coated with a platinum using sputter coater-platinum (Q150R Plus – Rotary Pumped Coater, Quorum technologies Ltd., Sussex, UK) at 20 mA for 90 s. Samples were observed with a field emission scanning electron microscope (FE-SEM Mira3, Tescan Ltd., Brno, Czech Republic).
2.5. Statistical analyses

Data of antifungal effect, disease severity, and rates of germination, appressoria of the conidia, and fluorescent sites on 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, Seoul, Korea). Images of fluorescent microscope equipped with a soft imaging solution (XC10, Olympus, Seoul, Korea) were edited and saved using an image acquisition software (Get It, Seoul, Korea).

3. Results

3.1. In vitro inhibition of mycelial growth of *C. orbiculare* by bio-sulfur

In order to investigate the inhibitory effect of bio-sulfur on mycelia growth of *C. orbiculare*, PDA medium added with bio-sulfur was compared with PDA without bio-sulfur. At 7 days after inoculation, growth of fungal mycelia on the PDA medium added with bio-sulfur was significantly reduced compared to those on untreated PDA medium. However, the inhibition of mycelial growth by bio-sulfur was not comparable to that by the commercial fungicide Benomyl® (Figures 2(A,C)).

Similarly, mycelial inhibition was observed on PDB medium added with bio-sulfur. 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 inoculation compared to that of the untreated control. Also, in the medium containing Benomyl®, fungal mycelia had hardly grown (Figure 2(B)).

Such growth inhibition of mycelia by bio-sulfur was apparently present on the fresh weight of the mycelium mass which was decreased to almost 90% than that of untreated one. Similar to fresh weight, dry weight of mycelia was also reduced by treatment with bio-sulfur (Figures 2(D,E)).

3.2. Suppression of cucumber anthracnose by pre-treatment with bio-sulfur

In order to investigate the suppression of anthracnose disease by bio-sulfur, the number of lesions on cucumber leaves pre-treated with bio-sulfur was compared to that on cucumber leaves pre-treated with commercial fungicide Benomyl® and that of...
untreated control leaves. On untreated leaves, some light yellow and irregular lesions appeared at 3 days after inoculation. Its color then turned gray. At 6 days after inoculation, the lesion area on leaves started to extend and some lesions have united together. Also, necrosis occurred on parts of the leaves (Figure 3(A)).

On bio-sulfur pre-treated cucumber leaves, some lesions did not develop well compared to typical anthracnose formed on untreated leaves. The number of lesions on such leaves was significantly decreased (at 53%) compared to that of untreated one at 7 days after fungal inoculation. The suspension of bio-sulfur was diluted 500 times. Concentrations of Benomyl® and pathogen were 0.7 g/L and $1.0 \times 10^5$ conidia/ml, respectively. Vertical bars indicate standard deviation of three replications. Different letters on columns indicate significant differences ($p < .05$) according to Duncan’s multiple test.

Figure 3. Reduced cucumber anthracnose by bio-sulfur pre-treatment. (A) Anthracnose symptom development on untreated control, pre-treated with bio-sulfur, and pre-treated with a commercial fungicide Benomyl® at 7 days after inoculation with anthracnose pathogen Colletotrichum orbiculare; (B) decreased number of anthracnose lesions by pre-treatment with bio-sulfur or Benomyl®. The suspension of bio-sulfur was diluted 500 times. Concentrations of Benomyl® and pathogen were 0.7 g/L and $1.0 \times 10^5$ conidia/ml, respectively. Vertical bars indicate standard deviation of three replications. Different letters on columns indicate significant differences ($p < .05$) according to Duncan’s multiple test.

3.3. Observation of onion-peel inoculated with Colletotrichum orbiculare using an optical microscope

At 12 h after inoculation, most conidia germinated and developed germ tube on untreated onion (Figure 4(A)). However, on bio-sulfur treated half, conidia did not germinate. Even germinated conidia could not develop their hyphae as those of untreated onion (Figure 4(B)), indicating that germination of fungi might be restricted by treatment with bio-sulfur. On Benomyl® pre-treated onion, germinated conidia were rarely observed (Figure 4(C)).

At 24 h after inoculation, on untreated onion inner peels, abundant hyphae were extended and tangled with each other. Some appressoria were also formed (Figure 4(D)), indicating the typical infection structure of anthracnose pathogen. However, on bio-sulfur pre-treated onion, hyphal growth was apparently suppressed. Furthermore, no appressorium was observed (Figure 4(E)). The strongest inhibition of fungal growth was observed on Benomyl® treated onion, although some conidia germinated. Hyphal growth was strongly suppressed by this fungicide (Figure 4(F)).

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

Infection structures of C. orbiculare on cucumber leaves untreated, pre-treated with bio-sulfur, or pre-treated with Benomyl® were observed using a fluorescence microscope at 1, 3, and 5 days after inoculation. On the surface of untreated leaves, most conidia germinated and formed germ tube at 1 day after inoculation. Several appressoria with a circular or oval shape and dark brown color were found (Figure 5(A)). At 3 days after inoculation, over 40% of germ tubes formed appressorium (Figures 5(D) and 6(B)). At 5 days after inoculation, hyphae grew broadly. Most of them were tangled with each other. The rate of appressorium was also higher than before, reaching about 60% (Figures 5(G) and 6(B)).

The rate of germination on bio-sulfur pre-treated leaves was not significantly different from that on untreated leaves at 1 day after inoculation. However, most germ tubes did not form appressorium like those on untreated leaves (Figure 5(B)). At 3 and 5 days after inoculation, the rate of conidia germination was slightly reduced than that of untreated leaves (Figure 6(A)). Remarkably, much less appressoria were found compared with the untreated one. Similarly, at 5 days after inoculation, appressorium formation was strongly suppressed on bio-sulfur pre-treated leaves (Figure 6(B)).

Likewise, the rate of appressorium formation on the surface of leaves pre-treated with commercial fungicide Benomyl® was similar to that on the surface of leaves pre-treated with bio-sulfur (Figures 5 and 6), indicating that bio-sulfur might exert an antifungal effect as a fungicide on leaf surfaces.
On the other hand, there was no apparent difference in the rate of fluorescent sites, indicating similar defense responses of plants among untreated, pre-treated with bio-sulfur, and pre-treated with Benomyl\textsuperscript{VR} (Figure 6(C)).

### 3.5. Observation of cucumber leaves inoculated with Colletotrichum orbiculate using a scanning electron microscope

On untreated leaves, some germinated conidia formed appressorium similar to those observed by a fluorescent microscope at 1 day after inoculation (Figures 7(A) and 5(A)). However, most hyphae on bio-sulfur treated leaves shrunk (Figure 7(B), arrow) and some of them floated from host leaves. Based on fluorescent microscopical observations, appressorium was hardly found on these leaves (Figures 7(B) and 5(B)). On fungicide pre-treated leaves, less fungal structures were found compared to those on bio-sulfur pre-treated leaves (data not shown). Even if a few conidia germinated, they were morphologically changed, showing a twisted shape (Figure 7(C), arrow).

At 3 days after inoculation, abundant hyphae were tangled with each other on untreated leaves on where some appressoria were found (Figure 7(D)). However, on bio-sulfur pre-treated leaves, some lengthwise growing hyphae were found without forming appressorium (Figure 7(E)). Likewise, a very few hyphae were observed on Benomyl\textsuperscript{VR} pre-treated leaves. No appressorium was found either (Figure 7(F)).

### 4. Discussion

Some previous studies have mentioned that sulfur treatments can lead to growth inhibition of fungal pathogen and reduced disease severity on plants. For example, fumigation with H$_2$S can significantly inhibit colonial growth of either \textit{Aspergillus niger} or \textit{Penicillium italicum} on yeast peptone dextrose (YPD) agar medium. Especially, the generation of reactive oxygen species (ROS) can be directly induced in \textit{A. niger} by H$_2$S treatment, causing oxidative damage to molecules vital to mycelial growth and spore germination [15]. Mechanism of the antifungal effect of sulfur treatment has been reported. Inorganic sulfur as fungicide can cause abnormal respiration of fungal mycelia by interrupting the electron transport system in mitochondria and producing antifungal H$_2$S [16]. In our study, pre-treatment with bio-sulfur showed the direct antifungal effect on \textit{C. orbiculate}. Growth of fungal mycelia on bio-sulfur medium was reduced both on its length and weight (Figure 2). However, the general mechanism of the antifungal activity by bio-sulfur has not been clearly illustrated yet.

Soil-applied S fertilization has a significant repressive effect on infection of grapes with powdery

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**Figure 4.** (A, D) Optical microscopic photographs of onion surfaces at 12 and 24h after inoculation with \textit{Colletotrichum orbiculare} for groups of untreated; (B, E) pre-treated with bio-sulfur, and; (C, F) pre-treated with a commercial fungicide Benomyl\textsuperscript{VR}. The suspension of bio-sulfur was diluted 500 times. Concentrations of Benomyl\textsuperscript{VR} and pathogen were 0.7 g/L and $1.0 \times 10^5$ conidia/ml, respectively. All Bar = 100 $\mu$m. ap: appressorium; c: conidium; gt: germ tube; h: fungal hyphae.
The occurrence of skin sooty dapple disease on Asian pear is decreased by treatment with lime-sulfur. On tomato leaves sprayed with loess-sulfur, the occurrence of tomato powdery mildew is decreased. Also, lime- or loess-sulfur has been used to prevent anthracnose by Colletotrichum gloeosporioides or circular leaf disease by Mycosphaerella nawae on sweet persimmon, respectively. Likewise, pre-treatment with bio-sulfur decreased anthracnose disease on cucumber plants in the present study (Figure 3).

In order to illustrate the general tendency of anthracnose suppression by bio-sulfur, infection structures on onion were observed with an optical microscope after inoculation with C. orbiculare. On onion peels, germination rate and hyphal growth were apparently decreased by bio-sulfur, very similar to observations with a fluorescent microscope (Figure 4). These observations indicate that bio-sulfur may suppress fungal growth not only on host plants but also on non-host plants like onion.

To illustrate mode-of-action involved in bio-sulfur-mediated reduced cucumber anthracnose, C. orbiculare-inoculated leaves with or without bio-sulfur pre-treatment were observed using a fluorescence microscope. Some sulfur materials are known to be fungicides that can control plant disease by suppressing spore germination of fungal pathogen. For example, conidial germination of Venturia nashicola pear scab fungus is decreased to 93.7% by...
treatment with organic sulfur [21]. Water-soluble sulfur compounds BTB® and Hwangstar® have shown strong inhibitory effects on spore germination of *Botrytis cinerea* [22]. In the present study, the rate of conidia germination on bio-sulfur pre-treated leaves was reduced at 3 and 5 days after inoculation (Figures 5 and 6). However, suppression of germination of conidia did not seem to play an important role in the inhibition of anthracnose disease.

There were a few appressoria of *C. orbiculare* on leaves pre-treated with bio-sulfur based on observation with a fluorescent microscope whereas there were lots of appressoria formed on untreated leaves at 3 and 5 days after fungal inoculation (Figure 6). Some fungi including *Colletotrichum* can form appressorium which is a specialized infection structure when they invade host leaves [23]. It has been known that turgor of appressorium is necessary for infecting plant cells physically. This structure can generate a penetration to protrude into the cuticle [24]. Many researchers have proved that suppression of appressorium formation can result in decreased disease severity caused by filamentous fungi. For example, lower formation of appressorium by pretreatment with an algae *Chlorella fusca* can remarkably reduce the number of lesions caused by anthracnose pathogen on cucumber leaves [25].

Figure 6. (A) Rates of germination of fungal conidia; (B) appressorium formation of fungal conidia, and; (C) fluorescent sites of host cells on cucumber leaves in groups of untreated, pre-treated with bio-sulfur, and pre-treated with a commercial fungicide Benomyl®. The suspension of bio-sulfur was diluted 500 times. Concentrations of Benomyl® and pathogen were 0.7 g/L and 1.0 × 10⁵ conidia/ml, respectively. Different letters on the columns indicate significant differences (p < .05) according to Duncan’s multiple test.
Formation of appressorium could also be reduced by treatment with soluble silicate by which the penetration of powdery mildew is halted [26]. Also, inhibition of appressorium formation of Venturia nashicola causing pear scab has been observed on pear leaves treated with commercial sulfur [21]. Thus, reduction of appressorium after bio-sulfur treatment may be the key to disturb the infection of some pathogens. Similarly, some chemicals could reduce appressorium formation of plant pathogens. Lime-sulfur can hinder appressoria formation of Venturia inaequalis at an early stage of infection [27]. In the present study, the rate of appressorium formation of C. orbiculare was decreased on leaves pre-treated with a commercial fungicide Benomyl®. Therefore, treatment with bio-sulfur might suppress appressorium formation and lead to the reduction in disease severity of anthracnose on cucumber plant.

Generally, callose formed in host cells indicates a defense response against fungal invasion [28]. Callose, also known as "papillae" forming on host secondary cell wall, may play a role as barriers during early infection stages of the pathogen [29]. Also, callose can be formed at penetration sites on grapevine plants whose resistance is mediated by β-aminobutyric acid (BABA) or jasmonic acid (JA) [30]. As expected, there were no differences in fluorescent cells at penetration sites on leaves pre-treated with bio-sulfur compared to untreated control (Figure 6(C)), indicating no defense response of host cells in this experiment.

To illustrate how bio-sulfur could suppress germination and growth of conidia, fine structures were observed with a scanning electron microscope. Morphological changes of the fungal pathogen after pre-treatment with bio-sulfur indicated the antifungal effect of bio-sulfur against this fungus (Figure 7) which might cause suppression of either germination rate or hyphal growth. Furthermore, some unattached hyphae to host leaves were observed on bio-sulfur pre-treated leaves (Figure 7). These were not found on untreated leaves. These observations suggest that unknown substances created by bio-sulfur might hinder interaction between the pathogen and the host.

In summary, bio-sulfur has a direct antifungal effect against anthracnose pathogen and results in the reduction of disease severity. On bio-sulfur treated leaves, germination of the fungus was not hindered. However, appressorium formation was remarkably reduced which might be the main reason for the decrease in disease severity. Also, anthracnose fungus shrunk after bio-sulfur treatment which might cause a decrease of appressorium formation. However, treatment with bio-sulfur did

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Figure 7. (A, D) Scanning electron microscopic photographs of cucumber leaves at 12 and 24 h after inoculation with Colletotrichum orbiculare for groups of untreated; (B and E) pre-treated with Bio-sulfur, and; (C and F) pre-treated with a commercial fungicide Benomyl®. The suspension of bio-sulfur was diluted 500 times. Concentrations of Benomyl® and pathogen were 0.7 g/L and 1.0 × 10⁵ conidia/ml, respectively. All Bar = 20 μm. ap: appressorium; c: conidium; gt: germ tube; h: fungal hyphae.
not seem to induce plant defense responses. These results may be useful for plant protection, especially for eco-friendly farms where the application of commercial chemicals is limited.

**Disclosure statement**

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