Menadione Sodium Bisulfite-Protected Tomato Leaves against Grey Mould via Antifungal Activity and Enhanced Plant Immunity

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Tomato grey mould has been one of the destructive fungal diseases during tomato production. Ten mM of menadione sodium bisulfite (MSB) was applied to tomato plants for eco-friendly control of the grey mould. MSB-reduced tomato grey mould in the 3rd true leaves was prolonged at least 7 days prior to the fungal inoculation of two inoculum densities (2×10^4 and 2×10^5 conidia/ml) of *Botrytis cinerea*. Protection efficacy was significantly higher in the leaves inoculated with the lower disease pressure of conidial suspension compared to the higher one. MSB-pretreatment was not effective to arrest oxalic acid-triggered necrosis on tomato leaves. Plant cell death and hydrogen peroxide accumulation were restricted in necrotic lesions of the *B. cinerea*-inoculated leaves by the MSB-pretreatment. Decreased conidia number and germ-tube elongation of *B. cinerea* were found at 10 h, and mycelial growth was also impeded at 24 h on the MSB-pretreated leaves. MSB-mediated disease suppressions were found in cotyledons and different positions (1st to 5th) of true leaves inoculated with the lower conidial suspension, but only 1st to

3rd true leaves showed decreases in lesion sizes by the higher inoculum density. Increasing MSB-pretreatment times more efficiently decreased the lesion size by the higher disease pressure. MSB led to inducible expressions of defence-related genes *SIPR1a*, *SIPR1b*, *SIPIN2*, *SIACO1*, *SIChi3*, and *SIChi9* in tomato leaves prior to *B. cinerea* infection. These results suggest that MSB pretreatment can be a promising alternative to chemical fungicides for environment-friendly management of tomato grey mould.

Keywords : *Botrytis cinerea*, eco-friendly, fungicidal, menadione sodium bisulfite, plant defence, tomato grey mould

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Grey mould disease by *Botrytis cinerea* has been a great trouble in culturing important horticultural crops including grape, strawberry and tomato plants, and chemical fungicides were widely used for the disease management (Petrash et al., 2019; Steel et al., 2013; Williamson et al., 2007). Occurrence of chemical fungicide-resistant *B. cinerea* isolates in many countries and environment concerns due to frequent fungicide usages have urged to find eco-friendly alternatives for controlling grey mould for a sustainable horticulture (Fan et al., 2016; Jacometti et al., 2010; Saito et al., 2019). Antagonistic microbes (*Bacillus subtilis*, *Streptomyces philanti*, *Fusarium equiseti*), plant essential oils (origanum oil, thyme oil) and plant defence-priming agents (β -aminobutyric acid, hexanoic acid) have been suggested to manage tomato grey mould on leaves and fruits eco-friendly (Ben-Jabeur et al., 2015; Boukaew

et al., 2017; Chen et al., 2019; Luna et al., 2016; Mónaco et al., 2009; Soyulu et al., 2010; Vicedo et al., 2009).

A variety of vitamins such as vitamin B1 and vitamin C have shown *in vitro* antifungal activities and/or plant defence activation, and vitamins have been exogenously applied to reduce plant diseases. Rice and cucumber plants pretreated with vitamin B1 (thiamine) showed reduced lesions on leaves infected by rice blast and cucumber anthracnose, respectively, although *in vitro* antifungal activity of vitamin B1 was not found against *Magnaporthe grisea* and *Colletotrichum orbiculare* (formally *C. lagenarium*) (Ahn et al., 2005). Dose-dependent decreases in germ-tube elongation and appressorium formation of *M. grisea* were demonstrated by vitamin C (ascorbic acid) treatment (Egan et al., 2007). Ascorbic acid also arrested *in vitro* mycelial growth of *Alternaria brassicicola* and *Fusarium ananatum* (Barral et al., 2017; Botanga et al., 2012). Deficiency of vitamin C in Arabidopsis plants resulted in enhanced disease susceptibility to *A. brassicicola* infection suggesting an active role of ascorbic acid during the plant immunity response (Botanga et al., 2012). Vitamin K3 is one of fat-soluble vitamin groups and often applied to fungi and plants as a form of water-soluble menadione sodium bisulfite (MSB). MSB had *in vitro* antifungal activity against a variety of phytopathogenic fungi such as *Alternaria alternata*, *Fusarium graminearum* and *Ustilago maydis* (Nikolaou et al., 2009; Yu et al., 2016). Stem canker, fungal disease caused by *Leptosphaeria maculans*, could be controlled in oilseed rape plants locally and systemically by MSB pretreatment via *pathogenesis-related protein 1* (*PR1*) and *ascorbate peroxidase* (*APX*) gene expressions showing MSB-enhanced plant defences (Borges et al., 2003). MSB-regulated hydrogen peroxide accumulation and defence-related gene transcription in Arabidopsis plants suggested that MSB can modulate defence-associated metabolisms in response to pathogen invasions as well as environmental stresses (Borges et al., 2009). Indeed, MSB can confer enhanced tolerance to Arabidopsis plants to deal with environmental stresses such as high salinity via controlling genes associated with proline metabolism (Jiménez-Arias et al., 2015a, 2015b). These results suggest that MSB can be applied to crops as a priming agent to cope with pathogen attacks as well as adverse environmental conditions (Borges et al., 2014a).

MSB treatment directly arrested *in vitro* conidial germination and mycelial growth of *B. cinerea* in dose-dependent manners in our previous study (Hong et al., 2016). Pretreatment with increasing MSB (2 to 20 mM) at 1 day before the fungal challenge-inoculation resulted in dose-dependent reductions in necrotic lesion sizes on tomato true

leaves inoculated by conidial suspension (1×10^5 conidia/ml) of *B. cinerea* compared to control leaves (Hong et al., 2016). In this study, we compared protection efficacies of 10 mM MSB on tomato grey mould in cotyledons and true leaves with different leaf positions against different disease pressures caused by two inoculum densities of *B. cinerea* (2×10^4 and 2×10^5 conidia/ml). Arrested plant cell death, hydrogen peroxide and fungal growths of *B. cinerea* were observed on MSB-pretreated tomato leaves. Repeated MSB-pretreatment was also evaluated to improve the protection efficacies against tomato grey mould. In addition, MSB-inducible expression of defence-related genes was analysed to decipher possible molecular role of MSB as a plant defence priming agent in tomato leaves.

Materials and Methods

Plant growth and fungus culture. Tomato seeds (cv. Cupirang) (Nongwoo Bio Co., Ltd., Suwon, Korea) were sown in pots (90 mm in diameter, 70 mm in height) containing commercial soil mixtures ‘Toshil’ (Shinan Growth Co., Ltd., Jinju, Korea) and grown under controlled environments in walk-in plant growth rooms for 4- or 5-weeks previously described (Hong et al., 2016). Third true leaves were detached from 5-leaf stages of the tomato seedlings and two primary leaflets of the third true leaves were used for the *Botrytis cinerea* inoculation unless otherwise noted.

B. cinerea KACC 40573 was provided by the Korean Agricultural Culture Collection established in Rural Development Administration, Republic of Korea, and the fungal isolate was virulent to the tomato cultivar ‘Cupirang’ in which spreading necrotic lesions were developed on the inoculated leaves. The fungal isolate was cultured on 1/2-strength potato dextrose agar media at 25°C under dark condition for 14 days to prepare conidial suspension. Quarterly diluted potato dextrose broth was poured in Petri dishes with the 14-day-old fungal cultures, scraped with sterile spatula and filtered with double-layered gauzes to collect conidial suspension. Concentration of the conidial suspension was adjusted to 2×10^4 and 2×10^5 conidia/ml with haemocytometer under a light microscope.

MSB treatment. Ten mM of MSB (Sigma-Aldrich Co., St. Louis, MO, USA) prepared in distilled water were foliar sprayed onto the tomato seedlings in the pots (10 ml per one tomato seedling) at 1, 3, or 7 days prior to challenge-inoculation of *B. cinerea*. To examine enhanced plant protection efficacies by repeated MSB pretreatment, four different pretreatment regimes (twice pretreatment: 7 and 3 days, 7 and 1 days, 3 and 1 days prior to the fungal inocula-

tion; thrice pretreatment: 7, 3 and 1 days prior to the fungal inoculation) were compared to single pretreatment at 7 days before the fungal challenge-inoculation.

Plant inoculation and disease evaluation. Three tomato leaflets were placed on a sterile water-saturated gauze in a plastic box (13 × 9 × 5 cm). One drop (10 µl) of *B. cinerea* conidial suspension was inoculated onto the centre of each tomato leaflet, and plastic lid was tightly covered to keep the moist condition. The plastic boxes containing the *B. cinerea*-inoculated tomato leaflets were transferred to an incubator at 20°C under continuous darkness for grey mould symptom progress. At 3 days after the fungal inoculation, diameter of necrotic lesions was measured to evaluate the disease severity.

Oxalic acid (OA) (20 mM), one of secreted pathogenicity factors of *B. cinerea*, was prepared in distilled water, and was applied to the detached tomato leaflets in the plastic boxes as a droplet (10 µl). The OA-treated tomato leaflets were incubated at 20°C under continuous moist and dark conditions for 3 days, and diameter of necrotic spots was measured.

Tomato leaflets staining with Evans blue and 3,3'-diaminobenzidine (DAB). Dying cells and hydrogen peroxide (H₂O₂) accumulation in *B. cinerea*-inoculated tomato leaflets were detected by staining with Evans blue and DAB solutions, respectively, according to modified methods of our previous study (Sung and Hong, 2010).

Tomato leaflets were stained in 0.25% Evans blue solution prepared in 10 mM CaCl₂ (pH 5.6 adjusted by HCl) for 1 h and washed with distilled water three times to detect blue colour-stained plant dead cells. To remove leaf chlorophylls, the leaflets were placed in boiling 95% ethanol for 15 min. Tomato leaflets were stained in 0.1% DAB solution overnight under a dark condition and chlorophylls were removed by boiling the leaflets in 95% ethanol for 15 min. H₂O₂ accumulation was detected as a red-brown coloured precipitates.

H₂O₂ treatments and leaf chlorophyll measurement. Mock- and MSB-treated tomato leaflets were cut with cork borer (10 mm in diameter) and the leaf discs were floated onto different concentrations (0, 0.0625, 0.125, 0.25, 0.5 and 1 M) of H₂O₂ solution in 4 × 6 well-plates. At 3 days after H₂O₂ treatments, chlorophyll contents in the leaf discs were measured by the method in our previous study and relative chlorophyll contents were calculated as percentages (Sung and Hong, 2010).

Staining of *B. cinerea* on the tomato leaflets and observation under a light microscope. At 10 and 24 hpi, *B. cinerea*-inoculated tomato leaflets were cut in pieces of 5 mm × 5 mm and stained with lactophenol-trypan blue solution, and conidial germination and mycelial elongation of *B. cinerea* on the leaf tissues were observed under a light microscope described previously (Hong et al., 2018). Conidial numbers of *B. cinerea* on the leaflet fragments were counted and expressed per leaf mm². Ten leaflet fragments were prepared for mock and MSB treatment, respectively, and five independent experiments were conducted.

Plant total RNA isolation and gene expression analyses.

Total RNA was isolated from tomato leaflets and gene expression analyses were performed via semi-quantitative reverse transcription polymerase chain reaction as described previously (Lee et al., 2019). Expression of tomato genes, *SIPR1a*, *SIPR1b*, *SIPIN2*, *SiChi3*, *SiChi9*, *SLACO1*, *SLAPX1*, and *SIPAL3* were evaluated for MSB-mediated defence responses. Tomato glyceraldehyde 3-phosphate dehydrogenase (*SIGAPDH*) gene expression was investigated as an internal control. PCR primer pair for each gene was shown in Supplementary Table 1. Three independent experiments were conducted for each gene expression and representative results were demonstrated.

Statistical analyses. An analysis of variance (ANOVA) was conducted to determine the effects of MSB pretreatments on the lesion diameters caused by *B. cinerea* inoculation and OA treatment. An ANOVA was also conducted to determine the effects of MSB pretreatments on sensitivity of tomato leaflets to H₂O₂. Means were compared using least significant difference tests. Statistical analysis was performed with the SAS software version 8.1 (SAS Institute Inc., Cary, NC, USA). For conidial numbers on the tomato leaflets, Student's *t*-test was used to compare the means. A difference was statistically significant when *P* < 0.05. Error bars in figures represent standard errors of the means.

Results

MSB pretreatment reduced grey mould lesion but not OA stress on detached tomato leaflets. Two concentrations (2 × 10⁴ and 2 × 10⁵ conidia/ml) of *B. cinerea* conidial suspension as different disease pressures were challenge-inoculated onto the leaflets from tomato plants pretreated with mock and MSB at 1, 3 and 7 days prior to the fungal inoculation (Fig. 1A). Three different timings of MSB pretreatment were equally sufficient for tomato leaflet protec-

Fig. 1. Effects of pretreatment with menadione sodium bisulfite (MSB) onto tomato plants on leaf grey mould lesion development by *Botrytis cinerea* challenge inoculation. (A) Necrotic lesion diameters on *B. cinerea*-inoculated tomato leaflets at 3 dpi. The MSB (10 mM) was foliar sprayed onto the 4-week-old tomato plants at 1, 3, and 7 days before the fungal inoculation, and two concentrations (2×10^4 and 2×10^5 conidia/ml) of the conidial suspensions were drop-inoculated on the primary leaflets of detached third leaves from the 5-week-old tomato plants. For mock-treatment, sterile water was used instead of MSB. (B) Plant protection (%) against tomato grey mould by the MSB pretreatment at 1, 3, and 7 days before the fungal inoculations. Lesion diameters on the MSB-pretreated leaflets were relatively compared to lesion diameters on the mock-pretreated ones. (C) Effects of MSB pretreatment onto tomato plants on oxalic acid (OA; 20 mM) stress-triggered tissue necrosis. Lesion diameters on the OA-treated tomato leaflets were measured at 3 days after treatment. Error bars represent the standard errors of the mean lesion diameter of four independent experimental replications. Each experiment had four replicates. Means followed by the same letter are not significantly different at 5% level by least significant difference tests. The same letter above bars represented no significant difference between treatments.

tion against two inoculum densities of *B. cinerea* shown as reduced lesion diameter on the inoculated leaflets. However, protection efficacies against the lower inoculum density was higher than those against higher inoculum density (Fig. 1B). MSB pretreatment at 1, 3 and 7 days prior to OA (20 mM) stress did not confer tolerance of tomato plants (Fig. 1C). No difference in lesion sizes caused by OA was found in the leaflets pretreated with mock and MSB.

Decreased cell death and hydrogen peroxide generation in the *B. cinerea*-inoculated tomato leaflets by MSB pretreatment. Plant cell death and hydrogen peroxide accumulation in the *B. cinerea*-inoculated tomato leaflets were decreased by MSB pretreatment (Fig. 2).

By *B. cinerea* infection in the mock-pretreated leaflets, necrotic grey mould lesions gradually enlarged with the fungal pathogenesis for 3 days. However, the lesion sizes

were relatively reduced during the MSB-mediated protection against *B. cinerea* compared to ones occurred in the mock-pretreated leaflets at the same time points. Plant cell deaths and hydrogen peroxide detected by Evans blue and DAB staining, respectively, were accordance with increasing necrotic lesion sizes (Fig. 2A). In MSB-pretreated leaflets, plant cell deaths and H_2O_2 were distinctly diminished compared to those in the mock-pretreated leaflets at the same time points.

No difference was found in chlorophyll contents in mock- and MSB-pretreated leaves in response to exogenous H_2O_2 (Fig. 2B). Increasing concentrations (0.0625 to 1 M) of H_2O_2 caused significant decreases in chlorophyll contents in dose-dependent manner compared to untreated control (0 mM) in the mock-pretreated leaves. Same doses of H_2O_2 resulted in reduced chlorophyll contents in the MSB-pretreated leaves without any difference with the

Fig. 2. Menadione sodium bisulfite (MSB)-reduced hydrogen peroxide (H₂O₂) production in *Botrytis cinerea*-inoculated leaves. (A) Arrested tomato leaf cell death and H₂O₂ accumulation in MSB-pretreated tomato leaflets challenge-inoculated by *B. cinerea*. Mock- and MSB-pretreated tomato leaflets were inoculated by two concentrations of *B. cinerea* conidial suspensions (2×10^4 and 2×10^5 conidia/ml), and were stained with Evans blue and 3,3-diaminobenzidine (DAB) for plant cell death occurrence and H₂O₂ accumulation, respectively. (B) Responses of mock- and MSB-pretreated tomato leaf discs to different concentrations of exogenous H₂O₂ treatment. Error bars represent the standard errors of the mean total chlorophyll contents of three independent experimental replications. Each experiment had four replicates. Means followed by the same letter are not significantly different at 5% level by least significant difference tests. The same letter above bars represented no significant difference between treatments.

chlorophyll contents in the mock-pretreated leaves.

Suppressed *B. cinerea* development on the MSB-pretreated tomato leaflets. Retarded early growth of *B. cinerea* by MSB pretreatment was observed on the tomato leaflets under a light microscope (Fig. 3).

Numbers of *B. cinerea* conidia reduced significantly on

Fig. 3. Microscopic observation of *Botrytis cinerea*-inoculated tomato leaflet pretreated with menadione sodium bisulfite (MSB). (A) Number of conidia observed on the tomato leaflets at 10 h. Error bars represent the standard errors of the mean conidial number of four independent experimental replications. Asterisk indicates significant difference as determined by the Student's *t*-test ($P < 0.05$). (B) Different fungal growth of *B. cinerea* on tomato leaflets with or without MSB pretreatment at 1 day prior to the fungal challenge inoculation. Conidial suspension (2×10^5 conidia/ml) of *B. cinerea* was drop-inoculated on the detached tomato leaflets treated with mock and MSB (10 mM). Conidial germination and mycelial growth of *B. cinerea* on the leaf tissues observed at 10 and 24 h under a light microscope after fungal staining using lactophenol-trypan blue. Size bars = 25 μ m.

the MSB-pretreated leaflets compared to mock-pretreated ones at 10 hpi (Fig. 3A). Conidia were germinated and occupied ca. 389.5 per leaf mm² on the mock-pretreated leaflets, which was comparably decreased number of conidia as 71.6 per leaf mm² on the MSB-pretreated leaflets. Most conidia germinated and formed appressorium on the mock-pretreated leaflets, whereas only a few conidia initiated to germinate on the MSB-pretreated ones at 10 hpi (Fig. 3B). Fungal hyphae were proliferated on the mock-pretreated leaflets at 24 hpi. But reduced germ-tube elongation and appressorium formation of *B. cinerea* was found on the MSB-pretreated ones at 24 hpi, and some conidia was still ungerminated (Fig. 3B).

Fig. 4. Leaf age- and inoculum density-dependent disease protection of tomato grey mould by menadione sodium bisulfite (MSB) pretreatment. The MSB (10 mM) were foliar sprayed onto the 4-week-old tomato plants 7 days before the fungal inoculation, and two concentrations (2×10^4 and 2×10^5 conidia/ml) of the *B. cinerea* conidial suspensions were inoculated on the detached leaves of different positions. Mock means sterile water. Lesion diameters on the *B. cinerea*-inoculated tomato leaflets were measured at 3 dpi. Error bars represent the standard errors of the mean lesion diameter of four independent experimental replications. Means followed by the same letter are not significantly different at 5% level by least significant difference tests. The same letter above bars represented no significant difference between treatments.

Leaf position-dependent protective efficacy of MSB pretreatment on tomato grey mould. Grey mould lesion formations on tomato plants with or without MSB pretreatment were dependent on both plant leaf position and inoculum densities of *B. cinerea* (Fig. 4).

Without MSB pretreatment, grey mould lesion sizes gradually decreased in tomato leaves as leaves were younger by both low and high inoculum densities. Cotyledons showed largest lesions by both inoculum densities. As true leaf numbers increased from 1° to 5° (from lower to upper position), lesion sizes were reduced by both inoculum densities.

Grey mould suppressions by MSB-pretreatment were different in tomato leaves by both low and high inoculum densities. By low inoculum density, distinct decline in lesion sizes were found in cotyledons and true leaflets from 1° to 5° without significant difference. However, only true leaflets from 1° to 3° showed MSB-pretreated reductions in grey mould lesions by high inoculum density compared

Fig. 5. Effect of repetitive pretreatments with menadione sodium bisulfite (MSB) on grey mould lesion development against high disease pressure by conidial suspension (2×10^5 conidia/ml) of the *Botrytis cinerea*. Error bars represent the standard errors of the mean lesion diameter of four independent experimental replications. Means followed by the same letter are not significantly different at 5% level by a least significant difference test. The same letter above bars represented no significant difference between treatments.

to mock-pretreated control in each leaf position. MSB pretreatment-mediated protections were not obviously observed in cotyledons and relatively younger 4° and 5° leaflets.

Enhanced protective efficacies against tomato grey mould by repetitive MSB pretreatments. Increased frequency of MSB pretreatment increased protection to high inoculum density (2×10^5 conidia/ml) of *B. cinerea* (Fig. 5). MSB pretreatment twice within 7 days prior to the fungal inoculation showed significantly increased tomato protection compared to MSB pretreatment once at 7 days prior to the fungal inoculation, nevertheless pretreatment timings. No difference was found in 3 different regimes of MSB pretreatment twice: 7 days and 3 days, 7 days and 1 day, 3 days and 1 day. MSB pretreatment thrice (7 days, 3 days, and 1 day) prior to the fungal inoculation increased protection efficacy compared MSB pretreatment once. But the protection efficacy was not different from ones derived by MSB pretreatment twice.

MSB-inducible expression of tomato defence-associated genes. Expressions of defence-related tomato genes were investigated in tomato leaflets treated with MSB at 6 and 24 h to investigate whether or not involvement of MSB in enhanced plant immunity to protect tomato plants against grey mould (Fig. 6).

Ten mM of MSB resulted in induction of plant defence-

Fig. 6. Expression of defence-related genes in tomato leaves in response to a menadione sodium bisulfite (MSB) treatment. Total RNA was isolated from the leaflets of 3rd true leaves at 6 and 24 h after foliar spray of mock or MSB (10 mM). Gene expressions were determined by semi-quantitative reverse transcription PCR analysis, and *SIGAPDH* was used as an internal control. The number of PCR cycles of each result is indicated on the right side in parentheses. Four independent experiments were conducted and one representative result was demonstrated.

related gene expressions in tomato leaflets compared to the mock treatment. *SIPR1a*, *SIPR1b*, *SIPIN2*, *SIChi3*, *SIChi9*, and *SIACO1* genes were up-regulated by MSB application within 24 h. However, *SIAPX1* and *SIPAL3* were not regulated in the tomato leaf tissues by the MSB.

Discussion

MSB has shown its antifungal activities during *in vitro* conidial germination and mycelial growth of *B. cinerea* (Hong et al., 2016). Pretreatment with the antifungal MSB efficiently protected tomato leaves against the two different disease pressures by *B. cinerea* infection. As expected, the protection efficacy against the low disease pressure (2×10^4 conidia/ml) was significantly higher than that against high disease pressure (2×10^5 conidia/ml). Increasing inoculum concentrations of *B. cinerea* was associated with augmenting stem end decay of pear fruits as well as grey mould in stem and flower of tomato plants (Carisse et al., 2015; Spotts et al., 2008). In the present study, necrotic lesion developments in the mock-pretreated tomato cotyledons and true leaves (1st to 5th) by the high disease pressure of *B. cinerea* were faster than those caused by the

low disease pressure, although statistical data with significant difference between low and high disease pressures were not demonstrated (Fig. 4). It needs to be investigated whether MSB was still effective to cope with much higher disease pressure caused by more than 2×10^5 conidia/ml of *B. cinerea*, because much higher disease pressure of *B. cinerea* can diminish protective effect of MSB on tomato leaf grey mould. More efficient grey mould control by MSB-pretreatment against the lower diseases suggests that reducing inoculum density of *B. cinerea* by applying other eco-friendly agents such as beneficial microbes and/or plant defence activators, may improve MSB-mediated grey mould control efficacy during tomato production under a protected greenhouse environment (Elad et al., 1994; Gao et al., 2018; Sivakumaran et al., 2016; Vicedo et al., 2009). It also suggested that repetitive pretreatment with MSB at least two times efficiently increased protection efficacy against tomato grey mould without phytotoxic side effect, showing more decreased grey mould lesions by MSB-pretreatment. Second MSB pretreatment may increase its residual antifungal activities against conidial germination and mycelial growth of *B. cinerea* on tomato leaves.

Preventive efficacies of MSB pretreatment was prolonged for at least 7 days in responses to the *B. cinerea* challenge inoculations by the low and high concentrations of *B. cinerea* under controlled ambient conditions. Preventive effects of MSB for 7-1 day prior to *B. cinerea* inoculation under a greenhouse remains investigated for stable and reliable grey mould control. Different control efficacies on grey mould in tomato stems were demonstrated by preventive and curative treatments with different commercial microbes (Utkhede and Mathur, 2006). Hexanoic acid pretreatment was more protective to control tomato grey mould as compared to its treatment after *B. cinerea* inoculation (Leyva et al., 2008). Prolonged curative effect of MSB treatment to control tomato grey mould remains evaluated in a further study.

OA was secreted from several fungal species including *B. cinerea* and played roles as a pathogenicity factor via decreasing ambient pH during the fungal invasion (Lee et al., 2019; Mbengue et al., 2016). In the present study, pretreated MSB could not mitigate tomato leaf tissue necrosis caused by OA application, indicating that MSB-mediated grey mould decline may not be related to the direct elimination of OA or enhanced plant defences to OA secreted from *B. cinerea*.

MSB-mediated decreases in grey mould lesions were closely associated with decreases in dying cells and H_2O_2 accumulation in the tomato leaflets. Invading *B. cinerea* led to plant cell deaths and H_2O_2 accumulation in tomato

leaf tissues and suspension cultured cells (Asselbergh et al., 2007; Pietrowska et al., 2015). H_2O_2 produced in the grey mould lesions was limited in biologically-protected leaves of tomato plants in response to *B. cinerea* infection (Herrera-Téllez et al., 2019). *N*-Nitro-L-arginine methyl ester (nitric oxide synthase inhibitor) reduced both tomato grey mould necrotic lesions and H_2O_2 generation in the lesions (Sivakumaran et al., 2016). H_2O_2 generation in the necrotic plant tissues provide favours to growth and virulence of *B. cinerea* (Govrin and Levine, 2000; Rossi et al., 2017). We investigated whether or not MSB confer tolerance to tomato leaves to H_2O_2 -mediated oxidative stress. MSB could not limit the tissue damages of tomato leaves by exogenously applied excess H_2O_2 , although MSB confer enhanced tolerances to plants abiotic stresses such as chilling and high salt stresses and is involved in reactive oxygen species (ROS)-dependent signalling (Borges et al., 2014a). The lowest H_2O_2 concentration of 0.0625 M used in this study was sufficient to decrease chlorophyll contents to ca. 50% in mock- and MSB-pretreated leaves without any difference. It remains investigated whether MSB pretreatment can alter stress sensitivity of tomato leaves in response to the relatively lower H_2O_2 . We also cannot exclude the possibility that MSB as a superoxide anion generator can trigger microscopic ROS-associated cellular events before necrotic symptom development to combat *B. cinerea* infection.

Early growth behaviour of *B. cinerea* during the fungal pathogenesis was distinctly arrested on the MSB-pretreated tomato leaf surfaces compared to the mock-pretreated one as observed under a light microscope. Conidial numbers on the MSB-pretreated leaf tissues were significantly reduced at 10 h, indicating that MSB may directly show its antifungal activity via mechanical disruption of *B. cinerea* conidia. In our previous study, 10 mM of MSB completely suppressed the *in vitro* conidial germination of *B. cinerea* at 20°C after 6 h-incubation, and reduced number of conidia treated with MSB was not recognised on the glass slides (Hong et al., 2016). Cell-free supernatant of *Pseudomonas* sp. QBA5 directly disrupted *B. cinerea* conidia by destroying plasma membrane integrity, and inhibited conidial germination and germ-tube elongation (Gao et al., 2018). *In vitro* and *in planta* destructive nature of MSB to *B. cinerea* conidia as well as conidial stress responses remains elucidated.

MSB-mediated grey mould protection was dependent on inoculum density and tomato leaf position. By both low and high inoculum densities, lesion diameters in the mock-pretreated leaves were decreased as leaves were younger in our present study. In contrast, grey mould in tomato stems

less developed in basal parts of tomato plants compared to middle and top parts (Borges et al., 2014b). Leaf age-dependent grey mould development was also found in strawberry leaves, and the plant basal immunity was highly correlated with biochemical changes including H_2O_2 , total flavonoids and ascorbate peroxidase activity in different leaves (Meng et al., 2019). Biochemical changes in tomato leaves of different ages with and without MSB pretreatment will provide basis on age-dependent plant immunity against *B. cinerea* infection. MSB-pretreatment resulted in constant protection efficacies against the low inoculum density of *B. cinerea* regardless of leaf positions. However, protection efficacies of MSB-pretreatment could not be different in highly susceptible cotyledons and moderate resistant true leaves 4° and 5° by the high inoculum density.

Recently MSB was suggested as a defence elicitor in host plants against challenging pathogen invasions (Borges et al., 2014a). MSB treatment differentially regulated transcriptions of stress-associated genes such as glutathione *S*-transferases, G-box-regulating transcription factors and cytochrome P450 in Arabidopsis plants without any challenging stress (Borges et al., 2009). In this present study, MSB was found to induce plant defence-related genes like *SIPR1a*, *SIPR1b*, *SIPIN2*, and *SLACO1* in tomato leaves within 24 h prior to *B. cinerea* inoculation. Tomato *SIPR1a*, *SIPR1b*, *SIPIN2*, and *SLACO1* were known to be *B. cinerea* infection-inducible genes involved in the defence responses to the fungal infection (Harel et al., 2014; Li et al., 2014). Both *SIPR1a* and *SIPR1b* gene expressions activated by either salicylic acid (SA) or ethylene application, but MSB-induced *SIPR1a* and *SIPR1b* in the tomato leaves may be mediated by activated ethylene-signalling not by SA-signalling, because SA down-regulated basal immunity of tomato leaves against *B. cinerea* (El Oirdi et al., 2011; Schuegger et al., 2006; Tomero et al., 1994, 1997). *SIPIN2* and *SLACO1* were responsive to methyl jasmonate and ethylene, respectively, and both gene expressions implied activation of jasmonic acid (JA) and ethylene defence signalling, respectively (Blume and Grierson, 1997; Li et al., 2002). JA-insensitive mutant *jail* and jasmonate synthesis tomato mutant *def1* showed enhanced susceptibility to grey mould in leaves (Díaz et al., 2002; Du et al., 2017). Treatments with ethylene and its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were sufficient to reduce lesions in tomato leaves inoculated by *B. cinerea* (Díaz et al., 2002; Nambeesan et al., 2012). It was opposite from results that exogenous ethylene accelerated necrotic lesion expansion and silver thiosulfate ethylene action blocker reduced susceptibility in tomato leaves inoculated by *B. cinerea* (Elad, 1990). *SlChi3* and *SlChi9* encoding acidic

and basic chitinases, respectively, showed differential gene expressions by defence-related chemicals, ethylene, methyl jasmonate and SA (Puthoff et al., 2010). *SlChi3* expression was strongly induced in tomato leaves by ethylene and moderately increased by SA and methyl jasmonate, whilst *SlChi9* expression was moderately inducible by methyl jasmonate and slightly increased by ethylene but not by salicylic acid. Pretreated MSB may mediate enhanced plant immunity in tomato leaves via activating JA and/or ethylene defence-signalling pathways to halt *B. cinerea* infection. *SlACO1* is one of seven ACC oxidase genes in tomato genome, and may convert ACC into ethylene during the wounding response and onset of senescence in tomato leaves (Barry et al., 1996; Houben and Van de Poel, 2019). It will be interesting to decipher defensive role of ethylene generated by MSB-mediated *SlACO1* gene expression, because ethylene may play roles positively or negatively during the tomato grey mould lesion development depending on different ambient conditions. *SlPR1a*, *SlPR1b*, *SlChi3*, and *SlChi9* encode different putative antimicrobial peptides, and we cannot exclude that possibility that *B. cinerea* invading tomato leaf tissues can be directly limited by these antimicrobial peptides induced by MSB pretreatment.

Tomato grey mould occurs in different organs of tomato plants like leaves, flowers and fruits, and protective role of MSB against the grey mould was demonstrated only in the tomato leaves in this study. Studies on MSB-controlling tomato grey mould in most vulnerable flowers and postharvest fruits may increase further horticultural application to extend shelf-life. Molecular and biochemical investigation on mode-of-action of MSB to enhance tomato plant immunity also will pave the ways to protect tomato plants against other destructive pathogens.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (<http://www.ppjonline.org/>).

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