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Hydrogen Peroxide- and Nitric Oxide-mediated Disease Control of Bacterial Wilt in Tomato Plants

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Reactive oxygen species (ROS) generation in tomato plants by *Ralstonia solanacearum* infection and the role of hydrogen peroxide (H₂O₂) and nitric oxide in tomato bacterial wilt control were demonstrated. During disease development of tomato bacterial wilt, accumulation of superoxide anion (O₂^{•-}) and H₂O₂ was observed and lipid peroxidation also occurred in the tomato leaf tissues. High doses of H₂O₂ and sodium nitroprusside (SNP) nitric oxide donor showed phytotoxicity to detached tomato leaves 1 day after petiole feeding showing reduced fresh weight. Both H₂O₂ and SNP have *in vitro* antibacterial activities against *R. solanacearum* in a dose-dependent manner, as well as plant protection in detached tomato leaves against bacterial wilt by 10⁶ and 10⁷ cfu/ml of *R. solanacearum*. H₂O₂- and SNP-mediated protection was also evaluated in pots using soil-drench treatment with the bacterial inoculation, and relative 'area under the disease progressive curve (AUDPC)' was calculated to compare disease protection by H₂O₂ and/or SNP with untreated control. Neither H₂O₂ nor SNP protect the tomato seedlings from the bacterial wilt, but H₂O₂ + SNP mixture significantly decreased disease severity with reduced relative AUDPC. These results suggest that H₂O₂ and SNP could be used together to control bacterial wilt in tomato plants as bactericidal agents.

Keywords : antibacterial agents, hydrogen peroxide, nitric oxide, *Ralstonia solanacearum*, tomato bacterial wilt

Bacterial wilt disease caused by *Ralstonia solanacearum* occurs in many plant species and leads to destructive and economical damages, especially, in tomato production in tropical, subtropical and warm temperate regions (Hayward,

1991). To invade and colonize host tissues successfully, soil-borne *R. solanacearum* has developed biochemical weapons; extracellular polysaccharides, cell-wall-degrading enzymes and type III secreted effectors (Macho et al., 2010; Saile et al., 1997; Valls et al., 2006). However, during infection process the bacterium experience reactive oxygen species (ROS) produced in host plants to arrest the bacterial growth (Flores-Cruz and Allen, 2009). Thus, the bacterium should overcome ROS-mediated host defenses by the activation of ROS-scavenging enzymes and the expression of oxidative stress tolerance gene (Colburn-Clifford et al., 2010; Flores-Cruz and Allen, 2011; Loprasert et al., 1996), and lead to compatible interactions between host plants and virulent bacterial strains. Virulence factors originated from *R. solanacearum* have been investigated to understand molecular machinery of the bacterial pathogenesis and to develop efficient disease control strategy in recent decades (Brown and Allen, 2004; Franks et al., 2008; Schell, 2000).

For the disease control of tomato bacterial wilt disease, multidirectional controls including cultural, biological and chemical methods have been applied so far. Chloropicrin was suggested as the most promising chemical for reducing tomato bacterial wilt when it was used as soil fumigant before transplanting (Enfinger et al., 1979). However, it can be irritating and phytotoxic under incomplete vaporization conditions in the treated soil. Antibiotic validamycin A inhibited *in vitro* growth of *R. solanacearum* and delayed symptom development of tomato bacterial wilt (Ishikawa et al., 1996). Commercial chemical pesticides containing copper hydroxide, copper hydroxide-oxadixyl, copper oxychloride-dithianon and streptomycin-validamycin A, delayed tomato bacterial wilt when the bacterial pathogen was inoculated at the same time of pesticide application (Lee et al., 2012). Appearance of pesticide resistance has been concerned although there is no report on the resistance of *R. solanacearum* to antibiotics or chemical pesticides in tomato

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fields. In recent years, soil amendment, essential oils, antagonistic bacteria, plant growth-promoting rhizobacteria (PGPR) and plant defense-activating chemical agents have been tried to control tomato bacterial wilt as environmentally sustainable disease management (Anith et al., 2004; Ji et al., 2005; Nakaune et al., 2012; Nguyen and Ranamukhaarachichi, 2010; Park et al., 2007). More eco-friendly control methods need to be investigated for the integrated management of tomato bacterial wilt.

To minimize damages from pathogen infections, host plants have established sophisticated defense mechanisms including cell wall enforcement, accumulation of pathogenesis-related proteins and accelerated cell death (Deepak et al., 2010; Greenberg et al., 2004; van Loon et al., 2006). Plants can memorize prior pathogen infection and be ready for challenging pathogens. For the establishment of induced and systemic acquired resistance, a variety of plant defense signaling pathways are involved and cross-talked (Grant and Lamb, 2006; Koornneef and Pieterse, 2008; Liu et al., 2011; Pieterse et al., 2009). So far, several small chemicals are known to be *de novo* synthesized and accumulated during pathogenesis and hypersensitive defense responses in host plants. Salicylic acid, jasmonic acid and ethylene are among most distinguished defense signal molecules, but there is limited information whether these are mobile signals for systemic defense or not. More recently, H₂O₂ and nitric oxide have been demonstrated defense signal molecules which play roles in activation of plant resistance against pathogen attacks. H₂O₂ mediated plant defense responses by induced resistance against pathogen infection (Byun and Choi, 2004; Hafez et al., 2012). Elevated endogenous nitric oxide level in plants also resulted in enhanced disease resistance against viral, bacterial, oomycete and fungal pathogens (Chun et al., 2012; Fu et al., 2010; Guo et al., 2004).

Relatively high doses of H₂O₂ and nitric oxide can exert direct antimicrobial activities to kill microbes including plant pathogens. H₂O₂ showed direct antibacterial activity against *Xanthomonas campestris* pv. *vignicola* causing cowpea bacterial blight, and pretreatment of cowpea seeds and seedlings with H₂O₂ reduced the disease severity (Kotchoni et al., 2007). *In vitro* germination of sporangiospores of *Peronospora tabacina* causing tobacco blue mildew was gradually inhibited by increasing H₂O₂ concentration exogenously applied (Peng and Kuć, 1992). Endogenous generation of H₂O₂ in tobacco leaves mediated by peroxidase and cofactors simultaneous applications in the presence of NADH or NADPH significantly reduced disease severity of tobacco blue mold by *P. tabacina* infection (Peng and Kuć, 1992). Nitric oxide treatment inhibited *in vitro* spore germination, sporulation and mycelial growth of *Aspergillus niger*, *Monilinia fructicola* and *Penicillium italicum*, and fruits become rotten by these fungal infection

during postharvest storage and marketing (Lazar et al., 2008). *In vitro* inhibition of spore germination and germ tube elongation was observed in grey mold fungus *Botrytis cinerea* treated with nitric oxide (Lai et al., 2011). Exogenous nitric oxide treatment of tomato fruits delayed the symptom development by direct antifungal activity as well as by indirectly enhancing resistance (Lai et al., 2011). These findings suggest that H₂O₂ and nitric oxide can be prevalently used for disease control including tomato bacterial wilt, although antibacterial activities of H₂O₂ and nitric oxide against *R. solanacearum* and phytotoxic effects on tomato plants have not been demonstrated yet. Genetically modified biocontrol bacterium, *Pseudomonas fluorescens* producing higher content of nitric oxide could confer much stronger disease suppression capability against tomato bacterial wilt (Wang et al., 2005). These studies promoted us to investigate whether H₂O₂ and nitric oxide can be applied directly in tomato plants to control bacterial wilt disease caused by *R. solanacearum*.

In this study, accumulation of ROS in tomato leaf tissues during the bacterial pathogenesis was investigated. *In vitro* antibacterial activity of H₂O₂ and nitric oxide, response of tomato plants to both chemicals and disease control of tomato bacterial wilt by these chemicals were evaluated.

Materials and Methods

Plant growth and bacterial culture. Tomatoes (cv. Cupirang) were raised in commercial soil mixtures in pots (80 mm in diameter, 75 mm in height) in a walk-in growth chamber, where 12-h light illuminated and temperature conditions of day and night were described in previous study (Lee et al., 2012). Four- and five-week-old tomato plants were used for soil drenching and petiole dipping of bacterial inoculation, respectively. *R. solanacearum* strain GMI1000 cultured overnight in TTC (1% peptone, 0.1% casamino acid, 0.5% glucose and 0.0005% 2,3,5-triphenyl tetrazolium chloride broth) at 30°C was centrifuged and the bacterial pellet was resuspended (Nakaune et al., 2012). Bacterial cell number in the suspension was adjusted to 0.15 at OD_{600nm} with a 10⁸ cfu/ml in sterile water using a spectrophotometer. For *in vitro* antibacterial activity test of H₂O₂ and SNP, 40 µl of the bacterial suspension (10⁷ or 10⁸ cfu/ml) was added to 4 ml of TTC broth in 15-ml conical tube and incubated with shaking overnight (17 ± 0.5 h duration) at 30°C. Bacterial growth with or without chemical treatment was indirectly measured spectrophotometrically at OD_{600nm}. Relative growth of the bacteria in response to H₂O₂ and SNP treatments was expressed as percentage (%) compared to that of the untreated bacterial culture.

Plant inoculation and disease assessment. For inoculation

by petiole dipping of tomato leaves, the 3rd leaves were detached from five-week-old plants and were transferred to 10 ml of the bacterial suspension (10^6 and 10^7 cfu/ml) in a 50 ml conical tube with or without antibacterial chemical treatment. Fresh weight (FW) of the detached leaves was measured before and after inoculation. Relative FW of the inoculated leaves were expressed for wilting symptom development with loss of FW.

For soil drench inoculation, 10 ml of bacterial suspension (2×10^8 cfu/ml) was evenly poured into soil mixture in pot (200 ml in volume) containing four-week-old tomato seedlings to make final bacterial concentration 1×10^7 cfu/ml soil mixture. Disease severity was evaluated daily based on disease index and area under disease progress curve (AUDPC) was calculated based on previously described method (Lee et al., 2012).

H₂O₂ and nitric oxide treatments. Different concentration of H₂O₂ (0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1 and 0.2 mM) and SNP nitric oxide releasing agent (0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 mM) was added to the TTC broth at the beginning of the bacterial culture to investigate *in vitro* growth of *R. solanacearum* in response to both chemicals. For plant protection by these chemicals, H₂O₂ (1, 2, 5, 10, 20, 50 and 100 mM) and SNP (0.001, 0.01, 0.1, 0.2 and 0.5 mM) were added to the bacterial suspension before dipping the detached tomato leaves into the bacterial suspension. For whole seedling assay in pots, 10 ml of 200 mM H₂O₂ and/or 10 ml of 4 mM SNP were soil-drenched into the pots containing four-week-old tomato plants in the 200 ml of soil mixture to make final concentrations of 10 mM of H₂O₂ and/or 0.2 mM of SNP in the pots.

Histochemical staining. To observe ROS accumulation and lipid peroxidation of the tomato leaf tissues, different histochemical staining was performed. Superoxide anion (O₂⁻) accumulation was detected as blue-violet color precipitates when the plant tissues were stained with 0.1% nitroblue tetrazolium (NBT) prepared in 10 mM of potassium phosphate (pH 7.8) and 10 mM NaN₃ without vacuum infiltration (Bourmonville and Díaz-Ricci, 2011). H₂O₂ accumulation was visualized as red brown color by floating the leaves on 0.1% 3,3'-diaminobenzidine (DAB) solution overnight (Thordal-Christensen et al., 1997). Lipid peroxidation was detected in the tomato leaf tissues stained by Schiff's reagent indicating pink color development (Wang and Yang, 2005).

Statistical analyses. Data were analyzed for statistical significance by the SAS (Statistical Analysis System, version 9.1, Cary, NC, USA), and means were compared using

Duncan's multiple range tests. Graphing was conducted with SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA).

Results

Oxidative stress and lipid peroxidation of detached tomato leaves infected by *R. solanacearum*. We investigated accumulation of ROS and lipid peroxidation during disease progress in tomato plant inoculated by the wilt-causing bacteria *R. solanacearum*, which is known to overcome oxidative stresses from host plants and followed by bacterial proliferation and colonization (Flores-Cruz and Allen, 2009). Tomato leaves infiltrated with the bacterial suspension showed strong H₂O₂ accumulation (Flores-Cruz and Allen, 2009). However, *R. solanacearum* naturally invades host plants through vascular structures of root, stem and leaf veins (Genin, 2010). In this study, we tried to infect tomato plants through petiole-dipping method to investigate accumulation of O₂⁻ and H₂O₂ in the *R. solanacearum*-inoculated tomato leaves (Fig. 1).

To detect histochemically ROS such as O₂⁻ and H₂O₂ in the tomato tissues, tomato petioles inoculated by petiole dipping were stained with NBT and DAB solutions, respectively. Two different ROS were not generated within 1 day (data not shown) and detected on the infected petioles 3

Fig. 1. Accumulation of reactive oxygen species (ROS) and lipid peroxidation in tomato petioles during pathogenesis of *Ralstonia solanacearum* GMI1000 strain. Superoxide anion (O₂⁻) (dark blue colored), hydrogen peroxide (H₂O₂) (red brown colored) and lipid peroxidation (pink colored) in the tomato tissues were histochemically detected by stainings with NBT, DAB and Schiff's reagent, respectively. -, mock-inoculated; +, inoculated. dpi, days post-inoculation.

Fig. 2. Effect of H₂O₂ and sodium nitroprusside (SNP) nitric oxide releaser on the growth of detached tomato leaves. (A) Different responses of the tomato leaves to H₂O₂ and SNP treatments. Photos were taken at 3 and 5 days after petiole dipping in the different concentrations of H₂O₂ and SNP. (B) Relative fresh weight (FW) of detached tomato leaves treated with different concentrations of H₂O₂ and SNP treatments by petiole dipping at 3 and 5 days. Independent experiments were performed 4 times with similar results. The data points are the mean FW $\pm$ standard errors. Mean separation by Duncan's multiple range test at $P = 0.05$. The same letter above bars represented no significant difference between treatments.

days after bacterial inoculation, at which leaves began to wilt and petioles still remained normal without lipid peroxidation (Fig. 1). The infected petiole tissues were collapsed after 4–5 days after inoculation. Accumulation of O₂⁻ and H₂O₂ and lipid peroxidation were more distinguished as shown by strong color development by NBT and DAB.

Response of tomato leaves by H₂O₂ and SNP treatments.

Detached tomato leaves were treated with H₂O₂ and SNP by petiole feeding, and loss of FW was evaluated whether these chemicals caused phytotoxic damages on the tomato leaves (Fig. 2). Notable tissue damage was not observed in the detached tomato leaves by the whole range of H₂O₂ concentrations (1–100 mM) at 3 and 5 days after treatment

(Fig. 2A). By 0.001 to 0.2 mM of SNP treatment, no distinct visible change was found in the detached tomato leaves at 3 and 5 days after treatment. More than 0.5 mM of SNP caused drastic collapse of leaf tissues. Growth of the detached leaves was demonstrated by measuring FW after H₂O₂ and SNP treatments (Fig. 2B). After 3 days, 2 mM of H₂O₂ slightly increased FW and the FW returned to near value of the normal by 5–10 mM of H₂O₂. Higher dose of H₂O₂ (20–100 mM) gradually decreased the FW to ca. 93.0%. At 5 days, the leaf FW was slightly increased by 2–5 mM of low H₂O₂ but 5–20 mM was not effective to modulate the growth. Much higher doses of H₂O₂ (50–100 mM) limited the tomato leaf growth to ca. 91.8%. SNP application significantly reduced FW at 3 and 5 days.

Fig. 3. Effect of H₂O₂ on *Ralstonia solanacearum* growth and bacterial wilt disease of detached tomato leaves. (A) *In vitro* bacterial growth of *R. solanacearum* in liquid cultures supplemented with different concentration of H₂O₂. Initial inoculum doses for the bacterial cultures were shown in upper (10⁵ cfu/ml) and lower (10⁶ cfu/ml) graphs. Relative bacterial growth in response to H₂O₂ treatment was demonstrated as percentage (%) compared to that in untreated bacterial culture. (B) Relative fresh weight (FW) of detached tomato leaves inoculated with two different *R. solanacearum* doses (10⁶ and 10⁷ cfu/ml) in the absence or presence of H₂O₂ at 3 and 5 days post-inoculation (dpi). Independent experiments were performed 4 times with similar results. The data points are the mean FW $\pm$ standard errors. Mean separation by Duncan's multiple range test at $P = 0.05$. The same letter above bars represented no significant difference between treatments.

Treatment with 0.5 mM began to decrease FW to ca. 76.1% compared to that of untreated control. SNP (1 and 2 mM) much reduced ca. 68.5 and 62.0%, respectively. At 5 days,

drastically decreased FW by 0.5 mM to ca. 56.0% remained when 1 and 2 mM of SNP were treated.

The effect of H₂O₂ on *R. solanacearum* growth *in vitro* and tomato bacterial wilt control. H₂O₂ has been suggested as an antibacterial agent against animal and food-associated microbes (Cerioni et al., 2009; Larsen and White, 1995). Thus, different doses of H₂O₂ were applied to investigate whether it is effective or not to control bacterial wilt of tomato plants (Fig. 3). The *in vitro* bacterial growths were initiated from two different doses 10⁵ and 10⁶ cfu/ml of liquid culture broth, and shown to be regulated by H₂O₂ dose-dependently (Fig. 3A). When the initial bacterial dose was 10⁵ cfu/ml, bacterial growth was slightly arrested to ca. 85.9% by 0.0005 mM of H₂O₂ and the reduced growth was maintained to 0.01 mM of H₂O₂. Higher doses of 0.02 and 0.05 mM H₂O₂ decreased the growth to ca. 76.2 and ca. 33.8% compared to that of untreated control, respectively. No bacterial growth was found in the bacterial cultures treated with 0.1 and 0.2 mM of H₂O₂. Initial bacterial inoculation concentration was increased to 10⁶ cfu/ml for the liquid cultures with or without H₂O₂ treatment. H₂O₂-induced bacterial growth reduction (ca. 89.7%) first occurred by 0.002 mM. H₂O₂ dose of 0.005 mM decreased the bacterial growth to ca. 80.9% and the bacterial reduction was continued to 0.02 mM. Higher doses of 0.05 and 0.1 mM H₂O₂ decreased the growth to ca. 66.7 and ca. 4.7% compared to that of untreated control, respectively.

Higher concentration range of H₂O₂ was required for control of tomato bacterial wilt (Fig. 3B). By the inoculation with *R. solanacearum* suspension (10⁶ cfu/ml), FW of the detached leaves was significantly decreased to ca. 79% without H₂O₂ treatment at 3 days. One mM of H₂O₂ was ineffective to protect the tomato leaves. Higher than 2 mM of H₂O₂ was evidently protective against tomato bacterial wilt, by which bacterial wilt was not found in the inoculated leaves at 3 days. Slight decrease of FW occurred in the detached leaves treated with 2 mM of H₂O₂, but protective effect was still obvious even at 5 days after inoculation. Plant protection by more than 5 mM of H₂O₂ was still distinguished at 5 days after inoculation.

H₂O₂ treatment was also effective to control tomato bacterial wilt caused by the inoculation with relatively higher dose of *R. solanacearum* suspension (10⁷ cfu/ml). Two to five mM of H₂O₂ started to delay FW loss caused by the bacterial inoculation, and 5–10 mM range was the most effective for plant protection at 3 days after inoculation. However, 50–100 mM of H₂O₂ rather reduced FW of detached leaves inoculated. Protective effect of 2 mM of H₂O₂ at 3 days after inoculation diminished along with the disease progress at 5 days after inoculation. Higher than 5 mM of H₂O₂ was still effective to control tomato bacterial wilt.

Fig. 4. Effect of sodium nitroprusside (SNP) nitric oxide releaser on *Ralstonia solanacearum* growth and bacterial wilt disease of detached tomato leaves. (A) *In vitro* bacterial growth of *R. solanacearum* in liquid cultures supplemented with different concentration of SNP. Initial inoculum doses for the bacterial cultures were shown in upper (10⁵ cfu/ml) and lower (10⁶ cfu/ml) graphs. Relative bacterial growth in response to SNP treatment was demonstrated as percentage (%) compared to that in untreated bacterial culture. (B) Relative fresh weight (FW) of detached tomato leaves inoculated with two different *R. solanacearum* doses (10⁶ and 10⁷ cfu/ml) in the absence or presence of SNP at 3 and 5 days post-inoculation (dpi). Independent experiments were performed 4 times with similar results. The data points are the mean FW $\pm$ standard errors. Means followed by the same letter are not significantly different at 5% level by Duncan's multiple range test.

The effect of nitric oxide on *R. solanacearum* growth *in vitro* and tomato bacterial wilt control. The growth of *R. solanacearum* was also examined *in vitro* during liquid culture supplemented with different concentrations of SNP

nitric oxide donor spectrophotometrically (Fig. 4A). Two different initial bacterial numbers (10⁵ and 10⁶ cfu/ml) were used. When the lower bacterial inoculum dose was 10⁵ cfu/ml, the bacterial growth was decreased to ca. 86.3% even at least 0.0005 mM SNP treatment. Increasing SNP concentration gradually arrested the bacterial growth during its culture, and ca. 10.2% of bacterial growth was observed with 2 mM supplement. When the relatively higher bacterial inoculum dose (10⁶ cfu/ml) was cultured in the beginning, decrease of bacterial growth was more or less retarded. However, increasing SNP of more than 0.1 mM also distinctly suppressed the bacterial growth.

To investigate the potential of SNP as a protection agent for tomato bacterial wilt control, tomato petiole dipping inoculation was performed (Fig. 4B). With a concentration of 0.001 mM, SNP could not provide disease control efficacy at 3 and 5 days after inoculation with 10⁶ cfu/ml bacterial dose. Treatment with higher than 0.01 mM of SNP began to show protective effect against bacterial wilt, and the protection reached plateau at 0.2 mM. Higher SNP dose of 0.5 mM led to slight decrease of the FW of the detached leaves, presumably due to phytotoxic effect of high SNP dose. Low dose of SNP 0.1 mM did not reduce disease severity at 5 days after inoculation, but disease protection was achieved by relatively higher SNP concentration of 0.2–0.5 mM. By the relatively higher bacterial inoculation (10⁷ cfu/ml), protective effect of SNP slightly attenuated. At least higher than 0.1 mM of SNP was effective to decrease wilting symptom at 3 and 5 days after inoculation. But 0.5 mM of SNP rather decreased FW of the detached leaves.

Synergistic effect of H₂O₂ and nitric oxide on *in vitro* *R. solanacearum* growth and tomato bacterial wilt control.

Accelerated cellular damages of *R. solanacearum* occurred by simultaneous treatment with different dose combinations of H₂O₂ and SNP when 10⁵ and 10⁶ cfu/ml of the bacterial doses for liquid culture was initiated (Fig. 5A). Bacterial culture began with 10⁵ cfu/ml was affected by different combinations of H₂O₂ and SNP. Increasing doses of SNP, 0.001, 0.01, 0.1 and 1 mM, in the absence of H₂O₂, caused reduced bacterial growth showing ca. 91.1, 84.6, 67.7, 17.9%, respectively, compared to that in untreated control culture. By 0.001 mM of H₂O₂ alone, bacterial growth was slightly but significantly reduced to ca. 95.8% of the untreated control. However, treatment with increasing doses of SNP, 0.001, 0.01, 0.1 and 1 mM, in the presence of 0.001 mM of H₂O₂ did not alter the bacterial growth inhibition found in the bacterial cultures treated with different concentrations of SNP alone. By treatment with 0.01 mM of H₂O₂, the bacterial growth much more arrested by 0.01–1 mM of SNP was distinctly inhibited with ca. 74.5, 37.5 and 10.0% of relative growth, respectively. Higher dose of H₂O₂

Fig. 5. Protective effect of H₂O₂ and/or SNP on bacterial wilt disease of tomato seedling in pots. (A) *In vitro* synergistic bactericidal effect of H₂O₂ and SNP treatment on the growth of *Ralstonia solanacearum* in liquid cultures. Initial inoculum doses for the bacterial cultures were shown in upper (10⁵ cfu/ml) and lower (10⁶ cfu/ml) graphs. (B) Wilting symptoms of tomato plants in pots soil drench-inoculated by *R. solanacearum* in the presence or absence of H₂O₂, SNP and H₂O₂ + SNP treatment. Photo was taken 10 days after bacterial inoculation dpi, days post-inoculation. (C) Relative area under disease progress curve (AUDPC) used to evaluate tomato bacterial wilt by *R. solanacearum* for 14 days after soil-drench inoculation. Independent experiments were performed 4 times with similar results. The data points are the mean AUDPC $\pm$ standard errors. Mean separation by Duncan's multiple range test at $P = 0.05$. The same letter above bars represented no significant difference between treatments.

(0.1 mM) completely suppressed the bacterial growth irrespective of SNP concentrations. Similar synergistic growth inhibition was also demonstrated in liquid cultures started with 10⁶ cfu/ml. However, growth inhibitions by H₂O₂ and/or SNP treatment was more or less lower than those found in liquid cultures initiated with 10⁵ cfu/ml of inoculum. Plant protection against the bacterial wilt by H₂O₂ and/or nitric oxide was evaluated using four-week-old tomato seedlings in pots (Fig. 5B and 5C). On the basis of results shown in Fig. 3 and Fig. 4, we selected 10 mM H₂O₂ and/or

0.2 mM SNP solutions were evenly applied just before bacterial suspension was soil-drenched in pots, and relative AUDPC was calculated based on disease severities evaluated daily for 2 weeks. Either H₂O₂ or SNP treatment alone in the soil mixture was not effective to suppress the bacterial wilt compared to disease severity of untreated plants. By contrast, H₂O₂ and SNP mixture significantly delayed disease development of tomato bacterial wilt.

Discussion

To reduce tomato bacterial wilt caused by *R. solanacearum*, various control methods have been applied in greenhouses and fields. Chemical pesticides have been suggested for a long time for soil sterilization before tomato planting or treatment during plant growth (Enfinger et al., 1979; Lee et al., 2012). Recently, antibacterial activity of different plant essential oils against *R. solanacearum* was demonstrated and it was applied to tomato plants against tomato bacterial wilt. Clove oil was most effective to decrease the disease severity of tomato bacterial wilt in our previous study (Lee et al., 2012). In this study, we observed accumulation of ROS in the tomato leaf tissues infected by *R. solanacearum* and evaluated the potential of H₂O₂ and nitric oxide for tomato bacterial wilt control.

Induced ROS generation has been well demonstrated in diverse plant-pathogen interactions (Lamb and Dixon, 1997). In general, rapid and strong oxidative burst during incompatible interaction contributed to hypersensitive cell death and disease resistance of host plants, whereas gradual and minute production of ROS in the plant tissues during compatible interaction did not attribute to disease resistance (Choi et al., 2007; Wang et al., 2007). Lipid peroxidation was also one of the hallmarks indicating cellular damage especially in membrane structure (Deighton et al., 1999; Jalloul et al., 2002). H₂O₂ accumulation and lipid peroxidation were differentially found in susceptible and resistant tomato cultivars inoculated by *R. solanacearum* (Mandal et al., 2011). Increase in H₂O₂ was much faster and stronger in the resistant response, whereas lipid peroxidation occurred much preferentially in the susceptible response. These indicated that H₂O₂ accumulation confers disease resistance of tomato plants against *R. solanacearum*. But dramatic increase of lipid peroxidation in the susceptible cultivar was cellular sign for plant membrane degeneration by disease progress. During bacterial pathogenesis in susceptible tomato cultivar by *R. solanacearum*, we confirmed the accumulation of ROS (O₂⁻ and H₂O₂) and the occurrence of lipid peroxidation histochemically in the tomato detached leaves of tomato during the susceptible response. ROS accumulation and lipid peroxidation were only found in the relatively late infection stage, at which plant leaf tissues were collapsed

by the progressive infection and bacterial colonization. ROS contents in the infected susceptible plant tissues in this study may be not enough to hinder the bacterial proliferation, and thus, the invading bacteria can overcome the low level of ROS toxicity using their own antioxidant system. It led a notion that excessive ROS exogenously applied can disarm the bacterial antioxidant machinery and reduce tomato bacterial wilt symptom.

H₂O₂ treatment inhibited *in vitro* growth of various phytopathogenic bacteria (*X. campestris* pv. *vignicola*), oomycete (*P. tabacina*) and fungi (*B. cinerea*, *Cladosporium cucumerinum*, *Colletotrichum lagenarium*, *Rhizopus stolonifer*, *Penicillium digitatum* and *P. italicum*) (El-Mougy et al., 2008; Kotchoni et al., 2007; Peng and Kuć, 1992). Mutants of *Pseudomonas syringae* pv. *tomato* and *X. campestris* pv. *campestris* which lost catalase-peroxidase KatG activity were more sensitive to even low levels of H₂O₂ than wild-type strains of the bacteria (Gou et al., 2012; Jittawuttipoka et al., 2009). Rice blast fungus *Magnaporthe oryzae* and citrus green mold *P. digitatum* established plant defense-induced H₂O₂-detoxifying mechanism for self-protection and full virulence in their host plant tissues (Huang et al., 2011; Macarasin et al., 2007). To nullify H₂O₂-detoxifying ability of *R. solanacearum*, excess H₂O₂ was added during the bacterial culture in this study. H₂O₂ have shown *in vitro* antibacterial activity to *R. solanacearum* in a dose-dependent manner, indicating it has the potential of bacterial wilt control in tomato plants. Indeed, a range of 5–100 mM H₂O₂ could effectively reduce the bacterial wilt in the detached tomato leaves inoculated by the petiole dipping method (Fig. 3B). It was notably interesting that H₂O₂ concentration range effective to reduce bacterial wilt of the detached tomato leaves was remarkably higher than that for *in vitro* bacterial growth inhibition. Ineffective antibacterial activity of the low doses of H₂O₂ applied, enhanced bacterial growth and reduced plant protection efficiency can be mediated by unidentified host factors originated from petiole exudates of the detached tomato leaves. It is supported by the fact that a variety of *R. solanacearum* genes such as virulence genes *pehR*, *vsrB* and *hrcC* have been specifically induced in tomato plants (Brown and Allen, 2004). These bacterial gene expressions might be activated by the tomato petiole exudates to evade or overcome oxidative stress condition by H₂O₂, which could let bacterial growth and virulence promoted but wilting symptom more progressed. Treatment with 10 mM of H₂O₂ to detached tomato leaves by petiole feeding significantly protected the leaves to the bacterial invasion. However, the same dose of H₂O₂ soil-drenched in the pots was not effective to reduce the bacterial wilt symptom. It may be caused by insufficient contact between H₂O₂ and bacteria in the soil environment, or by different chemical compositions such as amino acids, organic acids

and sugars of leaf petiole and root exudates (Kamilova et al., 2006; Lugtenberg et al., 1999).

High dose of nitric oxide may cause nitrosative stress in phytopathogenic bacteria (*Erwinia chrysanthemi*) and fungi (*A. niger*, *B. cinerea*, *M. fructicola*, *P. expansum* and *P. italicum*) as shown by reduced growths *in vitro* (Boccaro et al., 2005; Lazar et al., 2008). Nitric oxide donor SNP treatment also suppressed *R. solanacearum* growth *in vitro* in this study, suggesting that SNP may reduce tomato bacterial wilt by direct antibacterial effect. Response of the detached tomato leaves treated with SNP without *R. solanacearum* inoculation was also examined. More than 0.5 mM of SNP showed phytotoxic effect and significantly reduced plant growth of the detached leaves. SNP significantly decreased bacterial wilt in detached tomato leaves inoculated by petiole dipping. However, the 0.2 mM of SNP was not enough for the protection against tomato bacterial wilt in pots with soil environments. It also may be derived from insufficient contact between SNP and the bacteria, or detoxification of SNP by the phytopathogenic bacteria in the soil mixtures.

Interestingly, treatment with H₂O₂ and SNP together significantly increased *in vitro* bactericidal activity as well as protective effect against bacterial wilt in tomato plants in the soil mixtures. Nitric oxide donor diethylenetriamine nitric oxide inhibited *in vitro* growth of *Escherichia coli* and accelerated H₂O₂-induced cell death of the bacteria, implying distinct cellular target proteins of H₂O₂ and nitric oxide in the bacteria (Brandes et al., 2007; Pacelli et al., 1995). H₂O₂ and SNP may exert their antibacterial activities against *R. solanacearum* via distinctly different mode-of-action, which support the idea that H₂O₂ and SNP work together to suppress the bacterial growth of *R. solanacearum* and following tomato wilting synergistically. Activation of defense responses of tomato plants treated with H₂O₂ and/or SNP against *R. solanacearum* invasion remains investigated in further studies. The use of H₂O₂ and SNP together will be obviously useful in agricultural field for tomato protection against bacterial wilt through bactericidal activity and/or probable defense activation. Additionally, increases in dose of H₂O₂ and/or SNP without phytotoxic effects also remains to be evaluated in the tomato plants for the better protection efficiency against the bacterial wilt.

Taken together, both H₂O₂ and nitric oxide had direct bactericidal effects against *R. solanacearum*, which is one of the prominent activities to protect tomato plants against bacterial wilt. However, we cannot exclude the possibility that H₂O₂ and/or nitric oxide can be associated in the enhanced disease resistance to the tomato bacterial wilt by defense activating or priming in tomato plants, because it is evident that these two chemicals played roles as plant defense signal molecules in many plant species. Synergistic effect of H₂O₂ and nitric oxide for controlling tomato

bacterial wilt may be applicable for other plant disease management. Application of H₂O₂ and nitric oxide together with other disease controlling agent like clove oil treatment shown in our previous study should be further considered for better integrated management of tomato bacterial wilt.

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