RESEARCH ARTICLE

In vitro Conidial Germination and Mycelial Growth of *Fusarium oxysporum* f. sp. *fragariae* Coordinated by Hydrogen Peroxide- and Nitric Oxide-signalling

Yu Jin Do[1,†], Do Hyeon Kim[1,†], Myung Sung Jo[1], Dong Gi Kang[1], Sang Woo Lee[1], Jin-Won Kim[2] Jeum Kyu Hong[1,*]

[1]Department of Horticultural Science, Gyeongnam National University of Science and Technology (GNTech), 33 Dongjin-ro, Jinju 52725, Korea

[2]Department of Environmental Horticulture, The University of Seoul, Seoul 02504, Korea

*Corresponding author: jkhong@gntech.ac.kr

[†]These authors contributed equally to this work.

ABSTRACT

Chemicals related to hydrogen peroxide (H_2O_2) and nitric oxide (NO) generations were exogenously applied to *Fusarium oxysporum* f. sp. *fragariae* (*Fof*) causing Fusarium wilt disease in strawberry plants, and regulations of *in vitro* conidial germination and mycelial growth of the fungus by the chemical treatments were evaluated. H_2O_2 drastically reduced the conidial germination of *Fof* in a dose-dependent manner, and treatment with 3-amino-1,2,4-triazole (3-AT) catalase inhibitor also led to dose-dependent inhibition of conidial germination but relatively moderately. Gradual decreases in mycelial growth of *Fof* were found by high concentrations of H_2O_2, whilst exogenous 3-AT slightly increased the mycelial growth. Increasing sodium nitroprusside (SNP) NO donor, N^G-nitro-l-arginine methyl ester (L-NAME) NO synthase (NOS)-inhibitor and tungstate nitrate reductase (NR) inhibitor led to dose-dependent reductions in conidial germination of *Fof* in quite different levels. SNP conversely increased the mycelial growth but increasing L-NAME moderately decreased the mycelial growth. Tungstate strongly enhanced mycelial growth. Differentially regulated *in vitro* mycelial growths of *Fof* were demonstrated by SNP, L-NAME and tungstate with or without H_2O_2 supplement. Superoxide anion production was also regulated during the mycelial growth of *Fof* by nitric oxide. These results show that H_2O_2 and NO-associated enzymes can be suggested as fungal growth regulators of *Fof* as well as eco-friendly disease-managing agents in strawberry production fields.

Keywords: Antifungal, *Fusarium oxysporum* f. sp. *fragariae*, Nitric oxide, Reactive oxygen species

pISSN : 0253-651X
eISSN : 2383-5249

Kor. J. Mycol. 2019 September, 47(3): 219-32
https://doi.org/10.4489/KJM.20190026

Received: September 02, 2019
Revised: September 20, 2019
Accepted: September 22, 2019

INTRODUCTION

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *fragariae* (*Fof*) causes crown and root rots of strawberry plants and progressive wilting, followed by severe fruit losses. The germinated conidia of *Fof*

invaded roots and growing fungal hyphae colonised vascular tissues of strawberry roots, which caused vessel blockage, wilting and subsequent death of the plants [1]. During runner propagation in strawberry nursery beds, daughter plants could be infected by the fungal pathogen originated from diseased mother plants [2]. Although there is an increasing threat of Fusarium wilt to strawberry fruit production worldwide, only limited information has been available for fungal pathogenesis by *Fof* and its control. Differentially accumulated proteins were identified in mycelia of several *Fof* isolates showing different virulence on the strawberry plants to reveal the *Fof* fungal pathogenesis and to suggest novel disease control method [3].

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) levels were endogenously regulated in phytopathogenic fungi and involved in the fungal development such as conidial germination, mycelial growth and conidation, as well as in their virulence on the host plants [4-7]. However, only a little evidence on the ROS and RNS during the fungal development of *F. oxysporum* has been shown. Transcription factor SNT2 of *F. oxysporum* f. sp. *melonis* was related to inducible superoxide dismutase activity in response to H_2O_2 regulation of alternative oxidation for the fungal respiration [8].

ROS and RNS were accumulated in different tissues of plant species infected by various pathovars of *Fusarium oxysporum*. Temporal accumulation of H_2O_2 was found in cucumber root tissues inoculated by *F. oxysporum* f. sp. *cucumerinum*, yellow lupine seeds inoculated by *F. oxysporum* f. sp. *lupini*, banana plantlets inoculated by *F. oxysporum* f. sp. *cubense,* and flax suspension cells inoculated by *F. oxysporum* f. sp. *lini* [9-12]. An increase in H_2O_2 content in common bean root tissues was found only in resistant cultivar but not in susceptible cultivar against *F. oxysporum* f. sp. *phaseoli* inoculation [13]. Nitric oxide (NO) accumulation was only detected in *Arabidopsis* roots inoculated by *F. oxysporum* [14]. It remains to be elucidated whether each ROS and RNS inducible during the fungal pathogenesis is detrimental or beneficial to each invading fungal pathogen. Plant-invading fungal species have to overcome or utilise inducible ROS and/or RNS in host plant tissues to establish stable infection [4].

Exogenous application of ROS and RNS has shown direct *in vitro* antimicrobial activity against several phytopathogenic fungi and/or mediated induced host plant defences, which could pave eco-friendly ways for disease management of economically important crops. Pretreatment with H_2O_2 reduced anthracnose lesion development on cucumber leaves challenge-inoculated by *Colletotrichum orbiculare* [15]. Tomato seeds and seedlings treated with H_2O_2 have shown reduced Fusarium wilt under protected house and field conditions [16-18]. Seed soaking faba beans in H_2O_2 solution decreased damping-off and root rot by *F. oxysporum* infections in greenhouses and fields during growing seasons after sowing [19]. NO also has shown direct *in vitro* antimicrobial activity against several phytopathogenic fungi and/or mediated crop protection via enhancing host defence responses. NO donor sodium nitroprusside (SNP) suppressed *in vitro* conidial germination and mycelial growth of *Fusarium sulphureum* and prevented dry rot in potato tubers [20]. SNP-treated fungal spores of *F. oxysporum* f. sp. *quitoense* have shown reduced *in vitro* germination, and SNP also regulated plant defence-related gene expressions of *Solanum quitoense* leaves during the fungal pathogenesis as well [21]. Treatment with NO led to reduced brown rot and Rhizopus rot of harvested peach and tomato fruits infected by *Monilinia fructicola* and *Rhizopus nigricans*, respectively [22, 23]. These findings suggest that fungal diseases can be managed environmentally friendly manner by

exogenous H_2O_2 and NO application during crop production and post-harvest periods. However, limited fungal growth of *Fof* and/or controlled strawberry Fusarium wilt by regulating ROS and RNS is rarely found.

In this study, we applied the H_2O_2 and 3-amino-1,2,4-triazole (3-AT) to *Fof* to investigate the role of exogenous and endogenous H_2O_2 during the *in vitro* conidial germination and mycelial growth. NO donor SNP, NO synthase (NOS)-inhibitor N^G-nitro-l-arginine methyl ester (L-NAME) and nitrate reductase (NR)-inhibitor tungstate were applied to examine whether the *in vitro* mycelial growth was also regulated by nitric oxide contents. To investigate coordinated regulation of the *Fof* mycelial growth by H_2O_2 together with NO, *Fof* was treated with SNP, L-NAME and tungstate in the absence or presence of H_2O_2 during the mycelial growth. H_2O_2 and superoxide anion productions were analysed in *Fof* mycelia and its growing media histochemically during the differential mycelial growth.

MATERIALS AND METHODS

Fungal culture

Fusarium oxysporum f. sp. *fragariae* (*Fof*) isolate SFW-10 was cultured on 1/2-strength potato dextrose agar (PDA) media at 25°C under dark condition as previously described [24].

Chemical treatments

Hydrogen peroxide (H_2O_2) and 3-amino-1,2,4-triazole (3-AT) were used as one of ROS and an inducer of endogenous H_2O_2 level by inhibiting activity of catalase decomposing H_2O_2, respectively. SNP was used as an exogenous NO donor [20, 25, 26]. *N*-Nitro-*L*-arginine methyl ester (L-NAME) and sodium tungstate dihydrate (tungstate) were prepared as inhibitors of fungal NO synthase and nitrate reductase (NR), respectively, which were suggested to be involved in separate NO biosynthetic pathways in diverse living organisms [27, 28].

In vitro conidial germination and mycelial growth of *Fof*

In vitro conidial germination of *Fof* was analysed on glass slides according to modified methods previously described in our studies [24, 29]. Concentration of conidial suspension was adjusted to 5×10^5 conidia/ml using a haemacytometer under a light microscope. Different concentrations of chemicals were added and gently mixed to the conidial suspension, and 4 drops of the suspension (20 μl each) were placed on two glass slides without cover glass under humid condition in plastic square dishes. After incubation at 25°C for 10 hours under darkness and moist conditions, both ungerminated and germinated conidia were stained with lactophenol-trypan blue solution and counted under a light microscope. Relative conidial germination by chemical treatments was expressed as percentage (%) compared to that in the untreated control.

For analysing fungal mycelial growth affected by the chemical treatments, mycelial discs (5 mm in diameter) were cut from growing edge of 7-day-old fungal colonies and placed upside down at the center

of 1/2-strength PDA supplemented with different concentrations of the chemicals. Colony diameters of *Fof* were measured 7 days after culture at 25°C under darkness. Relative fungal colony diameters by the chemical treatments were expressed as percentage (%) compared to that in the untreated control.

Histochemical staining of H_2O_2 and superoxide anion in *Fof* colonies and growth media

Accumulation of H_2O_2 and superoxide anion was histochemically detected in the fungal colony and growth media by staining with 3,3-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT), respectively. Each staining solution was prepared according to methods of our previous study [30]. Staining solution was poured into culture plates and stained for 2 hours. To cease the colour development, the plates were rinsed with distilled water 3 times. Each plate was photographed for visible staining reactions. Culture media without fungal inoculation were stained with DAB and NBT solution to distinguish intracellular or secreted production of fungal H_2O_2 and superoxide anion from background of culture media. Red-brown and blue-purple colours indicate presence of H_2O_2 and superoxide anion, respectively.

Statistical analyses

An analysis of variance (ANOVA) was conducted to determine the effects of treatments with chemicals on the fungal growth. 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).

RESULTS

H_2O_2-regulated *in vitro* conidial germination of *Fof*

Both exogenous H_2O_2 and 3-AT decreased *in vitro* conidial germination of *Fof* in different levels (Fig. 1). The lowest concentration (0.5 mM) of H_2O_2 tested in this study was enough to suppress the conidial germination to ca. 64.6% compared to that in mock-treated control (ca. 89.5%). More than 1 mM of H_2O_2 completely suppressed conidial germination. Conidial germination was arrested to ca. 68.5% by at least 10 mM of 3-AT, and highest 3-AT dose (20 mM) led to much decreasing conidial germination to ca. 35.7%.

H_2O_2-regulated *in vitro* mycelial growth of *Fof*

H_2O_2 and 3-AT regulated mycelial growth of *Fof* differently (Fig. 2). Increasing H_2O_2 concentrations to 0.5, 1, 2, 5, 10 and 20 mM resulted in gradual reductions in relative mycelial growths to 100.9, 96.8, 95.2, 88.2, 78.9 and 64.3%, respectively, compared to that in mock-treated control. By contrast, 3-AT supplement in the media did not cause drastic changes in mycelial growth of *Fof*, but the fungal growth was significantly altered. The lowest concentration (0.5 mM) of 3-AT slightly promoted the mycelial growth (ca. 109.1%) and much mycelial growth was found to be ca. 112.7% by both 1 and 2 mM of 3-AT. Increasing 3-AT doses to 5, 10 and 20 mM slightly reduced to ca. 108.9, 105.4 and 104.2%, respectively. But these three

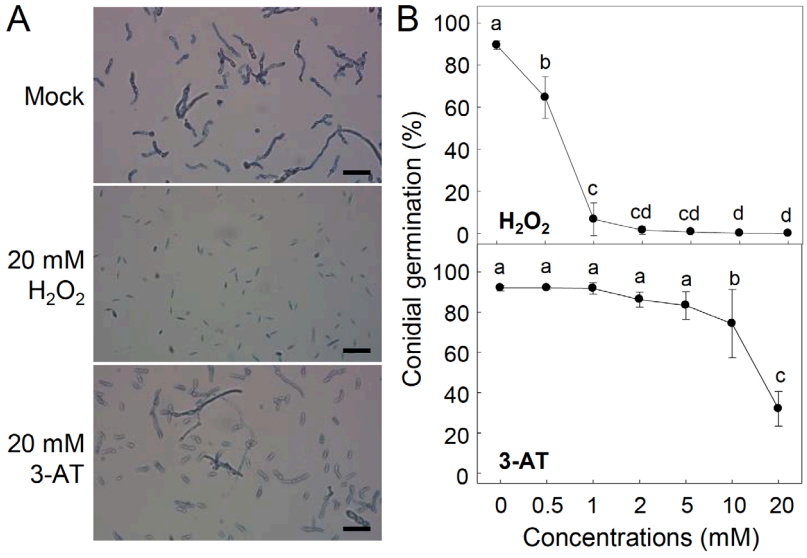

Fig. 1. Regulation of *in vitro* conidial germination of *Fusarium oxysporum* f. sp. *fragariae* by hydrogen peroxide (H_2O_2). (A) Germinated conidia of *F. oxysporum* f. sp. *fragariae* isolate SFW-10 on glass slides after treatment with 20 mM of ROS-related chemicals H_2O_2 and 3-amino-1,2,4-triazole (3-AT) were observed under a light microscope. (Scale bars: 25 μm). (B) Relative conidial germination (%) regulated by the chemical treatments was shown as a percentage of germinated conidia treated with different doses (0, 0.5, 1, 2, 5, 10 and 20 mM) of H_2O_2 and 3-AT compared to control. Error bars represent standard errors of means of five 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 the bars represented no significant difference between treatments.

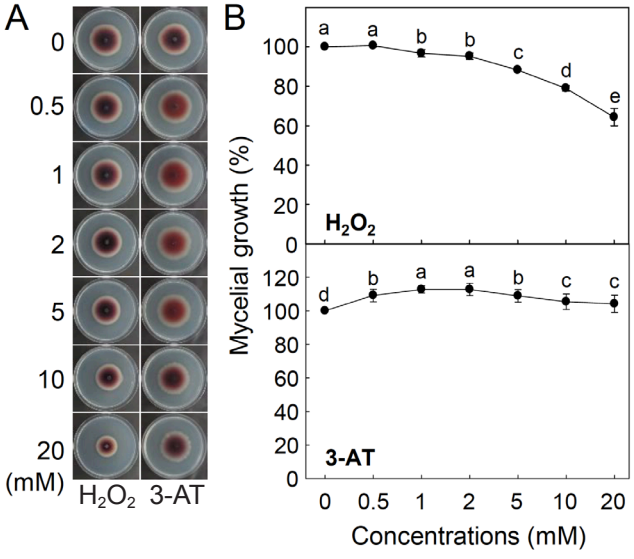

Fig. 2. Regulation of *in vitro* mycelial growth of *Fusarium oxysporum* f. sp. *fragariae* inhibited by hydrogen peroxide (H_2O_2). (A) Colony formation of *F. oxysporum* f. sp. *fragariae* isolate SFW-10 on PDA media supplemented with different doses (0, 0.5, 1, 2, 5, 10 and 20 mM) of ROS-related chemicals H_2O_2 and 3-amino-1,2,4-triazole (3-AT) was investigated. (B) Relative mycelial growths regulated by H_2O_2 and 3-AT are shown as a percentage of fungal colonies on PDA. Error bars represent standard errors of means of five 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 the bars represented no significant difference between treatments.

doses were still efficient to enhance the fungal mycelial growth compared to the untreated control.

NO-regulated *in vitro* conidial germination of *Fof*

Treatments with SNP, L-NAME and tungstate showed different reduction efficacies during the *in vitro* conidial germination (Fig. 3). SNP resulted in a linear decrease in the conidial germination in a dose-dependent manner. Increasing SNP concentrations to 0.5, 1, 2, 5, 10 and 20 mM lowered the conidial germination to ca. 80.0, 75.3, 68.7, 63.0, 53.6 and 49.5% compared to that of mock-treated control (ca. 89.3%). L-NAME treatment slightly reduced the conidial germination. Two mM of L-NAME began to inhibit the conidial germination to ca. 85.4%, and increasing L-NAME concentrations to 20 mM did not alter the conidial germination. Applied tungstate led to a drastic suppression of the conidial germination. At least 0.5 mM of tungstate was enough to reduce the conidial germination by ca. 69.7%, and 1 and 2 mM of tungstate suppressed the conidial germination to ca. 45.1 and 23.6%, respectively. Increasing tungstate

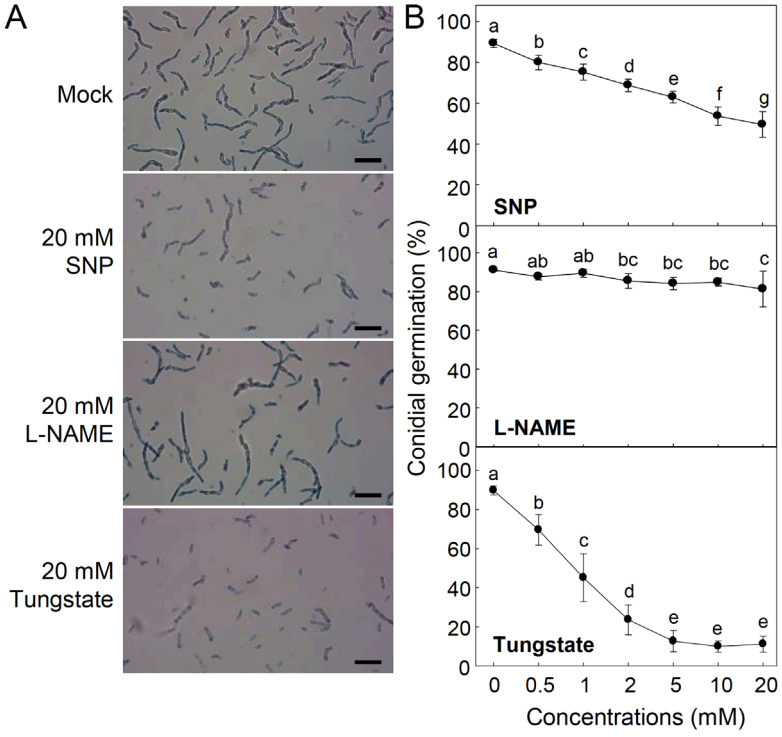

Fig. 3. Regulation of *in vitro* conidial germination of *Fusarium oxysporum* f. sp. *fragariae* by nitric oxide (NO) signalling. (A) Germinated conidia of *F. oxysporum* f. sp. *fragariae* isolate SFW-10 on glass slides after treatment with 20 mM of NO-related chemicals sodium nitroprusside (SNP) NO donor, *N*-nitro-*L*-arginine methyl ester (L-NAME) NO synthase inhibitor and sodium tungstate dihydrate (tungstate) nitrate reductase inhibitor were observed under a light microscope. (B) Relative conidial germination (%) regulated by the chemical treatments was shown as a percentage of germinated conidia treated with different doses (0, 0.5, 1, 2, 5, 10 and 20 mM) of NO-related chemicals compared to control. Error bars represent standard errors of means of five independent experimental replications. Means followed by the same letter are not significantly different at 5% level by least significant difference test. The same letter above the bars represented no significant difference between treatments.

concentrations to 5, 10 and 20 mM led to a decrease in the conidial germination to ca. 12.6, 10.0 and 11.1, respectively, but no significant difference was found among the germinations of conidia treated with the relatively higher concentration.

NO-regulated *in vitro* mycelial growth of *Fof*

The mycelial growth of *Fof* was evaluated in response to exogenous SNP, L-NAME and tungstate, which are NO production-associated chemicals (Fig. 4). SNP resulted in dose-dependent acceleration of mycelial growth (Fig. 4A). The purple colour of the fungal colony on the mock-treated media gradually receded by increasing SNP concentrations and became dark orange coloured by the higher SNP concentrations. Mycelial growth of *Fof* was distinctly enhanced as SNP concentrations increased (Fig. 4B). At least 0.5 mM of SNP significantly promoted mycelial growth by ca. 123.9%, and then increasing SNP to 1 - 2 mM maintained the enhanced mycelial growth. Increases in SNP concentrations to 5, 10 and 20 mM significantly increased the mycelial growth ca. 129.1, 130.4 and 131.7%. By contrast, L-NAME slightly

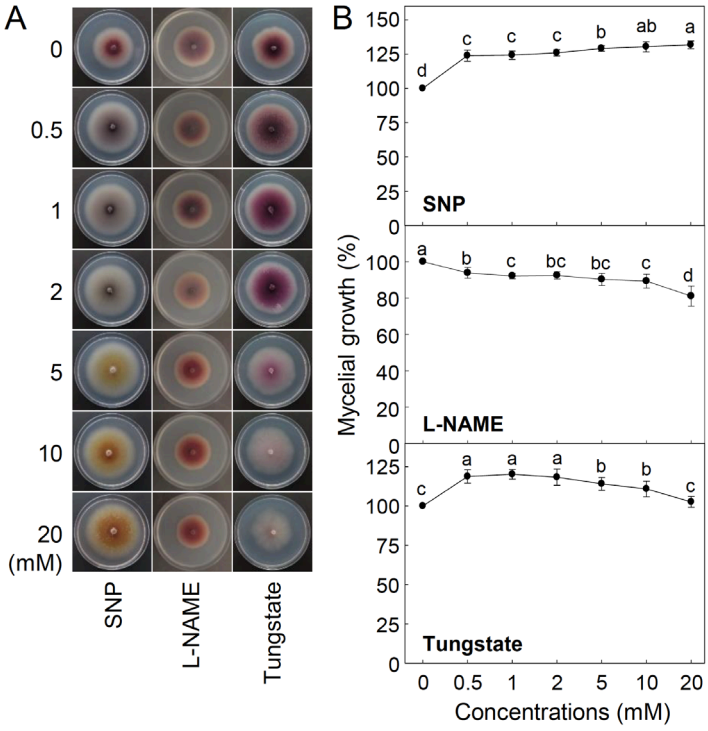

Fig. 4. Regulation of *in vitro* mycelial growth of *Fusarium oxysporum* f. sp. *fragariae* inhibited by nitric oxide (NO) signalling. (A) Colony formations of *F. oxysporum* f. sp. *fragariae* isolate SFW-10 on PDA media supplemented with different doses (0, 0.5, 1, 2, 5, 10 and 20 mM) of NO-related chemicals sodium nitroprusside (SNP) NO donor, *N*-nitro-*L*-arginine methyl ester (L-NAME) NO synthase inhibitor and sodium tungstate dihydrate (tungstate) nitrate reductase inhibitor were investigated. (B) Relative mycelial growths regulated by the chemical treatments are shown as a percentage of fungal colonies on PDA. Error bars represent standard errors of means of five 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 the bars represented no significant difference between treatments.

reduced the fungal mycelial growth without drastic changes in the fungal colony colours (Fig. 4). The mycelial growth was not altered by 0.5 mM of L-NAME, and then gradual retardation in the mycelial growth was found to be 93.1, 94.8, 96.4, 92.8, 91.2 and 86.4% by 1, 2, 5, 10 and 20 mM of L-NAME, respectively. Different tungstate concentrations differentially regulated the mycelial growth but the purple colony colour turned pale (Fig. 4). The lowest concentration of tungstate (0.5 mM) markedly enhanced the mycelial growth to ca. 118.8% compared to the mock-treated control, and both 1 and 2 mM of tungstate also led to the similar effects on the mycelial growth. But increasing tungstate to 5 and 10 mM reduced the mycelial growth to ca. 114.1 and 110.9%, respectively. The mycelial growth of *Fof* returned to the control level by the highest dose (20 mM) of tungstate.

Coordinately regulated *in vitro* mycelial growth of *Fof* by H_2O_2 and NO

The effect of NO on the H_2O_2-mediated growth inhibition of the *Fof* was investigated by co-treatment with SNP, L-NAME or tungstate during the fungal growth (Fig. 5). In the absence of antifungal H_2O_2 (10 mM), mycelial growth of *Fof* was enhanced by SNP (2 mM) and tungstate (2 mM) by ca. 127.0% and ca. 119.0%, respectively, whereas L-NAME (2 mM) slightly retarded the mycelial growth of *Fof* by ca. 92.5% as demonstrated in Fig. 4. Treatment with 10 mM of H_2O_2 alone led to a decrease in the mycelial growth of *Fof* by ca. 80.1% as demonstrated in Fig. 2. Combined treatment with H_2O_2 (10 mM) and SNP (2 mM) resulted in distinct reduction in the mycelial growth of *Fof* by ca. 3.7%. L-NAME (2 mM) together with H_2O_2 (10 mM) moderately limited the mycelial growth (ca. 71.2%). A supplement with tungstate (2 mM) in the H_2O_2 (10 mM)-containing media compromised tungstate-induced mycelial growth promotion.

During the differential growth responses of *Fof* to H_2O_2 and different NO levels, intracellular production or secretion of reactive oxygen species were investigated by histochemical staining (Fig. 5C, 5D). H_2O_2 accumulation was not distinctly found in *Fof* colonies treated with SNP, L-NAME and tungstate in the absence or presence of H_2O_2 (Fig. 5C). However, dark red-brown colour development was observed in culture media supplemented with both SNP and H_2O_2 regardless of the fungal inoculation and culture. A relatively bright red-brown colour was developed in culture media supplemented with both SNP and tungstate regardless of the fungal inoculation and culture. Superoxide anion accumulations were found to be different (Fig. 5D). Margins of *Fof* colonies treated with L-NAME and tungstate in the absence or presence of H_2O_2 were seen as a light purple colour, which was quite different from white colony edges (Fig. 5A). By contrast, visible purple colour development was not distinguished in the *Fof* colony treated with SNP in the absence or presence of H_2O_2.

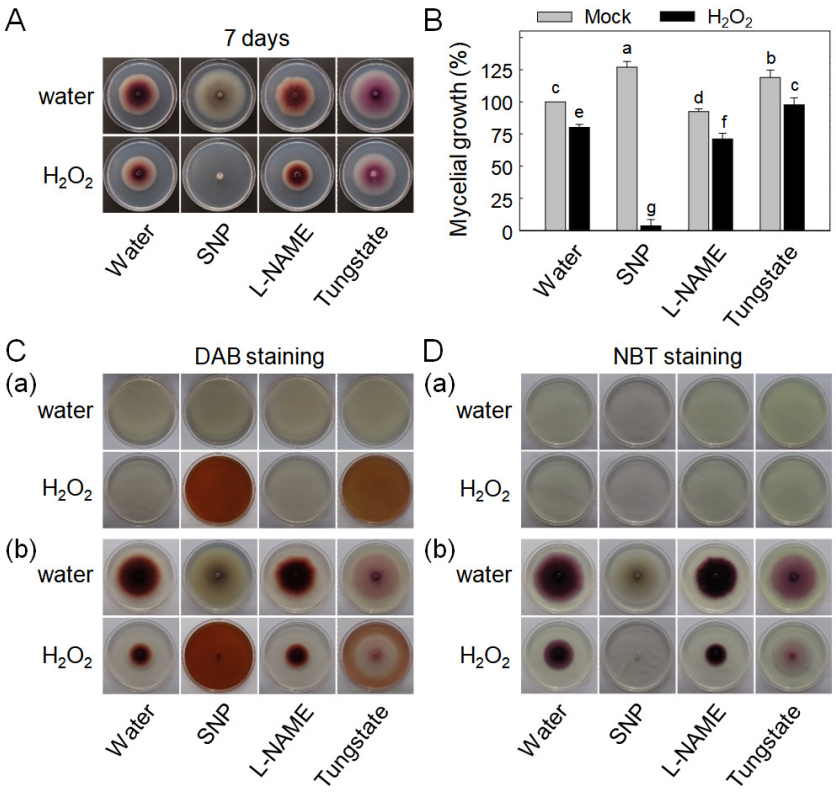

Fig. 5. *In vitro* mycelial growth of *Fusarium oxysporum* f. sp. *fragariae* regulated by hydrogen peroxide (H_2O_2) and/or nitric oxide (NO). (A) Colony formations of *F. oxysporum* f. sp. *fragariae* isolate SFW-10 on PDA media supplemented with sodium nitroprusside (SNP) (2 mM), *N*-nitro-*L*-arginine methyl ester (L-NAME) (2 mM) and sodium tungstate dihydrate (tungstate) (2 mM) were investigated in the absence or presence of H_2O_2 (10 mM). (B) Relative mycelial growths regulated by the chemical treatments are shown as a percentage of fungal colonies on PDA. Error bars represent standard errors of means of five 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 the bars represented no significant difference between treatments. Histochemical accumulation of (C(b)) H_2O_2 and (D(b)) superoxide anion of the 7-day old cultures of *F. oxysporum* f. sp. *fragariae* isolate SFW-10 detected by DAB and NBT staining, respectively. Fungal colonies were stained with DAB and NBT solution for 2 hours. Photos were taken after washed with distilled water. PDA media supplemented with different chemicals were stained without fungal cultures as controls of DAB and NBT staining shown in C(a) and D(a), respectively.

DISCUSSION

H_2O_2 has shown *in vitro* antifungal activity against the diverse taxonomic range of phytopathogenic fungi. *Fusarium* spp. including *F. fujikuroi, F. graminearum, F. moniliforme, F. oxysporum* and *F. solani* were also sensitive to exogenous H_2O_2 and their growth was limited in H_2O_2 dose-dependent manners [31, 32]. In addition to the direct antifungal activity of H_2O_2, exogenous H_2O_2 as a defence elicitor could activate plant defences in cucumber and wheat plants against anthracnose and Septoria blotch, respectively [15, 33]. Both direct and indirect plant protective roles of H_2O_2 against invading fungal pathogens have paved eco-friendly ways to control various plant diseases by applying H_2O_2 solution. In the present study, *in vitro* conidial

germination and mycelial growth of *Fof* were suppressed by H_2O_2 treatments, shown by decreasing conidial germination and mycelial growth accompanying with increasing H_2O_2 concentrations. It is interesting to note that conidial germination of *Fof* was more sensitive than mycelial growth to the exogenous H_2O_2, which indicates that H_2O_2 application can be more efficient to control strawberry Fusarium wilt during the very early stage of the fungal development.

3-AT inhibited catalase activity and was followed by increasing endogenous H_2O_2 concentrations in fungal species such as *Neurospora crassa* [34]. Decreased mycelial biomass of *Mycosphaerella fijiensis* by increasing H_2O_2 was more profound by additional 3-AT treatment [35]. Increasing 3-AT concentrations were applied to *Fof* during *in vitro* conidial germination and mycelial growth to investigate whether endogenously augmented H_2O_2 arrested the fungal development of *Fof* in this study. Increasing 3-AT concentrations resulted in arresting *in vitro* conidial germination, but only high doses of 10 and 20 mM of 3-AT showed inhibitory activity during the conidial germination. It is distinct that relatively lower H_2O_2 can be generated by high 3-AT doses to reduce the conidial germination, although endogenous H_2O_2 production in the fungal cells were not determined. By contrast, 3-AT treatment during the mycelial growth led to different activity depending on the concentrations, and fungal mycelial growth was enhanced by inhibiting fungal catalases at different levels. Low levels of H_2O_2 at least less than 0.5 mM mediated by inhibiting endogenous fungal catalases may contribute to enhancing fungal mycelial growth as a growth stimulator. This is supported by the fact that more than 0.5 mM of 3-AT began to increase the mycelial growth of *Fof*.

Fungi-generated NO also positively or negatively regulated their development and pathogenesis in plant tissues. NO was a critical signalling molecule for uredinial germination of wheat stripe rust fungus *Puccinia striiformis* f. sp. *tritici*, as well as conidial germination and germling development of rice blast fungus *Magnaporthe oryzae* [36, 37]. *Colletotrichum coccodes* causing anthracnose on tomato fruits produced NO during the *in vitro* conidial germination [38]. Interestingly, exogenous NO delayed the germination and the germination was accelerated by NO inhibition. These suggested that NO is a negative regulator for the conidial germination of *C. coccodes*. NO gas was suggested as a potent fungicide for postharvest fruits and vegetables by exerting its antifungal activities against *Aspergillus niger*, *Monilinia fructicola* and *Penicillium italicum* during their *in vitro* spore germination, mycelial growth and sporulation [39]. By contrast, *Fof* responded differently to SNP during *in vitro* conidial germination and mycelial growth. The conidial germination of *Fof* was drastically suppressed by SNP treatment, whilst mycelial growth was enhanced in dose-dependent manner. Increased mycelial growth was also found in *Neurospora crassa* by a certain range of SNP concentrations (0.0001 – 0.01 mM), but increasing the SNP dose to 0.1 mM was ineffective in increasing the mycelial growth [40]. SNP application alone in strawberry plant fields should be avoided because it can cause an increase in occurrence of Fusarium wilt by promoting the mycelial growth.

L-NAME and tungstate were applied during *in vitro* conidial germination and mycelial growth to underpin the involvement of NO synthase and NR enzymes for the fungal development of *Fof*, respectively. L-NAME slightly reduced both conidial germination and mycelial growth in dose-dependent manners, indicating that NO production mediated by NO synthase may attribute to promoting both conidial

germination and mycelial growth of *Fof*. Involvement of NO synthase in conidial germination and mycelial growth of filamentous fungus has been rarely found so far. It has been reported that NO was highly accumulated at an early stage of *in vitro* conidation of mycoparasite *Coniothyrium minitans,* and L-NAME suppressed its conidation suggesting the presence of NO synthase in *C. minitans* indirectly [41]. By contrast, roles of NR in different fungal species have been frequently investigated in relation to nitrate assimilation. But identification and characterization of NR in phytopathogenic fungi have been rarely described. Interestingly, *Fof* responded quite differently to the NR inhibitor during *in vitro* conidial germination and mycelial growth. Highly suppressed *in vitro* conidial germination by tungstate suggests that NR-mediated NO generation might be involved in the *in vitro* conidial germination. However, the NR-mediated NO production seems to limit the mycelial growth of *Fof* by a certain range of NO concentrations. Increasing tungstate concentrations from 5 to 10 mM slightly reduced the mycelial growth compared to 0.5 mM of tungstate treatment. But the fungal mycelial growth was still enhanced compared to the mock-treated control. These implied that highly suppressed NR activity might be regulated by feedback control to compensate NO production during the *in vitro* mycelial growth. Negative feedback regulations of NR gene expression and enzyme activity were uncovered in many plant species and different molecular cues seemed to be involved in the processes that were hardly explained in fungi.

NO was often found to interact with ROS to play a coordinate role in fungal development. Scavenging NO production or inhibiting ROS generation resulted in reduced germ tube elongation of uredospores of *Puccinia striiformis* f. sp. *tritici* [37]. Simultaneous decreases of NO and ROS in the uredospores synergistically suppressed the germination and indicated that NO and ROS both coordinately regulate the uredospore germination. *Fof* mycelial growth was more retarded by antifungal H_2O_2 (10 mM), together with L-NAME or tungstate treatment. Interestingly, simultaneous treatment with H_2O_2 and SNP drastically suppressed the mycelial growth; this was comparable to the slightly enhanced mycelial growth by SNP alone. Exogenous SNP-released NO seems to promote *Fof* mycelial growth by activating antioxidant machinery to scavenge excess ROS harmful to usual fungal growth, but NO adversely limited the mycelial growth via working with exogenous excess H_2O_2. Interaction of NO with catalase enzyme can be involved in the different fungal growth responses with or without excess H_2O_2. NO molecule can bind to catalase and form ferricatalase-NO complex with inhibited catalase activity [42-44]. Inhibited activity of constitutive catalases in *Fof* cells by SNP-released NO can lead to induce endogenous H_2O_2 production at a minute level, which can mediate a mycelial growth increase in the absence of exogenous H_2O_2. However, exogenous H_2O_2 might elevate its antifungal activity together with endogenously generated H_2O_2 by NO-mediated inhibiting catalase, and it may result in markedly suppressed *Fof* mycelial growth.

H_2O_2 was not detectable in culture media supplemented with H_2O_2 alone or with H_2O_2 and L-NAME after 7 days. H_2O_2 in the media may be gradually degraded to an undetectable level for 7 days, but SNP may delay H_2O_2 decline via an unknown mode-of-action. The prolonged action of H_2O_2 may result in highly suppressed mycelial growth of *Fof* compared to arrested mycelial growth on culture media containing H_2O_2 alone. Moderate H_2O_2 shown as a bright red-purple colour in culture media with H_2O_2 and tungstate rather confer enhanced fungal growth. H_2O_2 together with SNP may be an alternative method for chemical

fungicides to control Fusarium wilt.

In conclusion, the application of several H_2O_2 and NO-associated chemicals differentially modulated *in vitro* conidial germination and mycelial growth of *Fof*, and synergistic activity of H_2O_2 and NO to limit the fungal growth suggested efficient eco-friendly management for strawberry Fusarium wilt.

ACKNOWLEDGMENTS

This research was supported by Gyeongnam National University of Science and Technology (GNTech) Grant 2018 to Jeum Kyu Hong. We are grateful to professor Randy Green (GNTech) for critical English proofreading our manuscript.

REFERENCES

1. Fan, X, Kuo J, You MP, Finnegan PM, Barbetti MJ. Comparative root colonisation of strawberry cultivars Camarosa and Festival by *Fusarium oxysporum* f. sp. *fragariae*. Plant Soil 2012;358:75-89.
2. Nam MH, Kang YJ, Lee IH, Kim HG, Chun C. Infection of daughter plants by *Fusarium oxysporum* f. sp. *fragariae* through runner propagation of strawberry. Kor J Hort Sci Technol 2011;29:273-7.
3. Fang X, Barbetti MJ. Differential protein accumulations in isolates of the strawberry wilt pathogen *Fusarium oxysporum* f. sp. *fragariae* differing in virulence. J Proteomics 2014;108:223-37.
4. Arasimowicz-Jelonek M, Floryszak-Wieczorek J. Nitric oxide in the offensive strategy of fungal and oomycete plant pathogens. Front Plant Sci 2016;7:252.
5. Cánovas D, Marcos JF, Marcos AT, Strauss J. Nitric oxide in fungi: is there NO light at the end of the tunnel? Curr Genet 2016;62:513-8.
6. Gessler NN, Aver'yanov AA, Belozerskaya TA. Reactive oxygen species in regulation of fungal development. Biochemistry 2007;72:1091-109.
7. Heller J, Tudzynski P. Reactive oxygen species in phytopathogenic fungi: signaling, development and disease. Annu Rev Phytopathol 2011;49:369-90.
8. Denisov Y, Yarden O, Freeman S. The transcription factor SNT2 is involved in fungal respiration and reactive oxidative stress in *Fusarium oxysporum* and *Neurospora crassa*. Physiol Mol Plant Pathol 2011;76:137-43.
9. Ding J, Shi K, Zhou Y-H, Yu J-Q. Effects of root and foliar applications of 24-epibrassinolide on fusarium wilt and antioxidant metabolism in cucumber roots. Hort Science 2009;44:1340-5.
10. Morkunas I, Bednarski W, Kozłowska M. Response of embryo axes of germinating seeds of yellow lupine to *Fusarium oxysporum*. Plant Physiol Biochem 2004;42:493-9.
11. Olivain C, Trouvelot S, Binet M-N, Cordier C, Pugin A, Alabouvette C. Colonization of flax roots and early physiological responses of flax cells inoculated with pathogenic and nonpathogenic strains of *Fusarium oxysporum*. Appl Environ Microbiol 2003;69:5453-62.
12. Subramaniam S, Maziah M, Sariah M, Puad MP, Xavier R. Bioassay method for testing Fusarium wilt disease tolerance in transgenic banana. Sci Hort 2006;108:378-89.
13. de Quadros FM, Garcés-Fiallos FR, de Borba MC, de Freitas MB, Stadnik MJ. *Fusarium oxysporum* affects differently the hydrogen peroxide levels and oxidative metabolism in

susceptible and resistant bean roots. Physiol Mol Plant Pathol 2019;106:1-9.
14. Gupta KJ, Mur LAJ, Brotman Y. *Trichoderma asperelloides* suppresses nitric oxide generation elicited by *Fusarium oxysporum* in Arabidopsis roots. Mol Plant-Microbe Interact 2014;27:307-14.
15. Byun H-J, Choi S-J. Hydrogen peroxide induces resistance against anthracnose in cucumber (*Cucumis sativus*). J Kor Soc Hort Sci 2004;45:21-6.
16. Abdel-Monaim MF. Induced systemic resistance in tomato plants against fusarium wilt disease. Int Res J Microbiol 2012;3:14-23.
17. Biswas SK, Pandey NK, Rajik M. Inductions of defense response in tomato against fusarium wilt through inorganic chemicals as inducers. J Plant Pathol Microbiol 2012;3:128.
18. Hatem MW. Evaluation of the efficiency of hydrogen peroxide against *Fusarium oxysporum*, which causes fusarium wilt on tomato plant. Curr Res Microbiol Biotechnol 2017;5:1112-6.
19. Abdel-Monaim MF. Improvement of biocontrol of damping-off and root rot/wilt of faba bean by salicylic acid and hydrogen peroxide. Mycobiology 2013;41:47-55.
20. Hu L, Li Y, Bi Y, Li J, Bao G, Liu J, Yu X. Effects of nitric oxide on growth of *Fusarium sulphureum* and its virulence to potato tubers. Eur Food Res Technol 2014;238:1007-14.
21. Ávila AC, Ochoa J, Proaño K, Martínez MC. Jasmonic acid and nitric oxide protects naranjilla (*Solanum quitoense*) against infection by *Fusarium oxysporum* f. sp. *quitoense* by eliciting plant defense responses. Physiol Mol Plant Pathol 2019;106:129-36.
22. Fan B, Shen L, Liu K, Zhao D, Yu M, Sheng J. Interaction between nitric oxide and hydrogen peroxide in postharvest tomato resistance response to *Rhizopus nigricans*. J Sci Food Agric 2008;88:1238-44.
23. Shi JY, Liu N, Gu RX, Zhu LQ, Zhang C, Wang QG, Lei ZH, Liu YY, Ren JY. Signals induced by exogenous nitric oxide and their role in controlling brown rot disease caused by *Monilinia fructicola* in postharvest peach fruit. J Gen Plant Pathol 2015;81:68-76.
24. Park JY, Kim SH, Kim NH, Lee SW, Jeun Y-.C, Hong JK. Differential inhibitory activities of four plant essential oils on *in vitro* growth of *Fusarium oxysporum* f. sp. *fragariae* causing Fusarium wilt in strawberry plants. Plant Pathol J 2017;33:582-8.
25. Kim YJ, Lee YH, Lee H-J, Jung H, Hong JK. H_2O_2 production and gene expression of antioxidant enzymes in kimchi cabbage (*Brassica rapa* var. *glabra* Regel) seedlings regulated by plant development and nitrosative stress-triggered cell death. Plant Biotechnol Rep 2015;9:67-78.
26. Lai T, Li B, Qin G, Tian S. Oxidative damage involves in the inhibitory effect of nitric oxide on spore germination of *Penicillium expansum*. Curr Microbiol 2011;62:229-34.
27. Kaplan D, Roth-Bejerano N, Lips H. Nitrate reductase as a product-inducible enzyme. Eur J Biochem 1974;49:393-8.
28. Sarkar TS, Biswas P, Ghosh SK, Ghosh S. Nitric oxide production by necrotrophic pathogen *Macrophomina phaseolina* and the host plant in charcoal rot disease of jute: Complexity of the interplay between necrotroph-host plant interactions. PLoS One 2014;9:e107348.
29. Hong JK, Yang HJ, Jung H, Yoon DJ, Sang MK, Jeun Y.-C. Application of volatile antifungal plant essential oils for controlling pepper fruit anthracnose by *Colletotrichum gloeosporioides*. Plant Pathol J 2015;31:269-77.
30. Sung CH, Hong JK. Sodium nitroprusside mediates seedling development and attenuation of oxidative stresses in Chinese cabbage. Plant Biotechnol Rep 2010;4:243-51.
31. Abdou El-S, Galal AA. Sensitivity of *Fusarium moniliforme*, *F. oxysporum* and *F. solani* to superoxide anion and hydrogen peroxide *in vitro*. Egypt J Microbiol 1997;32:523-35.

32. Youn K, Choi H-W, Shin DB, Jung B, Lee J. Resistance to *Fusarium fujikuroi* isolates to hydrogen peroxide and its application for fungal isolation. Res Plant Dis 2015;21:227-30.
33. Shetty NP, Mehrabi R, Lütken H, Haldrup A, Kema GHJ, Collinge DB, Jørgensen HJL. Role of hydrogen peroxide during the interaction between the hemibiotrophic fungal pathogen *Septoria tritici* and wheat. New Phytol 2007;174:637-47.
34. Yoshida Y, Iigusa H, Wang N, Hasunuma K. Cross-talk between the cellular redox state and the circadian system in *Neurospora*. PLoS One 2011;6:e28227.
35. Beltrán-García MJ, Manzo-Sanchez G, Guzmán-González S, Arias-Castro C, Rodríguez-Mendiola M, Avila-Miranda M, Ogura, T. Oxidative stress response of *Mycosphaerella fijiensis*, the causal agent of black leaf streak disease in banana plants, to hydrogen peroxide and paraquat. Can J Microbiol 2009;55:887-94.
36. Samalova M, Johnson J, Illes M, Kelly S, Fricker M, Gurr S. Nitric oxide generated by the rice blast fungus *Magnaporthe oryzae* drives plant infection. New Phytol 2013;197:207-22.
37. Yin S, Gao Z, Wang C, Huang L, Kang Z, Zhang H. Nitric oxide and reactive oxygen species coordinately regulate the germination of *Puccinia striiformis* f. sp. *tritici* urediniospores. Front Microbiol 2016;7:178.
38. Wang J, Higgins VJ. Nitric oxide has a regulatory effect in the germination of conidia of *Colletotrichum coccodes*. Fungal Genet Biol 2005;42:284-92.
39. Lazar EE, Wills RBH, Ho BT, Harris AM, Spohr LJ. Antifungal effect of gaseous nitric oxide on mycelium growth, sporulation and spore germination of the postharvest horticulture pathogens, *Aspergillus niger*, *Monilinia fructicola* and *Penicillium italicum*. Lett Appl Microbiol 2008;46:688-92.
40. Pengkit A, Jeon SS, Son SJ, Shin JH, Baik KY, Choi EH, Park G. Identification and functional analysis of endogenous nitric oxide in a filamentous fungus. Sci Rep 2016;6:30037.
41. Gong X, Fu Y, Jiang D, Li G, Yi X, Peng Y. L-Arginine is essential for conidation in the filamentous fungus *Coniothyrium minitans*. Fungal Genet Biol 2007;44:1368-79.
42. Brown GC. Reversible binding and inhibition of catalase by nitric oxide. Eur J Biochem 1995;232:188-91.
43. Candelaresi M, Gumiero A, Adamczyk K, Robb K, Bellota-Antón C, Sangal V, Munnoch J, Greetham GM, Towrie M, Hoskisson PA, Parker AW, et al. A structural and dynamic investigation of the inhibition of catalase by nitric oxide. Org Biomol Chem 2013;11:7778-88.
44. Purwar N, McGarry JM, Kostera J, Pacheco AA, Schmidt M. Interaction of nitric oxide with catalase: structural and kinetic analysis. Biochemistry 2011;50:4491-503.