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

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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

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 conidiation, 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 H2O2 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 H2O2 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 flux suspension cells inoculated by F. oxysporum f. sp. lini [9-12]. An increase in H2O2 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 H2O2 reduced anthracnose lesion development on cucumber leaves challenge-inoculated by Colletotrichum orbiculare [15]. Tomato seeds and seedlings treated with H2O2 have shown reduced Fusarium wilt under protected house and field conditions [16-18]. Seed soaking faba beans in H2O2 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$_2$O$_2$ and NO application during crop production and post-harvest periods. However, limited fungal growth of Fos and/or controlled strawberry Fusarium wilt by regulating ROS and RNS is rarely found.

In this study, we applied the H$_2$O$_2$ and 3-amino-1,2,4-triazole (3-AT) to Fof to investigate the role of exogenous and endogenous H$_2$O$_2$ during the in vitro conidial germination and mycelial growth. NO donor SNP, NO synthase (NOS)-inhibitor N$\text{G}$-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$_2$O$_2$ together with NO, Fof was treated with SNP, L-NAME and tungstate in the absence or presence of H$_2$O$_2$ during the mycelial growth. H$_2$O$_2$ 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$_2$O$_2$) and 3-amino-1,2,4-triazole (3-AT) were used as one of ROS and an inducer of endogenous H$_2$O$_2$ level by inhibiting activity of catalase decomposing H$_2$O$_2$, 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 $5 \times 10^5$ 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$_2$O$_2$ and superoxide anion in *Fof* colonies and growth media**

Accumulation of H$_2$O$_2$ 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$_2$O$_2$ and superoxide anion from background of culture media. Red-brown and blue-purple colours indicate presence of H$_2$O$_2$ 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$_2$O$_2$-regulated *in vitro* conidial germination of *Fof***

Both exogenous H$_2$O$_2$ and 3-AT decreased *in vitro* conidial germination of *Fof* in different levels (Fig. 1). The lowest concentration (0.5 mM) of H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$-regulated *in vitro* mycelial growth of *Fof***

H$_2$O$_2$ and 3-AT regulated mycelial growth of *Fof* differently (Fig. 2). Increasing H$_2$O$_2$ 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$_2$O$_2$). (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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$). (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$_2$O$_2$ and 3-amino-1,2,4-triazole (3-AT) was investigated. (B) Relative mycelial growths regulated by H$_2$O$_2$ 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](image)

**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.](image)
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.

**Coordiately regulated *in vitro* mycelial growth of *Fof* by H$_2$O$_2$ and NO**

The effect of NO on the H$_2$O$_2$-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$_2$O$_2$ (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$_2$O$_2$ alone led to a decrease in the mycelial growth of *Fof* by ca. 80.1% as demonstrated in Fig. 2. Combined treatment with H$_2$O$_2$ (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$_2$O$_2$ (10 mM) moderately limited the mycelial growth (ca. 71.2%). A supplement with tungstate (2 mM) in the H$_2$O$_2$ (10 mM)-containing media compromised tungstate-induced mycelial growth promotion.

During the differential growth responses of *Fof* to H$_2$O$_2$ and different NO levels, intracellular production or secretion of reactive oxygen species were investigated by histochemical staining (Fig. 5C, 5D). H$_2$O$_2$ accumulation was not distinctly found in *Fof* colonies treated with SNP, L-NAME and tungstate in the absence or presence of H$_2$O$_2$ (Fig. 5C). However, dark red-brown colour development was observed in culture media supplemented with both SNP and H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$. 
DISCUSSION

H$_2$O$_2$ 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$_2$O$_2$ and their growth was limited in H$_2$O$_2$ dose-dependent manners [31, 32]. In addition to the direct antifungal activity of H$_2$O$_2$, exogenous H$_2$O$_2$ 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$_2$O$_2$ against invading fungal pathogens have paved eco-friendly ways to control various plant diseases by applying H$_2$O$_2$ solution. In the present study, *in vitro* conidial
germination and mycelial growth of Fof were suppressed by H$_2$O$_2$ treatments, shown by decreasing conidial germination and mycelial growth accompanying with increasing H$_2$O$_2$ concentrations. It is interesting to note that conidial germination of Fof was more sensitive than mycelial growth to the exogenous H$_2$O$_2$, which indicates that H$_2$O$_2$ 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$_2$O$_2$ concentrations in fungal species such as Neurospora crassa [34]. Decreased mycelial biomass of Mycosphaerella fijiensis by increasing H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ can be generated by high 3-AT doses to reduce the conidial germination, although endogenous H$_2$O$_2$ 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$_2$O$_2$ 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 H₂O₂ (10 mM), together with L-NAME or tungstate treatment. Interestingly, simultaneous treatment with H₂O₂ 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 H₂O₂. Interaction of NO with catalase enzyme can be involved in the different fungal growth responses with or without excess H₂O₂. 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 H₂O₂ production at a minute level, which can mediate a mycelial growth increase in the absence of exogenous H₂O₂. However, exogenous H₂O₂ might elevate its antifungal activity together with endogenously generated H₂O₂ by NO-mediated inhibiting catalase, and it may result in markedly suppressed Fof mycelial growth.

H₂O₂ was not detectable in culture media supplemented with H₂O₂ alone or with H₂O₂ and L-NAME after 7 days. H₂O₂ in the media may be gradually degraded to an undetectable level for 7 days, but SNP may delay H₂O₂ decline via an unknown mode-of-action. The prolonged action of H₂O₂ may result in highly suppressed mycelial growth of Fof compared to arrested mycelial growth on culture media containing H₂O₂ alone. Moderate H₂O₂ shown as a bright red-purple colour in culture media with H₂O₂ and tungstate rather confer enhanced fungal growth. H₂O₂ together with SNP may be an alternative method for chemical
fungicides to control Fusarium wilt.

In conclusion, the application of several H$_2$O$_2$ and NO-associated chemicals differentially modulated in vitro conidial germination and mycelial growth of Fof, and synergistic activity of H$_2$O$_2$ and NO to limit the fungal growth suggested efficient eco-friendly management for strawberry Fusarium wilt.

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