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Nitric Oxide and Reactive Oxygen Species Coordinately Regulate the Germination of *Puccinia striiformis* f. sp. *tritici* Urediniospores

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Nitric oxide (NO) and reactive oxygen species (ROS) function as signaling molecules in a number of critical signal transduction pathways in plants, including plant biotic interactions. In addition to the role of plant-derived NO and ROS in plant resistance, which has been well documented, pathogen-produced NO and ROS have recently emerged as important players in fungal development and pathogenesis. However, the effects of pathogenic fungi-derived NO and ROS on signaling pathways during fungal pre-infection development remain unknown. Here, using a combination of pharmacological approaches and confocal microscopy, we investigated the roles of NO and ROS during the germination of *Puccinia striiformis Westend* f. sp. *tritici* (*Pst*) the wheat stripe rust pathogen. Both NO and ROS have a crucial role in uredinial germination. The scavengers of NO and ROS delayed spore germination and decreased the lengths of germ tubes. A similar phenotype was produced after treatment with the promoter. However, the spores germinated and grew normally when the levels of NO and ROS were simultaneously elevated by the application of a promoter of NO and a donor of ROS. Confocal laser microscopy indicated that both NO and ROS preferentially localized at the germ pores and apexes of growing germ tubes when the ROS/NO ratio in the spores was maintained in a specific range. We concluded that both NO and ROS are critical signaling molecules in the pre-infection development of *Pst* and that the polar growth of the germ tube is coordinately regulated by NO and ROS.

Keywords: NO, ROS, *Pst*

Abbreviations: CLSM, confocal laser scanning microscopy; c-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, 4-amino-5-(*N*-methylamino)-2, 7-difluorofluorescein diacetate; H_2DCF DA, 2′, 7′-dichlorodihydrofluorescein diacetate; DAF-FM T, 4-amino-5-(*N*-methylamino)-2, 7-difluorofluorescein triazole; DCF, dichlorofluorescein; DPI, diphenyliodonium iodide; HPG, hours post germination; L-NAME, *N*-nitro-L-arginine methyl ester; MFI, mean fluorescence intensity; MGL, mean germ tube lengths; MGR, mean germination rate; NADPH, nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; NO, nitric oxide; NOS, nitric oxide synthase; *Pst*, *Puccinia striiformis* f. sp. *Tritici;* RNS, reactive nitrogen species; ROS, reactive oxygen species.

INTRODUCTION

Two important types of free radicals, NO and ROS, are crucial signaling molecules involved in a number of signal transduction pathways. The roles of NO and ROS in mammals have been studied for many years. They are crucial messengers in the immune, nervous, and cardiovascular systems (Palmer et al., 1987). In plants, they are involved in several physiological processes, including seed germination and lateral leaf and root development, and have been implicated in both abiotic and biotic stress responses (Besson-Bard et al., 2008; Wilson et al., 2008; Swanson and Gilroy, 2010). Indeed, there is considerable evidence that plant-derived NO and ROS are important in initiating plant responses to pathogens or elicitors (Brisson et al., 1994; Levine et al., 1994; Delledonne et al., 1998; Chaki et al., 2009).

Evidence is also emerging that NO and ROS are important regulatory molecules in microbe, including plant pathogens. It has been found that ROS is involved in the germination and germ tube growth of conidia of *Cladosporium fulvum* (Lu and Higginsf, 1999) and the biofilm resistance of *Pseudomonas aeruginosa* (Elkins et al., 1999).

It was reported that pathogen-derived NO influences germination in *Colletotrichum coccodes* (Wang and Higgins, 2005), conidiation in *Coniothyrium minitans* (Gong et al., 2007) and sporangiophore development in *Phycomyces blakesleeanus* (Maier et al., 2001) and affects the formation of appressoria in the obligate biotrophic powdery mildew fungus *Blumeria graminis* (Prats et al., 2008) and pathogenicity in the rice blast fungus *Magnaporthe oryzae* (Averyanov and Lapikova, 1990).

Reactive oxygen species have been reported to be involved in fungal virulence and development (Heller and Tudzynski, 2011). There are various reports on the effects of ROS free radicals on spore germination. O_2^- and OH radicals were both detected during the germination of *Pyricularia oryzae*, and the radical scavengers superoxide dismutase (SOD), catalase and OH increased the percentage of germination (Averyanov and Lapikova, 1990). During spore germination in *Neurospora crassa*, an accumulation of catalase was observed, indirectly suggesting that H_2O_2 was generated in the process (Michan et al., 2002).

Wheat stripe rust, caused by *Pst*, is one of the most important diseases of wheat and can cause significant loss to wheat yield and grain quality (Chen, 2005). Although *Pst* is a macrocyclic rust pathogen, its propagation and spread occur primarily by means of urediniospores, which are capable of germination and infection under suitable environmental conditions immediately after release (Chen et al., 2014). Following the initiation of germination, the cytoplasm of a urediniospore moves into the germ tube until it reaches a stoma.

To our knowledge, no study has suggested a role for NO and ROS during urediniospore germination. Hence, CLSM was used to visualize NO and ROS generated by urediniospores *in vivo* and the role of NO and ROS in *Pst* development was investigated. We identify a regulatory role for NO and ROS during the germination of urediniospores and the apical growth of germ tubes in *Pst* using specific probes and the donor/promoter and scavengers of NO and ROS. The scavengers of NO and ROS delayed germination and decreased germ tube length. Moreover, a promoter of NO and a donor of ROS could also delay germination and decrease the germ tube length. Further study determined that the spores germinate when the ROS/NO ratio is maintained within a specific range and that NO and ROS primarily exist in the apex of the germ tube, suggesting that both NO and ROS are involved in apical germ tube growth.

MATERIALS AND METHODS

Pathogen and Reagents

Fresh urediniospores of *Pst* pathotype CYR31 used in this study were provided by the Institute of Plant Pathology, Northwest A&F University. The specific NO scavenger c-PTIO (Balcerczyk et al., 2005), the substrate of NO synthesis L-Arg (Bonilla et al., 2004; for clarity, L-Arg is elsewhere called "the promoter of NO"), the ROS donor triphosphopyridine nucleotide (NADPH), the NADPH scavenger DPI, NO-specific probe 4-amino-5-(*N*-methylamino)-2,7-difluorofluorescein diacetate (DAF-FM DA) and the ROS-specific probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF DA) were used in this study. All reagents used in this study were obtained from Sigma–Aldrich, USA.

Determination of Appropriate Reagent Concentrations

Different reagents at different concentrations (c-PTIO at 0, 50, 100, 150 μM; L-Arg at 0, 2, 3, 4 mM; DPI at 0, 10, 20, 30 μM and NADPH at 0, 2.5, 3.5, 4.5 mM) were tested in order to find the appropriate concentrations for use in the experiments.

Different probes at different concentrations (DAF-FM DA at 1, 2, 5, 10, and 15 μM and H_2DCF DA at 20, 30, 50, 70, and 100 mM) were tested in order to find the appropriate concentrations to detect the generation of NO and ROS in the study.

Finally, concentration of c-PTIO at 100 μM, L-Arg at 2 mM, DPI at 20 μM, NADPH at 2.5 mM, DAF-FM DA at 10 μM and H_2DCF DA at 50 mM were selected.

Effects of NO and ROS on Urediniospore Germination and Germ Tube Growth

Fresh urediniospores (0.6 mg) were added to 10 ml distilled water, 10 ml 100 μM c-PTIO, 10 ml 2 mM L-Arg, 10 ml 20 μM DPI, 10 ml 2.5 mM NADPH, 5 ml 20 μM DPI+5 ml 100 μM c-PTIO (c-PTIO +DPI), 5 ml 2 mM L-Arg+5 ml 2.5 mM NADPH (L-Arg +NADPH), 5 ml 20 μM DPI+5 ml 2 mM L-Arg (L-Arg +DPI) or 5 ml 2.5 mM NADPH+5 ml 100 μM c-PTIO (c-PTIO +NADPH) and allowed to germinate at 9°C in darkness.

Evidence for, and Localization of, Endogenous NO and ROS in Germinating *Pst* Urediniospores

The specific fluorescence probes-H_2DCF DA and DAF-FM DA were used to detect ROS and NO. In the presence of ROS and NO, H_2DCF DA and DAF-FM DA were converted to fluorescent

DCF and DAF-FM triazole (DAF-FM T), which could be detected separately by bright green fluorescence in CLSM.

Germinating urediniospores treated as described above (see Effects of NO and ROS on Urediniospore Germination and Germ Tube Growth) were collected at different time points and mixed with 10 μM DAF-FM DA or 50 mM H_2DCF DA prior to incubation in darkness at 25°C for 30 min. They were then immersed twice for 10 min in Tris-HCl buffer (50 mM, pH 7.4) to remove excess dye. A distilled water control without DAF-FM DA or H_2DCF DA staining and with the same other procedures was treated as CK to detect the autofluorescence of germinating urediniospores. Then, the urediniospores were placed on slides to detect the generation of NO and ROS by CLSM (LSM 510 META, Zeiss Corporation, Germany). Fluorescence was detected at an excitation frequency of 488 nm, and emission was filtered using a 515–530 nm barrier filter. NO and ROS were detected with the same parameters (gain, magnification and so on) across all treatments. Images were recorded after DAF-FM DA and H_2DCF DA staining, and observations of the CK enabled discrimination between autofluorescence (also excited by the argon laser) and fluorescence due to NO and ROS generation. The MFI values were measured in different locations of the urediniospores and germ tubes using Image Pro Plus software (IPP software, USA).

Rates of Germination and Germ Tube Lengths

Germinating urediniospores were collected at different time points and placed on slides to count the numbers of germinated urediniospores and to measure the lengths of germ tubes (a germ tube length greater than one-half the spore diameter was defined as germination) using an Olympus BX51 microscope (Olympus Corporation, Japan). The germination rate was expressed as a percentage based on 100 urediniospores.

Statistical Analysis

One hundred urediniospores were analyzed in every treatment at random, and all experiments were performed at least three times. Only representative images are shown in the paper. Differences in germination rates, germ tube lengths and mean pixel intensity among the treatments were analyzed by one-way ANOVA with the least significant differences (LSD) test at 0.05 probability level. All statistical tests were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Promoter and Scavenger of NO and ROS Affect Spore Germination

After treatment with c-PTIO and DPI, spore germination was significantly suppressed and delayed in germination time compared with spores treated with distilled water (**Figures 1** and **2**), as measured by MGL and MGR.

Urediniospores began to germinate after 1.5 h in distilled water, and the MGR and MGL were 8% and 21.3 μm, respectively; they increased over time, especially at 4–6 hpg, and peaked at 6 hpg (78.2% and 194.6 μm). By contrast, the urediniospores maintained dormancy for 1–1.5 hpg when treated with DPI and c-PTIO at 2 hpg. The MGR values were 7.7 and 8.3%, and the MGL values were 20.8 and 23.4 μm, respectively. Although the MGR and MGL increased over time, there were significant differences compared with the distilled water control. Thus ROS and NO had important roles in the germination of spores and in germ tube growth.

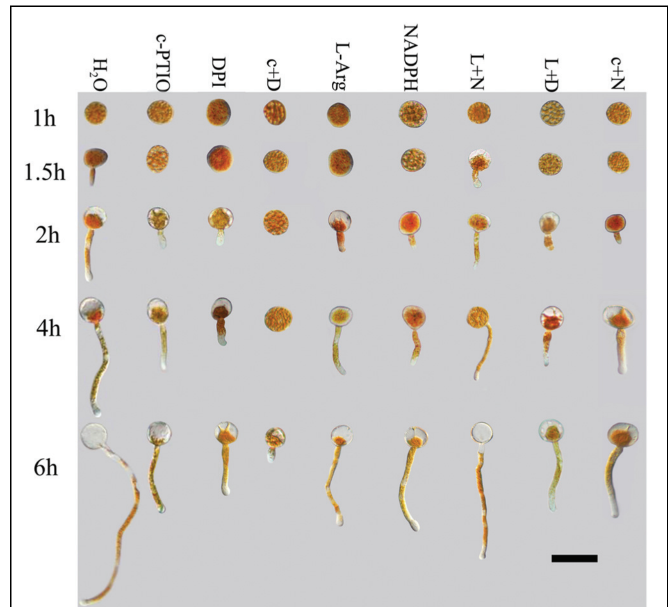

FIGURE 1 | Growth of *Pst* urediniospores after different treatments and time points. Deficiencies of ROS or NO inhibited germination of urediniospores and germ tube growth. Increases in ROS alone or NO alone did not promote germination or germ tube growth. Only increases in both ROS and NO led to increased spore germination. C+D, c-PTIO +DPI; L +N, L-Arg +NADPH; L+D, L-Arg +DPI; c+N, c-PTIO +NADPH. Scale bar, 50 μm.

After treatment with L-Arg and NADPH, urediniospore germination was also significantly suppressed (**Figures 1** and **2**). The data for MGR and MGL showed no differences between L-Arg, NADPH, c-PTIO, and DPI treatments at any time points, which suggested that an increase in ROS only or NO only could not promote spore germination or germ tube growth.

After treatment with L-Arg +DPI and c-PTIO +NADPH, the MGL and MGR were increased compared with the DPI and c-PTIO treatment (**Figures 1** and **2**). However, variance analysis showed no significant difference, indicating that excess NO or ROS did not promote spore germination or germ tube growth.

Germination was almost completely suppressed after treatment with c-PTIO +DPI (**Figures 1** and **2**), and variance analysis indicated significant differences compared with other treatments, especially distilled water.

In the presence of L-Arg +NADPH, spore germination was indistinguishable from that observed in distilled water (**Figures 1** and **2**). However, there was a significant difference between L-Arg +NADPH and distilled water at 4 hpg.

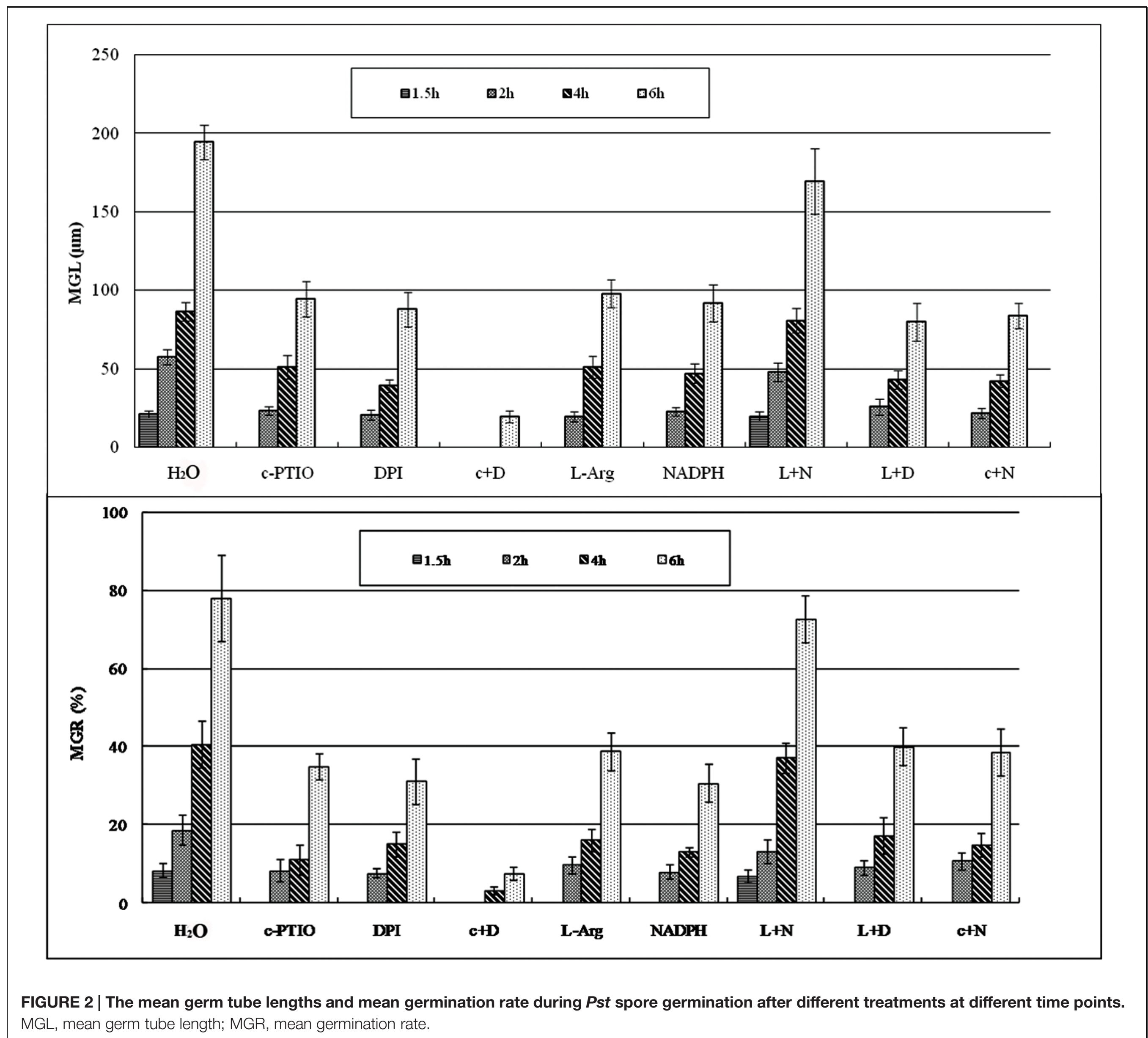

FIGURE 2 | The mean germ tube lengths and mean germination rate during *Pst* spore germination after different treatments at different time points. MGL, mean germ tube length; MGR, mean germination rate.

Thus, spore germination and fungal growth were significantly reduced when NO or ROS levels were decreased (**Figures 1** and **2**), and increases in ROS only or NO only failed to promote increases (**Figures 1** and **2**), suggesting that NO and ROS play a crucial role in spore germination and germ tube growth. This result suggested that an optimum ratio of ROS to NO possibly exist during urediniospore germination in *Pst*.

The Fluorescence Intensity of Endogenous NO Generated During Urediniospore Germination Following Different Treatments

Nitric oxide-specific fluorescent probe DAF-FM DA and CLSM were used to detect NO produced during urediniospore germination. The urediniospore and the germ tube were divided into three parts (urediniospore, base of germ tube and apex of germ tube), as shown in **Figure 3**.

CSLM performed after DAF-FM DA staining revealed the generation of NO in *Pst* spores and germ tubes.

Bright fluorescence was observed after treatment with distilled water and DAF-FM DA (**Figure 4A**); faint fluorescence was observed in distilled water without DAF-FM DA staining (CK; **Figure 4B**); c-PTIO-treated samples also displayed faint and uniform fluorescence throughout the study (**Figure 4C**). Combining the results of these three treatments, it could be confirmed that the fluorescence in **Figure 4B** is the autofluorescence of urediniospore, and the bright green fluorescence in **Figure 4A** is due to NO generation and not autofluorescence.

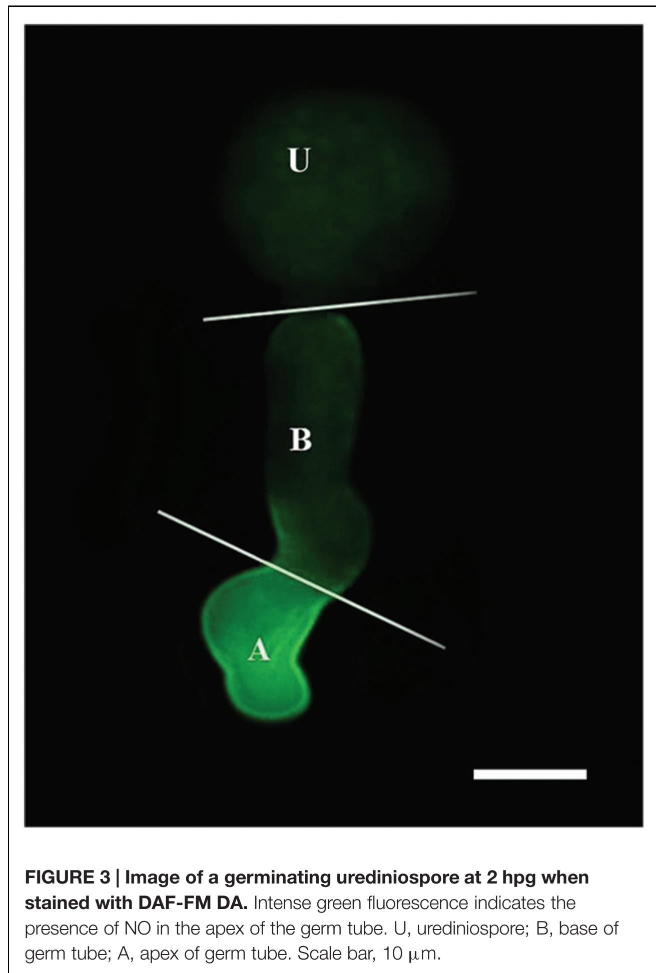

FIGURE 3 | Image of a germinating urediniospore at 2 hpg when stained with DAF-FM DA. Intense green fluorescence indicates the presence of NO in the apex of the germ tube. U, urediniospore; B, base of germ tube; A, apex of germ tube. Scale bar, 10 μm.

Bright green fluorescence was detected in germ pores, indicating that NO was generated around the germ pores during the urediniospore water-swelling stage at 1 hpg. However, germ tubes emerged from the germ pores at 1.5–2 hpg, and staining showed pronounced fluorescence in most short germ tubes, especially at their tips (**Figure 4A**). Further images showed intense fluorescence localized at the apices of germ tubes at 2–6 hpg, although faint fluorescence was also observed in the spores and bases of the germ tubes (**Figure 4A**). The same trends were observed after treatment with L-Arg +NADPH (**Figure 4H**), L-Arg (**Figure 4F**), and L-Arg +DPI (**Figure 4I**), although germination was delayed in the last two treatments.

After treatment with DPI or NADPH, visual inspection and staining with DAF-FM DA showed a constant faint fluorescence in the spores and germ tubes throughout the study (**Figures 4D,G**), showing that both the suppression and promotion of ROS inhibited NO.

When ROS and NO were both inhibited, the MFI of NO was decreased (**Figure 4E**), and spore germination was considerably delayed (**Figures 1** and **2**). After treatment with c-PTIO +NADPH, staining with DAF-FM DA showed faint fluorescence in the spores and germ tubes (**Figure 4J**).

Urediniospores treated with L-Arg, L-Arg +NADPH and L-Arg +DPI, when stained with DAF-FM DA, displayed a marked bright fluorescence correlated with the development of germ tubes (**Figures 4F,H,I**).

After treatment with L-Arg +DPI, the bright fluorescence at the spores and germ tubes were observed (**Figure 4I**), which suggested that although L-Arg could help to relieve ROS depression and produce more NO, it exhibited no enhancement on the elongation of germ tubes (**Figures 1** and **2**).

It was also observed that only when the ROS and NO were simultaneously added (**Figure 4H**) could the spore germinate normally, as in the distilled water treatment (**Figures 1** and **2**).

These results show that NO plays a crucial role in apical growth and that ROS is involved in the generation of NO. NO production was greatly increased by L-Arg, further suggesting a role for a NOS enzyme as the source of NO generation in the fungus.

Fluorescence Intensity of Endogenous ROS Generated During Germination After Different Treatments

Reactive oxygen species-specific fluorescent probe H_2DCF DA and CLSM were used to detect the ROS produced during urediniospore germination.

Bright green fluorescence was observed for the treatment with distilled water and H_2DCF DA (**Figure 5A**); faint fluorescence was observed in the distilled water without H_2DCF DA staining (CK; **Figure 5B**); DPI-treated samples also displayed faint and uniform fluorescence throughout the study (**Figure 5D**). Combining the results of these three treatments, it could be confirmed that the fluorescence in **Figure 5B** was the autofluorescence of urediniospores, whereas the bright green fluorescence in **Figure 5A** was due to ROS generation and not autofluorescence.

Bright green fluorescence indicated that ROS was generated around germination pores during the water-swelling stage at 1 hpg (**Figure 5A**). During the growth of the germ tubes, the characteristic of ROS generation were similar to DAF-FM DA staining (**Figure 5A**). The same trends were observed after treatment with L-Arg +NADPH (**Figure 5H**), NADPH (**Figure 5G**) and c-PTIO +NADPH (**Figure 5J**), although the germination was delayed in the last two treatments.

After treatment with the NO scavenger c-PTIO or the NO promoter L-Arg and staining with H_2DCF DA, only faint fluorescence was detected at the tips of germ tubes (**Figures 5C,F**). This result indicated that either the suppression or the promotion of NO decreased the concentration of ROS during spore germination.

Urediniospore treatment with NADPH, L-Arg +NADPH or c-PTIO +NADPH and staining with H_2DCF DA showed a markedly bright fluorescence during the germination of urediniospores (**Figures 5G,H,J**).

When the ROS and NO were both restrained, the MFI of ROS was decreased (**Figure 5E**) and spore germination was delayed (**Figures 1** and **2**) which was similar to DAF-FM DA staining.

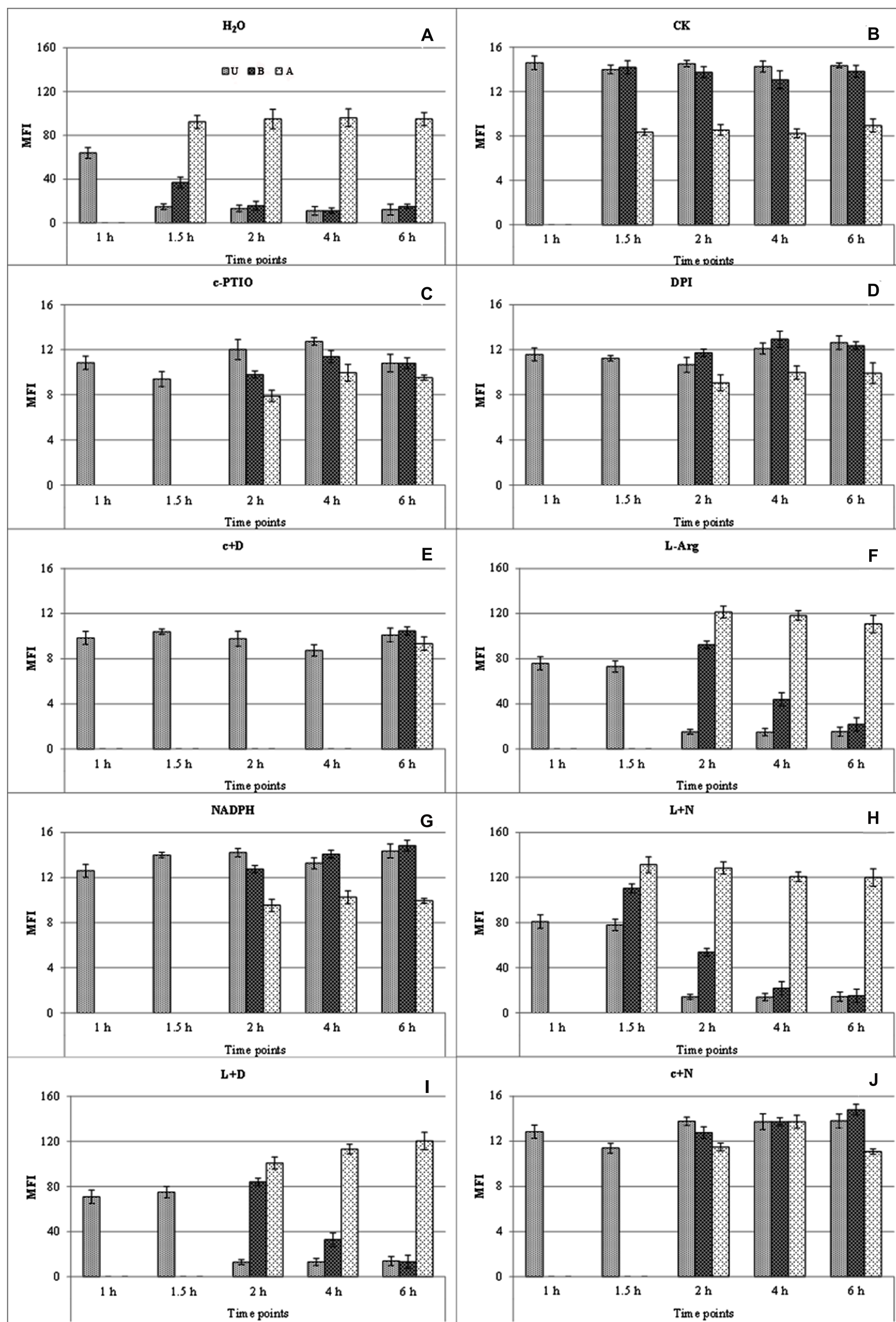

FIGURE 4 | Mean fluorescence intensity of endogenous NO generated during germination at different time points after different treatments. Bright fluorescence was observed after treatment with distilled water (H_2O) and DAF-FM DA **(A)**; faint fluorescence was observed in distilled water without DAF-FM DA staining (CK) **(B)**. U, urediniospore; B, base of germ tube; A, apex of germ tube. c+D, c-PTIO +DPI; L+N, L-Arg +NADPH; L+D, L-Arg +DPI; c+N, c-PTIO +NADPH.

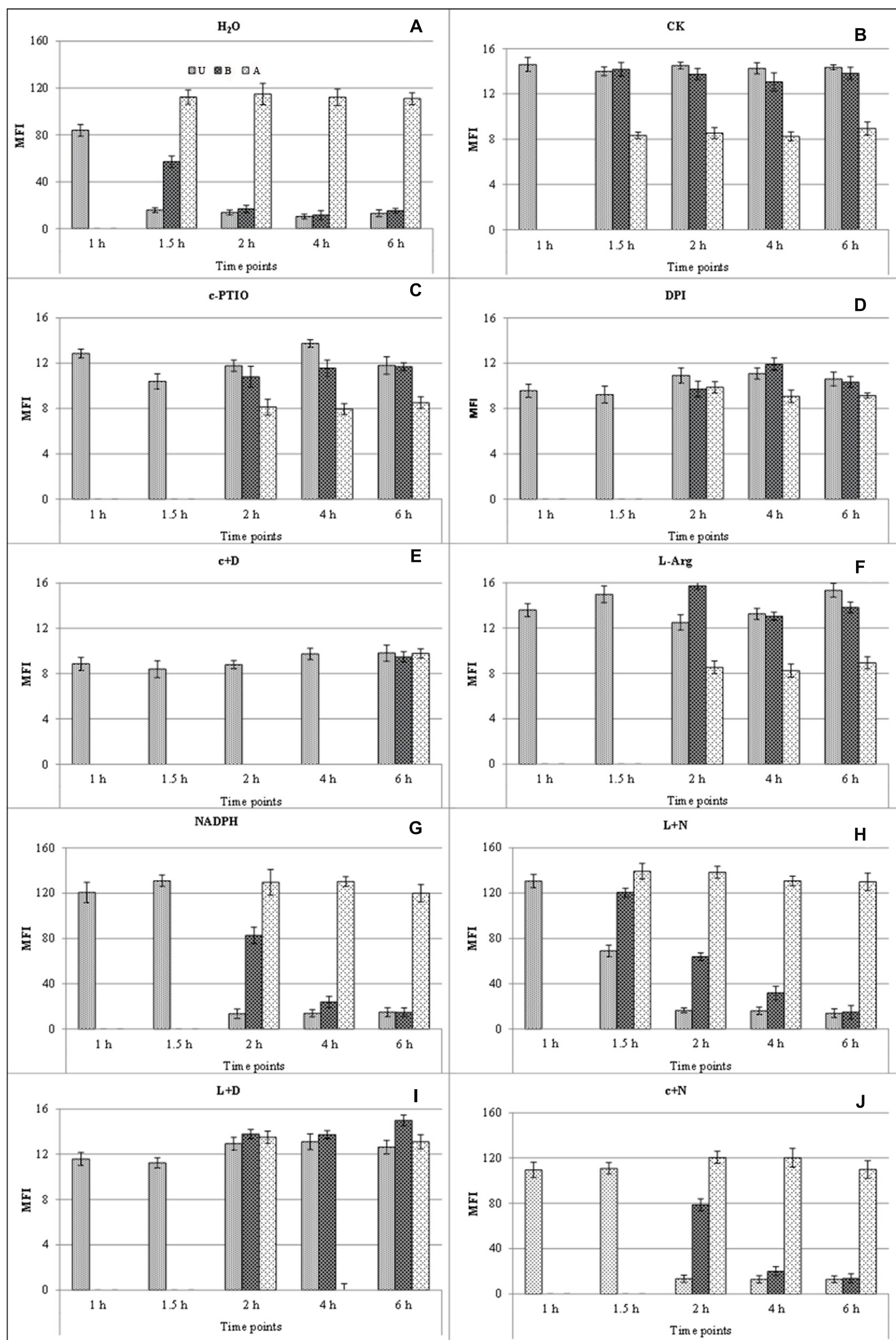

FIGURE 5 | Mean fluorescence intensity of endogenous ROS generated during germination at different time points and treatments. Bright fluorescence was observed after treatment with distilled water (H_2O) and H_2DCF DA **(A)**; faint fluorescence was observed in distilled water without H_2DCF DA staining (CK) **(B)**. U, urediniospore; B, base of germ tube; A, apex of germ tube. c+D, c-PTIO +DPI; L+N, L-Arg +NADPH; L+D, L-Arg +DPI; c+N, c-PTIO +NADPH.

After treatment with L-Arg +DPI and staining with H_2DCF DA, the urediniospore showed faint fluorescence in the spores and germ tubes (**Figure 5I**).

There was a significant difference between treatments with c-PTIO and c-PTIO +NADPH in the urediniospores at 1 hpg and in the apex of germ tubes at 2–6 hpg after H_2DCF DA staining (**Figures 5C,J**). However, the MGL were not increased (**Figures 1** and **2**), which suggested that although NADPH could help to relieve NO depression and produce more ROS, it did not enhance the elongation of germ tubes.

It was also observed that only when the ROS and NO were promoted simultaneously (**Figure 5H**), the spore could germinate normally as in the distilled water treatment (**Figures 1** and **2**).

These results show that ROS plays a crucial role in the apical growth of urediniospores and NO is involved in the generation of ROS. ROS production was greatly increased by NADPH, further suggesting a role for NADPH as the source of ROS generation in the fungus.

ROS/NO Ratio

The fluorescence intensity was directly proportional to the content of NO or ROS, so the ratio of ROS/NO fluorescence intensity could indirectly reflect the ratio of ROS/NO content in germinated urediniospores.

The MFI values of urediniospores treatment with H_2O and L-Arg +NADPH (the urediniospores after the two treatments could germinate normally) were recorded and analyzed (**Table 1**).

The ratio of ROS/NO at different locations and time points showed that spores germinated normally when the ROS/NO ratio maintained within the range of 0.9–1.6.

DISCUSSION

In eukaryotes, NO is generated from many oxynitrides such as nitrite (NO_2^-) under acid conditions (Castello et al., 2008). In addition to chemical synthesis, NO can be generated by enzymatic reactions. In animals, NO is synthesized from O_2 and L-Arg by different NO synthase (Bonilla et al., 2004) isoforms (Nowles and Moncada, 1994). Ninnemann and Maier (1996) reported NO synthase activities in fungi for the first time. NO synthase activity was detected during sporulation in *Blastocladiella emersonii* (Bonilla et al., 2004), and activity decreased significantly with the addition of L-NAME (Vieira et al., 2009). These results suggested that there was an enzymatic pathway of NO synthesis in fungi that was similar to that of mammals. In our study, endogenous NO increased sharply when the substrate of NO synthase L-Arg was added. L-Arg generates NO through the NOS catalysis pathway. Green fluorescence could still be observed after staining with the specific NO fluorescence probe, and the intensity fluorescence decreased dramatically after c-PTIO treatment. Therefore, endogenous NO might be generated by the NOS pathway during the germination of *Pst* urediniospores. It has been suggested that NO synthesis in phytopathogenic fungi is derived from an L-Arg-dependent pathway by a NOS-like system, as in the ascomycete fungi *C. coccodes* and *B. graminis* (Wang and Higgins, 2005; Prats

TABLE 1 | Reactive oxygen species/NO ratio after H_2O and L+N treatments.

Treatment	Localization	Free radicals	Time points				
			1 h	**1.5 h**	**2 h**	**4 h**	**6 h**
H_2O	U	ROS	83.8 ± 5.3	15.7 ± 2.1	13.9 ± 1.5	10.3 ± 2.7	13.0 ± 3.1
		NO	63.8 ± 4.1	14.7 ± 1.3	13.1 ± 1.3	11.0 ± 2.7	12.1 ± 3.0
		ROS/NO	1.3	1.1	1.1	0.9	1.1
	B	ROS	0	57.0 ± 5.1	16.8 ± 2.7	11.6 ± 3.0	15.3 ± 2.1
		NO	0	37.0 ± 5.2	15.7 ± 2.2	11.2 ± 1.4	15.0 ± 2.8
		ROS/NO	/	1.5	1.1	1.0	1.0
	A	ROS	0	112.2 ± 7.1	114.8 ± 8.4	112.0 ± 7.0	110.9 ± 7.1
		NO	0	92.2 ± 6.0	94.8 ± 8.4	96.0 ± 8.1	94.9 ± 6.2
		ROS/NO	/	1.2	1.2	1.2	1.2
L+N	U	ROS	130.9 ± 5.9	68.7 ± 5.0	16.5 ± 2.2	15.5 ± 3.2	14.0 ± 3.0
		NO	80.9 ± 5.9	78.0 ± 6.7	14.2 ± 1.9	14.0 ± 3.7	14.4 ± 4.0
		ROS/NO	1.6	0.9	1.2	1.4	1.0
	B	ROS	0	120.4 ± 5.2	63.9 ± 5.0	31.9 ± 3.2	14.9 ± 2.2
		NO	0	110.4 ± 4.4	53.9 ± 3.4	21.9 ± 5.9	15.2 ± 5.9
		ROS/NO	/	1.1	1.2	1.5	1.0
	A	ROS	0	139.3 ± 8.2	138.3 ± 9.5	130.7 ± 8.2	129.9 ± 9.2
		NO	0	131.3 ± 7.4	128.3 ± 5.3	120.7 ± 4.3	119.9 ± 7.7
		ROS/NO	/	1.1	1.1	1.1	1.1

The MFI values of ROS and NO of the spores treated with H_2O and L + N (spores after the two treatments could germinate normally) was collected. The ratio of ROS/NO was calculated at different locations at different time points. U, urediniospore; B, base of germ tube; A, apex of germ tube.

et al., 2008). However, fungi do not contain NOS-like sequences in their genomes, except for *Aspergillus* species and *Glomerella graminicola* (Turrion-Gomez and Benito, 2011). Genetic studies indicated that NO synthesis in *M. oryzae* was not associated with an arginine-dependent pathway, although relatively weak NOS-like sequences were present in the genome (Samalova et al., 2013). It should be feasible to validate this result by identifying the NOS in *Pst* using the available genome sequence (Zheng et al., 2013).

There is growing evidence that certain specific enzymes, such as NADPH oxidase (NOX), produce ROS to regulate cellular functions, such as immunity, cell proliferation, cell differentiation, signal transduction, and ion transport (Finkel, 2003; Foreman et al., 2003; Kwak et al., 2003; Lambeth, 2004). In this study, it was found that although spore germination was restrained after NADPH treatment, a mass of green fluorescence was still detected by the ROS-specific fluorescence probe, and the intensity of this green fluorescence decreased dramatically after DPI treatment. Thus, during urediniospore germination, endogenous ROS might be generated by the NADPH pathway.

During the study, we observe that the wax layer and the epidermal hairs on the surface of the wheat leaves would cause the gathering of ROS/NO fluorescence probes which could interfere with the accuracy of the results by CLSM. This causes the difficulty to the research of the role of NO and ROS during the germination of urediniospore. Fortunately, *Pst*, as an obligate biotroph urediniospores, can germinate and form a germ tube on the water surface which cause the feasibility of the research in the pre-infection process *in vitro* under controlled conditions.

After hydration, a urediniospore germinates and develops a germ tube that can extend along the water surface. During germination, it is believed that fungal spores undergo an initial period of isotropic expansion associated with the uptake of water. Upon the establishment of a polarity axis, a short germ tube emerges and grows by apical extension, which is a defining feature of the filamentous fungi (Harris, 2006; Riquelme, 2013). Thus, the pre-infection development of *Pst* essentially involves the transition from isotropic growth to polarized growth, and it has been proposed that polarized hyphal growth requires the establishment of polarity during spore germination and maintenance of polarity during germ tube elongation (Momany, 2002). In this study, NO and ROS preferentially localized to the spore pore and apical region of the germ tube, suggesting that they are associated with these processes. Accumulating evidence indicates that there is a correlation between ROS production by NADPH oxidase and the polarized growth of fungal cells (Glasauer and Chandel, 2013). Localized production of ROS at the growing hyphal tips was detected by NBT or H_2DCF DA staining for several fungal species, including *Epichlöe festucae*, *M. grisea,* and *Aspergillus nidulans* (Tanaka et al., 2006; Egan et al., 2007; Semighini and Harris, 2008). Further experiments performed in *M. grisea* showed that the inhibition or scavenging of ROS production by the NADPH oxidase inhibitor DPI or by the antioxidant ascorbate inhibited or impaired fungal polarized growth, which was detected as inhibition or delay of germination of the conidia and aberrant morphology of the germ tubes or appressoria (Egan et al., 2007). In the mutualistic, endophytic fungus *E. festucae*, it was demonstrated that ROS generation requires the functional assembly of a multisubunit complex composed of NoxA, a regulatory component, NoxR, and the small GTPase RacA (Takemoto et al., 2007; Tanaka et al., 2008), whereas BemA and Cdc24, well-characterized regulators of polarity in yeast, were identified as interacting with the Nox complex via NoxR (Takemoto et al., 2011). Significantly, GFP fusions of NoxR, RacA, Cdc24, and BemA preferentially localized to actively growing hyphal tips, where they functioned as an activated NADPH oxidase enzyme complex responsible for the production of ROS (Takemoto et al., 2011). These results together with our observations indicate that the NADPH oxidase-dependent production of ROS plays a conserved role in polarized hyphal growth. It is well known that the fungal cytoskeleton plays a crucial role in polarity establishment, maintenance and polar growth (Harris, 2006; Riquelme, 2013). This finding was validated in *Pst* by functional analysis of the actin gene *PsACT1* (Liu et al., 2012). A recent study revealed that ROS may regulate filamentous polarized fungal growth by remodeling the arrangement of the F-actin cytoskeleton, whereas the latrunculin-mediated depolymerization of fungal appressorial F-actin is competitively inhibited by fungal NADPH oxidases mediated by ROS (Rydera et al., 2013). Thus, we can speculate that the production of ROS by the *Pst* NADPH oxidase complex in *Pst* regulates polarized growth by reorganizing components of the cytoskeleton, such as F-actin.

In addition to ROS, we provide evidence that NO is involved in polarized growth during spore germination and subsequent germ tube growth. As with ROS, interference in NO production by application of scavenger or promoter delayed spore germination and impaired germ tube growth. A similar role has also been indicated in other fungi. For instance, the application of external NO to *C. coccodes* delayed spore germination, whereas treatment with L-Arg accelerated the germination and development of conidiospores (Wang and Higgins, 2005). Similarly, in the hemibiotrophic ascomycete *M. oryzae,* NO scavengers delayed germination and reduced lesion formation (Samalova et al., 2013). These data and our observations collectively indicate that NO may have a role in signaling in spore germination and polarized growth in fungi.

Significantly, NO seems to act in concert with ROS to control germination and germ tube growth because the elevation or reduction of NO or ROS alone has a negative effect on these processes, whereas the accumulation of high level of NO and ROS results in normal growth in *Pst.* It is likely that a balance between NO and ROS, rather than these molecules functioning alone, allows germination to proceed while ensuring that it does so only under ideal environmental conditions (Wang and Higgins, 2005).

It has been demonstrated that NO and ROS signaling pathways in plant biotic interactions are closely connected (Scheler et al., 2013). Furthermore, there is evidence showing that ROS can influence NO levels and vice versa (Moncada and Erusalimsky, 2002; Desikan et al., 2004). For example, the regulation of ROS production by NO is thought to modulate the development of the hypersensitive response (HR), a programmed cell death involved in plant defense (Yun et al., 2011; Rasul et al., 2012). NO and ROS crosstalk during fungal development may be resolved only after the characterization of all fungal NOS isomers

(Wang and Higgins, 2005). We found that upon treatment with either the promoter or scavenger of NO, ROS generation in *Pst* urediniospores was inhibited. Similarly, upon treatment with either the donor or scavenger of ROS, the generation of NO was inhibited. A critical balance of ROS and NO seems to be essential in regulating urediniospore germination and germ tube development in *Pst* and other fungi.

The generation of ROS during the interaction of fungus and its host has been repeatedly studied. There is evidence that the host produces ROS (Wang et al., 2007, 2010; Zhang et al., 2012) and NO (Romero-Puertas et al., 2004; Piterkova et al., 2009; Sedlářvá et al., 2010; Melillo et al., 2011) during the early stages of infection. In incompatible interactions, ROS was detected in the stomata and the necrotic mesophyll cells following fungal penetration and the induction of HR (Wang et al., 2007, 2010). Similar results were obtained by our laboratory with regard to NO (Yin et al., unpublished). The generation of ROS and NO is also involved in systemic acquired resistance (Gao et al., 2014; Wendehenne et al., 2014).

Combining all of the results, we deduced that in addition to supplying itself for apical growth, it was likely that *Pst* also produced a small amount of ROS and NO to adapt to the highly oxidative conditions in the infected plant. The small amount of ROS and NO might protect the growth and development of the germ tube and the expansion of hyphae in the intercellular space from the harm of highly oxidative condition in the infected plant.

In future studies, a higher priority should be given to defining the molecular identity of the genes involved in NO and ROS biosynthesis and the relationship between NO and ROS in signaling transduction during germination and germ tube growth in *Pst*.

AUTHOR CONTRIBUTIONS

ZK and HZ designed experiments; SY carried out experiments; ZG analyzed experimental results; CW and LH joined the discussion and gave the original ideas; SY wrote the manuscript.

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The reviewer JA and handling Editor declared a current collaboration and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

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