Spermine Is a Potent Plant Defense Activator Against Gray Mold Disease on *Solanum lycopersicum*, *Phaseolus vulgaris*, and *Arabidopsis thaliana*

Hamed S. Seifi,¹ Adel Zarei,¹ Tom Hsiang,² and Barry J. Shelp^{1,†}

¹ Department of Plant Agriculture, University of Guelph, Guelph, Ontario N1G 2W1, Canada

² School of Environmental Sciences, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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ABSTRACT

Polyamines (PAs) are ubiquitous aliphatic amines that play important roles in growth, development, and environmental stress responses in plants. In this study, we report that exogenous application of spermine (Spm) is effective in the induction of resistance to gray mold disease, which is caused by the necrotrophic fungal pathogen *Botrytis cinerea*, on tomato (*Solanum lycopersicum*), bean (*Phaseolus vulgaris*), and *Arabidopsis thaliana*. High throughput transcriptome analysis revealed a priming role for the Spm molecule in the genus *Arabidopsis*, resulting in strong upregulation of several important defense-associated genes, particularly those involved in systemic-acquired resistance. Microscopic analysis confirmed that Spm application potentiates endogenous defense responses in tomato leaves through the generation of reactive

oxygen species and the hypersensitive response, which effectively contained *B. cinerea* growth within the inoculated area. Moreover, co-application of Spm and salicylic acid resulted in a synergistic effect against the pathogen, leading to higher levels of resistance than those induced by separate applications of the two compounds. The Spm plus salicylic acid treatment also reduced infection in systemic nontreated leaves of tomato plants. Our findings suggest that Spm, particularly when applied in combination with salicylic acid, functions as a potent plant defense activator that leads to effective local and systemic resistance against *B. cinerea*.

Keywords: biochemistry, cell biology

Botrytis cinerea, the causal agent of the gray mold disease, is the most economically important necrotrophic fungal plant pathogen in the world (Dean et al. 2012). Gray mold causes significant losses to various crops, including those growing under greenhouse or vineyard conditions and during postharvest storage, which is estimated at ~\$10 to \$100 billion per year (Zhao et al. 2009). Although conventional fungicides against the gray mold disease are available, there are situations where they do not offer complete control, and negative health and environmental consequences have been ascribed to some of them. Furthermore, this fungal pathogen possesses a high level of genetic plasticity and has developed resistance against a variety of pre- and postharvest fungicides (Jurick et al. 2017; Williamson et al. 2007). Therefore, the development of novel resistance-enhancing and ecofriendly controls for such an economically impactful disease is important for the production of field and horticultural crops as well as organic production in particular.

In response to phytopathogens, plants have evolved a sophisticated innate immune system consisting of both constitutive and inducible defense mechanisms. Inducible defense mechanisms include the generation of reactive oxygen species (ROS) (e.g., H₂O₂), the hypersensitive response (HR), the fortification of cell walls, and de novo synthesis of antimicrobial compounds, such as pathogenesis-related (PR) proteins and phytoalexins (Heath 2000; Jones and Dangl 2006). These defenses are normally quiescent in healthy plants; however, they are rapidly and strongly induced in response to a pathogenic challenge after timely perception of

specific stress signals (Meldau et al. 2012). Some nonpathogenic stimuli, such as root colonization by specific rhizobacteria and contact with certain synthetic or natural compounds known as plant defense activators (PDAs), also induce defense mechanisms in plants, a phenomenon commonly referred to as induced resistance (Walters et al. 2005). A well-characterized type of induced resistance is systemic-acquired resistance (SAR), which results in durable protection against a broad spectrum of pathogens (Conrath et al. 2006). PDAs elicit SAR in plants by boosting key defense-associated pathways, particularly the salicylic acid (SA) pathway (Gaffney et al. 1993), which provokes a “priming” state in the host, thereby leading to stronger and quicker responses to future pathogenic attacks (Aranega-Bou et al. 2014).

Polyamines (PAs) are ubiquitous aliphatic amines that serve various physiological and developmental roles in plants (Galston and Sawhney 1990). The biosynthesis and degradation of PAs are very responsive to different abiotic and biotic stimuli, suggesting that they function in plant–stress interactions (Alcázar et al. 2006; Walters 2003b). Furthermore, the major plant PAs, such as putrescine (Put), spermidine (Spd), and spermine (Spm), as well as their catabolic product diaminopropane (DAP), are known to modulate plant defense responses to phytopathogens (Cona et al. 2006; Jiménez-Bremont et al. 2014; Minocha et al. 2014; Walters 2003b). A specific signaling role for Spm in the induction of plant defense responses has been proposed (Mitsuya et al. 2009). Spm is known to accumulate in the intercellular spaces of tobacco mosaic virus-infected plants (Yamakawa et al. 1998), and Spm application/accumulation induces mitochondrial membrane dysfunction and H₂O₂ generation; expression/accumulation of defense-related proteins, such as PR and protein kinases; and resistance of plants to tobacco mosaic virus, cucumber mosaic virus, *Pseudomonas syringae*, or *Hyaloperonospora arabidopsidis* (Marco et al. 2014; Mitsuya et al. 2009; Takahashi et al. 2003, 2004; Yamakawa et al. 1998). Here, we demonstrated that, (i) compared with other PAs, exogenous application of Spm specifically induces strong resistance to *B. cinerea* in tomato, bean, and *Arabidopsis* plants; (ii) application of Spm primes the defense response in *Arabidopsis*

†Corresponding author: B. J. Shelp; bshelp@uoguelph.ca

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through induction of SAR and HR; and (iii) coapplication of Spm and SA results in a synergistic interaction with respect to disease suppression in the tomato–*B. cinerea* pathosystem. These findings provide novel insights into the effectiveness of Spm as a potent PDA against the most economically important necrotrophic phytopathogen, *B. cinerea*. Our study provides valuable information that could be used in the development of novel ecofriendly methods for plant disease management in sustainable crop production systems.

MATERIALS AND METHODS

Plant and fungal growth conditions. Tomato (*Solanum lycopersicum* L. cultivar Moneymaker), *Arabidopsis* (*Arabidopsis thaliana* L. Heyhn ecotype Columbia), and bean (*Phaseolus vulgaris* L. cultivar Yellow Bush Bean) plants were grown on Sunshine mix LC1 (Sungro Horticulture Inc.) potting soil in a naturally lighted greenhouse supplemented with high-pressure sodium lighting ($\sim 65 \text{ mmol m}^{-2} \text{ s}^{-1}$ at pot level) and provided with 16-h light at 25°C and 8-h dark at 20°C. Plants were fertilized with 200 ml of N-P-K (20-7-20) solution once weekly.

B. cinerea isolate 1305 was grown on 2% potato dextrose agar at 20 to 22°C in the dark. Plates were completely covered in mycelium after 1 week, and then, they were shifted to a 12-h light/12-h dark regime for 1 week to stimulate sporulation. A highly concentrated stock of *B. cinerea* conidial suspension was freshly prepared by washing spores from fully sporulated plates with sterile water. The final *B. cinerea* inoculation suspension was composed of one quarter-strength potato dextrose broth (PDB) containing 5×10^5 and 10^5 spores ml^{-1} in leaf disk and whole/detached leaf infection trials, respectively.

Inoculation method and infection trials. Infection trials with floating leaf disks were conducted essentially following Asselbergh et al. (2007). Briefly, leaf disks (1 cm in diameter) were cut using a cork borer from the fifth and sixth leaves of 5-week-old tomato plants, the first and second leaves of 2-week-old bean plants, and rosette leaves of 4-week-old *Arabidopsis* plants, and they were individually floated overnight in a 24-well plate on 1 ml of aqueous solutions of the desired compounds (i.e., Spm, Spd, Put, and DAP at specific concentrations), whereas control leaf disks were floated on sterile deionized water. After 16 h, a 10- μl droplet of the inoculation suspension was placed on the adaxial surface of each leaf disk, and the plates were incubated at $\sim 22^\circ\text{C}$ under dark conditions. Lesion diameters were measured at 3 to 5 days postinoculation (dpi) using an electronic caliper.

Infection trials were also conducted with whole detached fifth and sixth leaves of 5-week-old tomato plants (Seifi et al. 2013a). Briefly, the abaxial surface of the leaves of intact tomato plants was sprayed until runoff with the desired mixture of compounds, including 0.05% nonylphenol ethoxylate as an adjuvant, and the leaves were placed on four layers of moistened paper towels in a plastic tray sealed with plastic wrap. After 16 h, droplets of the inoculum suspension (2×10 - μl droplets per leaflet and 10 droplets per leaf in total) were placed on the adaxial surface of each leaf. For systemicity trials, the first, second, and third leaves of intact plants were sprayed, and after 16 h, the fifth and sixth nonsprayed leaves were detached before inoculation as described above. Lesion diameters were measured at 3 dpi as described above.

Visualization of defense responses. Inoculated leaf disks were sampled at several time points (hours postinoculation [hpi]), and they were cleared and fixed using 100% ethanol. Then, the leaf disks were mounted in 50% glycerol for additional microscopic analyses. The defense response was evaluated macroscopically and microscopically using several staining techniques. Cell wall fortifications were monitored by incubating the leaf disks in 0.01% safranin-O in 50% ethanol for 3 min followed by several washes in deionized water (Seifi et al. 2013a). Likewise, fungal structures were stained with 0.1% trypan blue in 10% acetic acid for 30 s and then, thoroughly washed (Seifi et al. 2013a). H_2O_2 generation/accumulation was visualized as a brown reddish

polymer sedimented in the leaf tissue by adding 2 mg ml^{-1} of 3,3'-diaminobenzidine (DAB) to a final concentration of 1 mg ml^{-1} 3 h before the sampling time (Asselbergh et al. 2007).

Statistical analysis. A linear mixed effects model was used to test for differences among the response to exogenous application of the various compounds studied, wherein “treatment” and “block” were assigned as “fixed” and “random” effects, respectively. The infection trials with tomato and bush bean were conducted with a randomized complete block design containing three or four blocks (i.e., plates or trays), wherein each treatment and/or sampling time in the block consisted of leaf samples derived from at least three different plants. Assumptions of normality for the data were confirmed using a Shapiro–Wilk test, and mean comparisons were conducted using a one-way analysis of variance in combination with Tukey’s honest significant difference test ($P \leq 0.05$). All statistical analyses were performed using R version 3.4.4. (R Core Team).

RNA extraction, sequencing, and data analysis. *Arabidopsis*–*B. cinerea* infection assays were conducted as described above with two modifications. Detached rosette leaves were incubated on 10 mM Spm for 16 h and then, inoculated with multiple 10-ml droplets to cover maximal surface area of the leaves with *B. cinerea* conidial suspension. Also, leaves were mock inoculated with one quarter-strength PDB without spores. Leaves were sampled at 16 hpi, flash frozen in liquid nitrogen, and stored at -80°C . In total, four treatments were prepared: (i) Spm treated and *B. cinerea* inoculated designated as SI, (ii) Spm treated and mock inoculated designated as SM, (iii) water treated and *B. cinerea* inoculated designated as WI, and (iv) water treated and mock inoculated designated as WM. For each treatment, one leaf each was collected from nine different plants and eventually pooled into three replicates (i.e., $n = 3$; each replicate contained one leaf from three different plants at the time of sampling). Also, extra leaves (from three different plants) were similarly inoculated and incubated for at least 3 dpi to monitor the progress of the infection.

Leaf samples were ground with a chilled mortar and pestle, and total RNA was extracted using the Bio Basic Rapid Plant RNA Isolation Kit (catalog number PT4191; Bio Basic Inc.) following the manufacturer’s instructions. RNA quantity and quality were assessed using the Agilent 2100 BioAnalyser system (Agilent Technologies) at the Genomics Facility of the University of Guelph. High-quality RNA samples (RNA integrity number ≥ 6.5) were sequenced at the Genome Quebec Innovation Center at McGill University in Montreal, Canada.

All RNA-seq data were analyzed using the Galaxy platform (accessible at <https://usegalaxy.org/>). Briefly, quality control of data was performed using FastQC (Andrews 2010) (accessible at <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Quality and adaptor trimmings were done using the Trimmomatic tool (Bolger et al. 2014). The Tuxedo-Suite pipeline (i.e., Tophat2, Cufflinks, Cuffmerge, and Cuffdiff) (Trapnell et al. 2012) was used for read alignment and mapping against *A. thaliana* TAIR10 Col-0 reference genome, transcriptome assembly, merging of assembly data, and differential gene expression analysis. The R package CummeRbund was used for visualization of the data generated. Significantly differentially expressed genes were identified as those with P value ≤ 0.01 and $\log_2$ fold change ≥ 2 . This output was used for Venn diagram generation using jvenn (Bardou et al. 2014) (accessible at <http://jvenn.toulouse.inra.fr/app/example.html>) and gene ontology enrichment (GOE) analysis using ClueGO (Bindea et al. 2009) (accessible at <http://apps.cytoscape.org/apps/cluego>) and AgriGO (Tian et al. 2017).

RESULTS

Application of Spm strongly suppressed *B. cinerea* infection of tomato and bean leaf disks. Exogenous application of 1, 2.5, or 5 mM Spm was more effective than the corresponding level of Spd, Put, or DAP in suppressing *B. cinerea* infection of tomato leaf disks by 3 dpi; notably, there was 75% disease

reduction with 5 mM Spm compared with the inoculated control (Fig. 1A). The disease suppression level was not affected by lowered pH of the incubating solution, because similar results were obtained with the addition of 0.1 M 2-(*N*-morpholino)-ethane-sulfonate buffer (pH 6) to the incubation solution (data not shown). Macroscopic analysis of the samples treated with 5 mM Spm revealed that the pathogen was restricted to the challenged area (i.e., the area located beneath the inoculation droplet) as well as the presence of a presumed HR ring around that area (Fig. 1B). Histochemical analysis of fungal structures and cell wall modifications revealed that the HR ring, containing *B. cinerea* mycelium, was located in the mesophyll (Fig. 1C). Similarly, 1 mM Spm reduced infection by ~85% in bean leaf disks (Supplementary Fig. S1).

Application of Spm strongly suppressed *B. cinerea* infection of *Arabidopsis* leaves and upregulated genes controlling the immune response, particularly SAR. Exogenous application of Spm onto rosette leaves of the genus *Arabidopsis* suppressed *B. cinerea* infection of *Arabidopsis* leaves in a dose-dependent manner, with the 10 mM level resulting, on average, in 88% disease suppression (Fig. 2A and B). Microscopic analysis showed that the *B. cinerea* mycelium grew normally on Spm-treated leaves, but no lesions were induced, whereas water-treated samples showed severe lesion development (Fig. 2C). Visualization of the defense-associated H₂O₂ generation with DAB showed an early accumulation of ROS at 16 hpi in Spm-treated and

B. cinerea-inoculated leaf samples, which was not visible at 24 or 48 h (Fig. 2D). Subsequently, the effect of 10 mM Spm treatment on global patterns of gene expression in *Arabidopsis* rosette leaves at 16 hpi was evaluated through RNA-seq. A summary of read alignments and mapping coverage is provided in Supplementary Table S1. Supplementary Figures S2 and S3 summarize the density plot and principal component analyses of the transcripts detected across different treatment groups. The overall pattern of the two plots indicates that the Spm treatment induced a distinctive and strong transcriptional reconfiguration in the genus *Arabidopsis*. The number of shared and specifically upregulated genes (cuff offs: $P \leq 0.01$, log₂ fold change ≥ 2) in the WI, SM, and SI treatment groups versus the control treatment (WM) are shown in Figure 3A (a complete list of these genes is provided in Supplementary Table S2). A set of 2,599 genes that were exclusively upregulated in response to Spm but were not overlapping with the WI/WM susceptible interaction (i.e., SM-[SM $\cap$ WI]) (Fig. 3A, dashed area) was selected for the downstream GOE analysis. These differentially expressed genes (DEGs) highlighted significantly activated molecular functions related to defense signaling and response to environmental stimuli, such as programmed cell death, apoptosis, protein kinase activity, and DNA binding (Fig. 3B), as well as specific GOE terms related to plant immune responses and SAR (Fig. 3C). Notably, the H₂O₂-generating PA oxidase genes *PAO1* (AT5G13700.1), *PAO2* (AT2G43020.1), and *PAO4* (AT1G65840.1) were among the genes significantly induced in response to Spm, with *PAO1* showing the

Fig. 1. Impact of spermine (Spm), spermidine (Spd), putrescine (Put), and diaminopropane (DAP) on *Botrytis cinerea* infection of tomato leaf disks 3 days postinoculation. **A**, Dose-dependent disease suppression. Within each concentration, means not possessing the same letter are significantly different according to a one-way analysis of variance and Tukey's test ($P \leq 0.05$). **B**, Dose-dependent response to Spm at the macroscopic level. The red lines indicate the width of the lesion on a 1-cm-wide leaf disk. **C**, Induction of a mesophylllic hypersensitive response-like reaction (black arrow) around the site of pathogen ingress (yellow dashed line) by 5 mM Spm. The blue string-like network represents mycelial structures of *B. cinerea*, showing the restriction of pathogen growth within boundaries of the inoculation droplet. Scale bar = 50 μ m.

strongest upregulation in both SM/WM and SI/WM treatment groups (i.e., $\log_2$ fold change = 6.89 and 5.96, respectively). Moreover, several key SAR-associated genes, such as flavin-dependent monooxygenase 1 (*FMO1*; AT1G19250.1), isochorismate synthase 1 (*ICS1*; AT1G74710.1) and the transcription factor *WRKY70* (AT3G56400.1), were specifically identified to be strongly upregulated ($\log_2$ fold change = 9.47,

8.36, and 3.88, respectively) in response to Spm treatment (Fig. 3D and Supplementary Table S3), exhibiting distinctive expression pattern in Spm-treated samples (SM/WM and SI/WM) compared with the water-treated WI/WM group (Fig. 3E). A list of Spm-induced genes involved in host immune response is provided in Supplementary Table S4.

Fig. 2. Dose-dependent effect of spermine (Spm) on *Botrytis cinerea* infection of *Arabidopsis* rosette leaves 3 days postinoculation. **A**, Lesion size. Data represent mean $\pm$ standard error ($n = 4$). **B**, Disease suppression. Bars not possessing the same letter are significantly different according to a one-way analysis of variance and Tukey's test ($P \leq 0.5$). **C**, Macroscopic and microscopic symptoms after treatment with 10 mM Spm or water (Ctrl). The micrograph (lower panel) shows normal growth of *B. cinerea* mycelia, which is fully suppressed within the boundaries of the inoculation droplet (dashed yellow line). **D**, Pattern of reactive oxygen species accumulation in inoculated leaf disks.

Coapplication of Spm and SA synergistically suppressed *B. cinerea* infection on tomato leaf disks. Coapplication of 1 mM SA and 1 mM Spm resulted in 92, 80, and 63% suppression of disease severity in tomato leaf disks at 3, 4, and 5 dpi, respectively (Fig. 4A and B). The level of disease suppression with

the coapplication of 1 mM Spm and SA was markedly higher than that obtained with separate applications of either Spm or SA at the corresponding time points. Notably, mycelial growth in treated samples was generally restricted to the challenged area (Fig. 4C), and both epidermal (mosaic-shaped) and mesophyll

Fig. 3. Transcriptome analysis of spermine (Spm)-induced suppression of *Botrytis cinerea* infection on *Arabidopsis* rosette leaves. **A**, Venn diagram showing the unique and overlapping sets of genes significantly ($P \leq 0.01$) and differentially ($\log_2$ fold change ≥ 2) upregulated in the various treatments (i.e., SI: spermine treated and *B. cinerea* inoculated; SM: spermine treated and mock inoculated; WI: water treated and *B. cinerea* inoculated) compared with the water control (i.e., WM: water treated and mock inoculated). The set of 2,599 differentially expressed genes (DEGs) belonging to SM-(SM $\cap$ WI; dashed area) was selected for the downstream gene ontology enrichment analysis. **B** and **C**, Enriched "Molecular Function" and "Immune Response" gene ontology terms of the selected genes. **D**, DEG analysis of genes involved in the induction of systemic-acquired resistance (SAR). **E**, Principal component (PC) analysis of the SAR-associated DEGs detected in SI/WM, SM/WM, and WI/WM.

(cylinder-shaped) HR reactions were observed in most of the Spm/SA-treated samples (Fig. 4D). The coapplication of 0.5 mM Spm and 3 mM SA gave the highest synergism among the various concentration combinations tested (Fig. 4E). Furthermore, 0.5 mM

Spm did not show any inhibitory effect on in vitro growth rate and sporulation (not shown) of *B. cinerea*, whereas separate applications of 3 mM SA and a mixture of 0.5 mM Spm and 3 mM SA had weak fungistatic effects (Fig. 5A and C). However, 3 mM SA did not

Fig. 4. Impact of coapplication of spermine (Spm) and sodium salicylate (SA), applied singly and in combination (1 mM each), on *Botrytis cinerea* infection on tomato leaf disks 3 to 5 days postinoculation (dpi). **A**, Lesion size. Data typically represent mean $\pm$ standard error ($n = 4$). **B**, Disease suppression. For each dpi group, means not possessing the same letter are significantly different ($P \leq 0.05$). **C**, Disease suppression 3 dpi at the macroscopic level. **D**, Disease suppression 3 dpi at the microscopic level. Induction of both epidermal hypersensitive response (HR; red arrow) within the challenged area (indicated by the yellow dashed line) and mesophyll HR ring (black arrow) around the challenged area. Growth of *B. cinerea* mycelia (the blue string-like structures) is suppressed within the boundaries of the inoculation droplet. Scale bar = 50 μ m. **E**, Optimization of the synergistic response for disease suppression. This is expressed as a synergy index (i.e., ratio of disease suppression with the mixture divided by the sum of the disease suppression with the single compounds). Ctrl, water.

obviously negatively affect the growth of *B. cinerea* on tomato leaf disks (Fig. 5B). These findings indicate that the fungus responded differentially to applications or coapplications of Spm and SA in vitro and on the foliage (Fig. 5C).

Coapplication of Spm and SA potentiated early apoplastic ROS generation in tomato leaf disks inoculated with *B. cinerea*. The spatiotemporal pattern of H₂O₂ generation in tomato leaf disks revealed no accumulation in water- or SA-treated samples from 0 to 24 hpi (Fig. 6A, Ctrl and SA rows). However, strong H₂O₂ accumulation was evident within and outside the boundaries of the challenged area at 48 hpi with both treatments, which corresponded to severe lesion development by 72 hpi (Fig. 6A). Application of Spm induced moderate H₂O₂ accumulation beneath the challenged area at 8 and 12 hpi (Fig. 6A, Spm row), mainly in the apoplastic space of the leaf tissue (Fig. 6B, left panel). At 24 and 48 hpi, the Spm treatment showed H₂O₂ accumulation accompanied with dark brown HR-like spots within and slightly around the challenged area (Fig. 6A, Spm row), leading to slightly

spreading lesions (Fig. 6A, Spm row) (72 hpi). A similar situation was observed at 8 and 12 hpi with the coapplication of Spm and SA (Fig. 6A, Spm/SA row). However, a restricted, highly localized accumulation of H₂O₂ within the challenged area only was evident at 48 hpi with Spm/SA (Fig. 6A, Spm/SA row), lending support for the strong HR and full resistance response seen at 72 hpi (Fig. 6A, Spm/SA row). Also, Spm/SA-induced HR reactions could be detected in the epidermal layers of the challenged area at 48 hpi (Fig. 6B, right panel). Notably, the length of *B. cinerea* hyphae on the tomato leaf surface at 8 hpi, which is the typical time for cell penetration (Asselbergh et al. 2007), did not differ between the water- and Spm/SA-treated samples (Fig. 6C and D), suggesting that Spm and SA coapplication did not adversely affect *B. cinerea* growth.

Coapplication of Spm and SA suppressed *B. cinerea* infection on both sprayed and systemic (nonsprayed) detached leaves. Spraying of Spm and SA mixtures (1/3 mM) onto intact leaves of tomato plants 16 h before inoculation

Fig. 5. Impact of spermine (Spm; 0.5 mM) and 3 mM sodium salicylate (NaSA), applied singly and in combination, on the growth of *Botrytis cinerea* mycelium. **A**, Macroscopic examination of in vitro culture. **B**, Macroscopic examination on tomato leaf disks. **C**, Suppression in in vitro culture and on leaf disks. Data represent the mean $\pm$ standard error of three biological replicates. Ctrl, water.

suppressed disease severity in the sprayed leaves by 41% at 3 dpi (Fig. 7A to C). Similarly, when only the first, second, and third leaves of intact plants were sprayed with the same Spm/SA mixture, 40% disease suppression was observed in the upper systemic nonsprayed leaves (the fifth and sixth leaves) at 3 dpi (Fig. 7D to F).

DISCUSSION

In this study, we report that, among the major PAs in plants (i.e., Spm, Spd, and Put) and their catabolic product DAP, the exogenous application of Spm had the greatest potential to induce effective control of *B. cinerea* infection in tomato, bean, and the

Fig. 6. Impact of spermine (Spm; 1 mM) and sodium salicylate (SA; 1 mM), singly and in combination, on reactive oxygen species (ROS) accumulation and hyphal growth in tomato leaf disks infected with *Botrytis cinerea*. **A**, Time course of ROS accumulation as indicated by the brownish orange color/halo (red arrows). **B**, left panel, Apoplastic accumulation of H_2O_2 in Spm-treated samples at 8 hpi. **B**, right panel, Epidermal hypersensitive response reaction in Spm/SA-treated samples at 72 h postinoculation (hpi). Scale bar = 50 μ m. **C** and **D**, Hyphae growth at 8 hpi on water (Ctrl)- and Spm/SA-treated tomato leaf disks.

genus *Arabidopsis* and seemed to activate potent plant defense mechanism(s) rather than exert an antimicrobial effect. This was confirmed through RNA-seq-based transcriptome analysis showing that exogenous application of Spm induced immune response and SAR-associated genes in the genus *Arabidopsis*. Moreover, co-application of Spm and SA was found to induce even stronger protection than Spm alone in a synergistic manner in tomato plants. Additionally, the Spm/SA mixture induced resistance against *B. cinerea* in nontreated leaves detached from the same plants, suggesting that it acts in a systemic manner.

Our results suggest a special role for Spm in modulating plant defense response against *B. cinerea* infection. Previously, Yamakawa et al. (1998) reported that exogenous application of Spm reduces susceptibility of tobacco leaves to tomato mosaic virus in a dose-dependent manner. Of all of the PAs tested, including Put and Spd, only Spm induces strong upregulation of the classic defense marker gene *PR1* through an SA-independent pathway. Similarly, exogenous Spm fully suppresses cauliflower mosaic virus yellow in the genus *Arabidopsis*, whereas Spd and Put have little and no effect, respectively (Mitsuya et al. 2009). Other research indicated that Spm treatment strongly induces different defense-related genes in *Arabidopsis* seedlings, whereas similar doses of Put and Spd treatments are totally ineffective, and higher levels of Spm in Spm

synthase overexpression lines of the genus *Arabidopsis* are causally linked to higher tolerance to the bacterial pathogen *Pseudomonas syringae* and the oomycete *H. arabidopsidis* (Marco et al. 2014). Likewise, our transcriptome analysis indicated that Spm induced several defense-related genes in the genus *Arabidopsis*, particularly those involved with the establishment of SAR (Fig. 3C and D). *FMO1*, *ICS1*, and *WRKY70* in particular were strongly upregulated. Previous research has shown that *Arabidopsis fmo* mutants are impaired in the establishment of SAR triggered by both avirulent and virulent *Pseudomonas syringae* bacteria as well as the systemic accumulation of SA and systemic expression of several defense-related genes (Mishina and Zeier 2006). Furthermore, *ICS1* is essential for the biosynthesis of SAR-associated SA (Wildermuth et al. 2001), and *WRKY70* integrates signals from salicylate- and jasmonate-mediated defense pathways, which are mutually antagonistic (Li et al. 2004). Notably, *WRKY70* is involved in the regulation of SA biosynthesis and the SAR response (Wang et al. 2006). Additional research is clearly necessary to unravel molecular mechanisms that control Spm-induced SAR.

Although the exact function of the PAs, particularly Spm, during incompatible plant-pathogen interactions is not yet known, the favored hypothesis attributes the induction of cell death and HR and

Fig. 7. Impact of spermine (Spm; 1 mM) and sodium salicylate (SA; 3 to 4 mM) mixtures on *Botrytis cinerea* infection of directly sprayed and systemic (nonsprayed) tomato leaves 3 days postinoculation. **A**, Lesion size on sprayed leaves. Data represent mean $\pm$ standard error ($n = 4$). **B**, Disease suppression on directly sprayed leaves. Bars not possessing the same letter are significantly different according to a one-way analysis of variance (ANOVA) and Tukey's test ($P \leq 0.5$). **C**, Disease symptoms of directly sprayed leaves. **D**, Lesion size on systemic leaves. Data represent mean $\pm$ standard error ($n = 4$). **E**, Disease suppression on systemic leaves. **F**, Disease symptoms on systemic leaves. *Significantly different from the control (Ctrl) according to a one-way ANOVA and Tukey's test ($P \leq 0.5$).

consequently, the expression of defense genes to H₂O₂ production via PA oxidation (Jiménez-Bremont et al. 2014; Seifi et al. 2013b; Takahashi et al. 2004; Walters 2003a). In this study, we found traces of early apoplastic H₂O₂ accumulation in Spm- and Spm/SA-treated leaves, which did not coincide with the signs of epidermal or mesophyll HR at 4 and 8 hpi (Fig. 6), suggesting that the level of the early H₂O₂ burst was tightly controlled to prevent cell death. A similar situation was observed in Spm-treated *Arabidopsis* leaves (Fig. 2D), and it was associated with the induction of the H₂O₂-generating *PAO1* (AT5G13700.1), *PAO2* (AT2G43020.1), and *PAO4* (AT1G65840.1) genes at 16 hpi. Such early H₂O₂ generation impulses in response to pathogen ingress are generally known to serve a defense-associated signaling role, activating downstream defense mechanisms in the host tissue (Conrath et al. 2006). Notably, at later stages of the interaction (i.e., 24 and 48 hpi), clear signs of strong but highly localized HR reaction were detected in tomato leaf disks (Fig. 6). Presumably, restricting the extent of oxidative stress during early stages of the interaction followed by a highly localized HR prevents the cell death-favoring invasion strategy of the necrotrophic *B. cinerea* from being initiated on penetration of the host tissue. This finding is in accordance with previous studies showing that, despite early H₂O₂ generation in the *sitiens* tomato mutant, the extent of cell death is tightly controlled, leading to strong HR-dependent resistance against *B. cinerea* (Asselbergh et al. 2007; Curvers et al. 2010; Seifi et al. 2013a). Interestingly, the SA signaling pathway plays an important role in the *sitiens* resistance response to *B. cinerea* (Audenaert et al. 2002). Moreover, exogenous application of the SA analog, benzothiadiazole, reduces susceptibility to *B. cinerea* in tomato, bean, and cucumber (Azami-Sardooei et al. 2013). These observations may explain why coapplication of Spm and SA in tomato led to a synergistically higher level of resistance to *B. cinerea* (Fig. 2), presumably through the induction of a stronger SAR response in the host. An interaction between Spm and SA has been reported for the chickpea-*Fusarium oxysporum* pathosystem, where coapplication of Spm and SA elevates the activities of two defense-related enzymes, polyphenol oxidase and phenylalanine ammonia lyase (Raju et al. 2008). Notably, a wilt disease-resistant cultivar exhibits an additive interaction between Spm (0.01 mM) and SA (0.8 mM), whereas a susceptible cultivar does not (Raju et al. 2009). Likewise, the Spm and SA coapplication induces defense-associated protease inhibitors in the resistant cultivar only (Raju et al. 2009). In our study, however, the susceptible wild-type tomato was highly responsive to Spm and the Spm/SA mixture, resulting in strong resistance against *B. cinerea*. Collectively, our data highlight the importance of an SA-natured SAR response in the induction of resistance to *B. cinerea* and do not support the widely held view that SA-mediated defense pathways are specifically associated with immunity against biotrophic pathogens, whereas jasmonate-dependent defense responses are effective only against necrotrophs (Glazebrook 2005).

The economic importance of gray mold disease (Dean et al. 2012) highlights the necessity for effective but environmentally safe controls against *B. cinerea*. To our knowledge, this study is the first report of effective gray mold control on tomato, bean, and the genus *Arabidopsis* by exogenous application of the PA Spm as well as the synergistic interaction of Spm and SA coapplication on induced resistance in tomato. Considering the natural origin of the two compounds, this finding could be a significant step toward establishing novel ecofriendly methods to control not only *Botrytis* blight but possibly, many other important plant pathogens with a similar necrotrophic mode of infection, such as *Sclerotinia sclerotiorum* and *Alternaria solani*. Furthermore, our transcriptomics data provided a baseline for future mechanistic studies to further elucidate the molecular basis underlying Spm-induced defense activation in plants.

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