The Aspergillus flavus Spermidine Synthase (spds) Gene, Is Required for Normal Development, Aflatoxin Production, and Pathogenesis During Infection of Maize Kernels

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Aspergillus flavus is a soil-borne saprophyte and an opportunistic pathogen of both humans and plants. This fungus not only causes disease in important food and feed crops such as maize, peanut, cottonseed, and tree nuts but also produces the toxic and carcinogenic secondary metabolites (SMs) known as aflatoxins. Polyamines (PAs) are ubiquitous polycations that influence normal growth, development, and stress responses in living organisms and have been shown to play a significant role in fungal pathogenesis. Biosynthesis of spermidine (Spd) is critical for cell growth as it is required for hypusination-mediated activation of eukaryotic translation initiation factor 5A (eIF5A), and other biochemical functions. The tri-amine Spd is synthesized from the diamine putrescine (Put) by the enzyme spermidine synthase (Spds). Inactivation of spds resulted in a total loss of growth and sporulation in vitro which could be partially restored by addition of exogenous Spd. Complementation of the Δspds mutant with a wild type (WT) A. flavus spds gene restored the WT phenotype. In WT A. flavus, exogenous supply of Spd (in vitro) significantly increased the production of sclerotia and SMs. Infection of maize kernels with the Δspds mutant resulted in a significant reduction in fungal growth, sporulation, and aflatoxin production compared to controls. Quantitative PCR of Δspds mutant infected seeds showed down-regulation of aflatoxin biosynthetic genes in the mutant compared to WT A. flavus infected seeds. Expression analyses of PA metabolism/transport genes during A. flavus-maize interaction showed significant increase in the expression of arginine decarboxylase (Adc) and S-adenosylmethionine decarboxylase (Samdc) genes in the maize host and PA uptake transporters in the fungus. The results presented here demonstrate that Spd biosynthesis is critical for normal development and pathogenesis of A. flavus and pre-treatment of a Δspds mutant with Spd or Spd uptake from the host plant, are insufficient to restore WT levels.
INTRODUCTION

Mycotoxin contamination in food and feed crops is a global problem. Exposure to mycotoxins primarily occurs through the consumption of contaminated seeds/edible plant parts by humans and livestock. The majority of mycotoxin contamination in crop plants comes from the fungal genera, Aspergillus, Fusarium, and Penicillium, with Aspergillus causing the greatest adverse economic and health impacts (Ismaiel and Papenbrock, 2015; Mitchell et al., 2016; Umesha et al., 2016). Contamination of crops with aflatoxins has been shown to be intensified during episodes of drought (Kebede et al., 2012; Fountain et al., 2014). Increases in aflatoxin contamination in maize during episodes of drought and heat stress is believed to be due to the response of A. flavus to increased oxidative stress as well as impairment of host plant defense responses (Fountain et al., 2014, 2016). Although drought tolerance alone does not necessarily result in increased aflatoxin resistance in maize, drought tolerance accompanied with aflatoxin resistance would be ideal in reducing aflatoxin accumulation in maize during drought (Hamidou et al., 2014; Farfan et al., 2015; Fountain et al., 2015). Aflatoxin contamination in maize results in economic losses of almost $700 million/year in the U.S. based on a study conducted in 2013 (Mitchell et al., 2016). Based on global climate change predictions, it is estimated that losses resulting from aflatoxin contamination of maize could be as high as US$1.68 billion/year in the United States (Mitchell et al., 2016). The three predominant PAs widely found in living organisms are, Put (diamine), Spd (tri-amine), and Spm (tetramine). The diamine Put is produced by Odc (E.C.4.1.1.17) from Orn and/or by Adc (EC 4.1.1.19) from Arg. Higher PAs, Spd, and Spm are synthesized by the action of Spds (E.C.2.5.1.16) and Spms (E.C.2.5.1.22) from Put and Spm, respectively (reviewed in Shao et al., 2012). Both reactions require decarboxylated S-adenosylmethionine (dcSAM) that is produced by Samdc (E.C.4.1.1.50). Spm and Spd on the other hand can be back-converted to Spd and Put, respectively, by Spd/Spm N1-acetyltransferase (Sat; E.C.2.3.1.57 – mostly in animals) and Pao (E.C.1.5.3.11 – animals and plants). The predominant PAs that are often found in fungi are Put and Spd, while some fungal genera might lack Spm (reviewed in Valdés-Santiago et al., 2012). Given the requirement of PAs to maintain normal growth, development, and pathogenesis, the PA biosynthetic pathway (Figure 1) has often been a target to restrict fungal pathogenesis in both plants and animals (reviewed in Valdés-Santiago et al., 2012). In fungi, while Put is associated with hyphal growth, Spd has been implicated in cellular events associated with cell division, sporulation, and mycotoxin production. In the model fungus Aspergillus nidulans (A. nidulans), inactivation of spds altered fungal growth in the mutant (an auxotroph for Spd) and reduced sterigmatocystin production in vitro (Jin et al., 2002). In the human pathogenic fungus Penicillium marneffei (P. marneffei), impairment of Spd biosynthesis in a samdc (sada) mutant reduced growth, conidiogenesis, spore germination, and temperature-dependent dimorphic transition (Kummasook et al., 2013). Exogenous supply of Spd to the P. marneffei sada mutant could restore the WT phenotype. In wheat, early activation of the PA biosynthetic pathway has been reported in response to Fusarium head blight and PA pathway intermediates have been correlated with the production of deoxynivalenol (DON;
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FIGURE 1 | Polyamine (PA) pathway. Overview of the PA biosynthetic pathway (modified from Majumdar et al., 2015) in connection with amino acids (AAs) and tricarboxylic (TCA) cycle metabolites in plants and fungi. Dashed arrows indicate multiple steps. Abbreviations of enzymes, with EC numbers: AL, argininosuccinate lyase (EC 4.3.2.1); arginase (EC 3.5.3.1); AS, argininosuccinate synthase (EC 6.3.4.5); ODC, ornithine decarboxylase (EC 4.1.1.17); OTC, ornithine transcarbamylase (EC 2.1.3.3); PAO, polyamine oxidase (EC 1.5.3.11); SAMDC, S-adenosylmethionine decarboxylase (EC 4.1.1.50); SPDS, spermidine synthase (EC 2.5.1.16); SPMS, spermine synthase (EC 2.5.1.12); SSAT, Spd/Spm N1-acetyl-transferases (EC 2.3.1.57). Other abbreviations: dcSAM, decarboxylated S-adenosylmethionine; GABA, γ-aminobutyric acid; Glu, glutamate; OAA, oxaloacetic acid; P5C, Δ1-pyrroline-5-carboxylate; Pro, proline; Put, putrescine; SAM, S-adenosylmethionine; Spd, spermidine; Spm, spermine; TCA, tricarboxylic acid.

Polyamines are common to both plants and fungal pathogens, and their metabolism can play significant roles in host defense as well as successful pathogenesis and mycotoxin production during compatible host-pathogen interactions (Gardiner et al., 2009, 2010; Valdés-Santiago and Ruiz-Herrera, 2013; reviewed in Takahashi, 2016). It was demonstrated that the fungus Tapesia yallundae, rendered avirulent due to inactivation of the odc gene, had virulence restored upon re-introduction back into plants, possibly due to the uptake of PAs from the host thus compensating for PA depletion in the pathogen (Mueller et al., 2001). One of the important genes in higher PA biosynthesis is spds, whose product Spd, plays a critical role in mRNA translation due to its requirement for hypusination-mediated activation of eukaryotic translation initiation factor 5A (eIF5A) in all organisms, including fungi (Martinez-Rocha et al., 2016). The current study was undertaken to investigate if the A. flavus spds (AFLA_017920) gene could be a suitable target to reduce fungal pathogenesis during maize seed infection. Our results show that inactivation of spds in A. flavus significantly reduced fungal infection, sporulation and aflatoxin production in vitro and during maize seed infection.

MATERIALS AND METHODS

Fungal Strains, Media, and Growth Conditions

Aspergillus flavus CA14 (Δku70, ΔpyrG, ΔniaD) was used as host for transformation. Unless otherwise stated, CA14 pyrG-1, transformed with plasmid pPG2.8 (Cary et al., 2015) carrying the A. parasiticus pyrG gene was used as control for all experiments. A WT A. flavus 70 (AF70), capable of producing significantly higher levels of aflatoxins and sclerotia than CA14, was used to study the effect of PAs on SM and sclerotia production. A WT CA14 was used as the control for maize kernel infection studies. Fungal strains were cultured on Czapek-Dox (CZ) medium (Difco, BD). The medium was supplemented with Spd (0.5 or 0.25 mM) or Spm (0.2 or 0.1 mM) [both Spd and Spm were purchased from Sigma-Aldrich, St. Louis, MO,
United States] as required. As CZ medium is not conducive for aflatoxin production, A&M medium (Matales and Adye, 1965) supplemented with Spd and Spm was used to study the effects of PAs on aflatoxin production. Solid media were prepared by adding 15 g L\(^{-1}\) of agar. Fungal cultures were grown in light or dark at 30°C.

For maize kernel inoculation studies, an aflatoxin-producing A. flavus CA14 WT strain was obtained from the SRRC fungal collection (SRRC 1436; USDA Agricultural Research Service, New Orleans, LA, United States). The fungal strain was grown on CZ agar medium for 7 days at 30°C with illumination. Conidia were harvested by flooding each plate with 20 ml of 0.02% (v/v) sterile Triton X-100 solution and gently dislodging conidia from the surface mycelia using a sterile scraper. Conidial suspensions were adjusted to 4 x 10\(^6\) spores/ml prior to inoculation of kernels.

**Generation of Deletion and Complementation spds Strains**

The spds (AFLA_017920) deletion cassette was constructed by fusion PCR as described by Szewczyk et al. (2006). The 5' and 3' regions flanking the spds gene were PCR amplified from A. flavus CA14 genomic DNA using primer sets Spds_5F/Spds_5R and Spds_3F/Spds_3R, respectively (Supplementary Table S1). The middle fragment containing the pyrG marker gene was PCR amplified from the cDNA of A. parasiticus (BN9 strain), using the primers pyrG_F and pyrG_R (Supplementary Table S1). The three PCR fragments were then fused together through PCR using the nested primer pair Spds_nest-F and Spds_nest-R. The final PCR product (3739 bp) was used for polyethylene glycol-mediated transformation of A. flavus CA14 protoplasts as described by Cary et al. (2006). A number of putative Δspds transformants (Δku70, ΔniaD, pyrG+) were isolated and analyzed by PCR. Replacement of the spds coding region with pyrG selectable marker was confirmed through PCR using the primer pair Spds_5F and pyrG_R (Supplementary Figure S1). CA14 transformed with plasmid pPG2.8 (Cary et al., 2015) carrying the A. parasiticus pyrG gene was used as an isogenic control (referred to as the control).

To genetically complement the CA14 Δspds mutant, the spds gene region including 615 bp of the 5' UTR and 286 bp of the 3' UTR was PCR amplified from WT CA14 gDNA using prom_F2 and term_R primers. The A. parasiticus (BN9 strain) pyrG selectable marker gene was PCR amplified using primer pair pyrG_F1 and pyrG_R1, and was fused to the 2.6 kb spds PCR product through overlap fusion PCR using primer pairs prom_nest_F and pyrG_nest_R, that generated a 4.1 kb PCR product. The final PCR product (spds-pyrG, 4.1 kb) was used for polyethylene glycol-mediated transformation of Δspds CA14 (Δku70, ΔniaD, pyrG+) protoplasts. To select genetically complemented Δspds transformants (Δspds\(^C\), Δku70, ΔniaD, pyrG+), protoplasts (post-transformation) were plated onto regeneration medium (Cary et al., 2006) without Spd. Replacement of the Δspds coding region with the spds-pyrG complementation PCR product was confirmed through PCR using the primer pair 017920prom_nest-F and pyrG_nest-R (Supplementary Figure S2). PCR amplifications for creating the Δspds knockout construct were performed using ExTaq HS polymerase (Takara Bio, Inc., Mountain View, CA, United States), while the spds-pyrG complementation construct was generated using Phusion polymerase (New England BioLabs, Ipswich, MA, United States). The primers used in construction of the knockout mutant and complementation strain are listed in Supplementary Table S1.

**Nucleic Acid Isolation and Analysis**

Fungal genomic DNA was extracted from mycelia following 24 h incubation with shaking (250 rpm) at 30°C in CZ broth using a MasterPure Yeast DNA Purification Kit (Epicentre, Madison, WI, United States) according to the manufacturer's instructions. To confirm the successful integration of either knockout or complementation gene cassettes, genomic DNA isolated from transformants was PCR amplified using ExTaq HS polymerase and specific primer pairs listed in Supplementary Table S1.

**Morphological Analysis**

Conidia (10\(^6\) spores/ml) of the CA14 control and Δspds mutant strains were used to inoculate CZ agar plates with or without Spd supplementation and grown under illumination at 30°C. Six-millimeter diameter cores were collected from the center of each colony (from three replicate plates) at 5 days post-inoculation (dpi). The cores were homogenized, and conidia were counted using a Hemocytometer (Hauser Scientific, Horsham, PA, United States) and a Leitz Laborlux S bright-field microscope (Leica Microsystems, Inc., Buffalo Grove, IL, United States).

The AF70 strain was used to study the effects of PAs on sclerotia and SM production. Conidia (10\(^6\) spores/ml) of AF70 were point inoculated (2 μl) at the center of CZ agar plates supplemented with Spd (0.25 and 0.50 mM) or Spm (0.1 and 0.2 mM) along with control plates (CZ only) in duplicate. The culture plates were grown in the dark at 30°C. After 14 days, sclerotia were counted using a Leitz Laborlux S bright-field microscope under 10× optical lens. Plate images were captured using a PowerShot SD790 IS camera (Canon USA Inc., Melville, NY, United States).

**Maize Kernel Inoculation and Incubation**

Undamaged and roughly uniform sized maize (Zea mays var. B73) kernels were randomly assigned and processed according to a kernel screening assay (KSA; Rajasekaran et al., 2013). All kernels were surface sterilized using 70% ethanol, air dried, and stored under sterile conditions. Kernels were inoculated by immersion into a suspension of 4 x 10\(^6\) spores/ml of the CA14 WT (grown on CZ agar medium), CA14 WT w/Spd (CZ agar medium + 0.5 mM Spd), and Δspds w/Spd (CZ agar medium with ammonium sulfate + 0.5 mM Spd) strains followed by stirring for 3 min. Following removal of excess inoculum, the kernels were transferred to plastic caps that were placed in trays with a sheet of 3 MM paper on the bottom. The paper was saturated with sterile ddH\(_2\)O and the tray covered with a lid. The kernels were incubated under high RH (>90%) at 31°C in the dark for 8 days. The filter papers inside the trays were kept...
Quantification of Polyamines and Amino Acids

Freeze dried mycelia were subjected to three cycles of freezing (−20°C) and thawing (room temperature) in 5% PCA. After the final thaw, samples were vortexed for 2 min and centrifuged for 8 min at 14,000 × g. PAs and AAs were simultaneously dansylated and quantified using an HPLC method from Minocha and Long (2004) with following modifications. Samples were incubated at 60°C for 30 min, cooled for 3 min and then microcentrifuged at 14,000 ×g for 30 s. The reaction was terminated by the addition of 45 µl of glacial acetic acid. Sample tubes were kept open for 3 min under a flow hood to allow CO₂ bubbles to escape. Acetone used to dissolve dansyl chloride was evaporated using a SpeedVac Evaporator (Savant, Farmingdale, NY, United States) for 5 min. Finally, 1735 µl of filtered HPLC grade methanol was added to all tubes bringing the total volume to 2 ml. PAs and AAs were analyzed by HPLC method according to Minocha and Long (2004). The data were processed using Perkin Elmer TotalChrom software (version 6.2.1).

Secondary Metabolite Analysis

Maize kernels (~20–70 mg) inoculated with WT CA14 and Δspds mutant strains were homogenized and extracted in 1 ml of ethyl acetate/acetone (1:1)/0.1% formic acid solution at room temperature for 24 h. The extracts were filtered through cotton plugs and the filtrates were concentrated under N to dryness. Each extract was re-dissolved in acetonitrile (1 mg/ml), filtered through a 0.22 µm Spin-X centrifuge tube filter, and analyzed using a Waters ACQUITY UPLC system (40% methanol in water, BEH C18 1.7 µm, 2.1 × 50 mm column) using fluorescence detection (Ex = 365 nm, Em = 440 nm). Samples were diluted to 10-fold if the aflatoxin signal saturated the detector. Analytical standards (Sigma-Aldrich, St. Louis, MO, United States) were used to identify and quantify aflatoxins: aflatoxin B₁ (AFB₁); aflatoxin B₂ (AFB₂). Aflatoxin content was expressed in ng/mg fresh weight of homogenized kernels.

For analysis of other SMs, AF70 was grown on CZ agar with ammonium sulfate (supplemented with Spd at 0.5 and 0.25 mM or Spm at 0.2 and 0.1 mM) medium at 30°C in the dark for 14 days along with controls with no PAs. Fungal cultures were lyophilized and then extracted with ethyl acetate/acetone (5.0–7.5 min), then column equilibration 5% B (7.6–10.1 min). Peaks were identified using authentic standards. Cyclopiazonic acid (CPA) was purchased from Sigma-Aldrich (St. Louis, MO, United States). Aflavinine and aflatrem standards were kind gifts from Dr. James Gloer, University of Iowa, Iowa City, IA, United States.

RNA Isolation, cDNA Synthesis, and Gene Expression Analysis

Total RNA was isolated from homogenized A. flavus-infected maize kernels using ‘Spectrum™ Plant Total RNA kit’ (Sigma-Aldrich, St. Louis, MO, United States) and cDNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, United States) according to the manufacturer’s protocols. The ZR Fungal/Bacterial RNA MiniPrep™ kit (Zymo Research, Irvine, CA, United States) was used for RNA extraction from A. flavus mycelial samples. Quantitative RT-PCR (qRT-PCR) was performed using SYBR green I chemistry and iCycler iQ5 Multicolor real-time PCR detection system (Bio-Rad). The thermocycler conditions included a pre-incubation at 95°C for 3 min, dye activation at 95°C for 10 s, primer annealing at 55°C for 30 s, elongation at 55°C for 50 s followed by a dissociation curve from 65 to 95°C for 30 min (with 0.5°C increments). The primers used for qRT-PCR are shown in Supplementary Table S2. Gene expression was normalized by ΔΔCt analysis (Livak and Schmittgen, 2001) to A. flavus β-tubulin gene (AFLA_068620) or Zea mays ribosomal structural gene GRMZM2G024838 expression (Shu et al., 2015) utilizing the gene expression analysis software package of the BioRad iQ5.

Determination of fungal load in the maize kernels infected with the WT and Δspds A. flavus CA14 strains were performed on 8 dpi samples. Fungal load was measured (using a method similar to that of Thakare et al. (2017) as relative expression of A. flavus β-tubulin gene (AFLA_068620) to the maize ribosomal structural gene GRMZM2G024838 (Shu et al., 2015).

Statistical Analysis

Statistical significance between control and treatments were determined by Student’s t-test. Significant difference between control and treatment were analyzed at **P ≤ 0.05 and/or *P ≤ 0.10 as indicated in the legends of Figures and Table.

RESULTS

Phenotypic Analyses of A. flavus Δspds Mutant and Genetic Complementation of the Mutant

Disruption of the spds gene in the A. flavus CA14 strain was confirmed by PCR analysis, and a single representative knockout strain was selected for subsequent analyses (Supplementary Figure S1). Loss of spds gene expression in the selected knockout strain was confirmed by qRT-PCR (Supplementary Figure S1). Inactivation of A. flavus spds resulted in a total loss of growth and sporulation in the Δspds mutant as compared to the CA14 pyrG-1 control in the absence of exogenously supplied Spd in the CZ growth medium (Figures 2A,B). Addition of 0.5 mM Spd...
FIGURE 2 | The effect of mutation in the spds gene ($\Delta$spds) on colony growth and sporulation. (A) Colony morphology of control (CA14 pyrG-1) and $\Delta$spds mutant Aspergillus flavus CA14 strains after 5 days of growth on Czapek’s (CZ) solid medium; (B) Comparison of conidial production between control and $\Delta$spds strains; and (C) Colony morphology of control and $\Delta$spds complemented strains. The cultures were grown under light for 5 days at 30°C. Data are Mean ± SE of three replicates ($**P \leq 0.05$ between control and treatment, Student’s t-test).

restored sporulation in the $\Delta$spds mutant but it was still <50% of that observed in the control grown in 0.5 mM Spd. An increase of Spd concentration in the CZ medium to 1.0 mM from 0.5 mM reduced sporulation by 34% in the $\Delta$spds mutant. A significant increase (334%) in spore production was observed in the control strain in response to 0.5 mM Spd compared to the $\Delta$spds mutant that was 49% less than the control at this concentration. An increase in Spd concentration to 1.0 mM had less promotional effect than 0.5 mM Spd on spore production in the control strain. Genetic complementation of the $\Delta$spds mutant ($\Delta$spdsC) with a WT A. flavus spds gene restored host strain levels of sporulation and aflatoxin production without any exogenous supply of Spd (Figure 2C and Supplementary Figures S2, S3).

Polyamine Content
Among the three different PAs analyzed (Figure 3A) in A. flavus mycelia, the concentration of Spd was highest (1.730 μM/mg DW), followed by Put (0.4 μM/mg DW) and Spm (0.1 μM/mg DW) in the control strain (CA14 pyrG-1) at 8 dpi when grown in CZ liquid medium. Addition of 0.5 mM Spd in the growth medium significantly decreased Put content by 134%, whereas cellular contents of Spd increased by 108% in the control.
soluble AAs) of Arg + Thr constituted the highest (30%) followed by Ala (16%), Orn (11%), and Lys (10%). Among the remaining AAs whose cellular content was between ≥10% were GABA (9%), Asp and Glu (7% each), Ser and Gly (3% each), and Pro (2%). The AAs that were <1% included Val, Ile, Leu, and Cys.

In general, the AAs whose cellular content (nmol/g DW basis) decreased significantly in the Δspds mutant (vs. control) were Asp (42%), Glu (33%), Ser (22%), Lys (17%), and Cys (not detected in the Δspds mutant). The AAs that increased significantly in the Δspds mutant with Spd (vs. control) were Ala (16%) and His (not detected in any other treatments). The AAs that were changed in the control with 0.5 mM Spd vs. control (no Spd) were, Ala (36% decrease) and Orn (40% decrease).

**Effect of Exogenous Supply of Spd and Spm on Sclerotia Production**

To investigate if PAs promoted sclerotial production, CZ medium supplemented with PAs was used to inoculate the *A. flavus* AF70 strain (a high sclerotia-producing strain). Exogenous supply of PAs significantly increased the number of sclerotia and the percentage of mature (melanised) sclerotia when the AF70 strain was grown on CZ medium supplemented with Spd or Spm (Figures 4A,B). Supplementing CZ medium with 0.25 or 0.5 mM Spd increased the total number of sclerotia by 32 and 47%, respectively, as compared to the non-supplemented control (Figure 4B). The effect of Spd was more pronounced on the production of mature sclerotia resulting in a 61 and 57% increase with 0.25 and 0.5 mM Spd, respectively (vs. non-supplemented control). Supplementation of CZ medium with Spm significantly increased the number of mature sclerotia by 24% at 0.2 mM. No significant changes in sclerotia production were observed at any other concentration of Spm used.

**Effect of Exogenous Supply of Spd and Spm on Secondary Metabolites Production, and Associated Gene Expression in *A. flavus***

*Aspergillus flavus* 70, a producer of high levels of aflatoxins and sclerotia, was used to study the effects of PAs on SM production. UPLC-MS analysis of AF70 extracts grown on CZ medium (with ammonium sulfate) in the presence of Spd showed an overall significant increase in the production of SMs compared to the control (untreated) or Spm treated samples. Indole diterpenes, particularly aflavinines (1, 3, 6; Figure 5A) and aflatrems (4, 5; Figure 5A), and CPA (2; Figure 5A) were significantly increased when Spd was added to the culture medium. Spermine also significantly increased the production of aflavinines, aflatrems, and CPA that varied with the Spm concentration. The overall effect of Spd on the production of aflavinines and aflatrems was higher than Spm. CZ medium is not conducive for the production of aflatoxins, therefore, A&M medium, which contains ammonium sulfate and supports aflatoxin biosynthesis was used to study the effects of PAs on aflatoxin production. Spermine (as compared to Spd) had a greater impact on the production of aflatoxins. At 0.1 and 0.2 mM Spm there was a significant increase in AFB1 content of 44 and
with or without exogenous spermidine (Spd).

**Figure 5B**

significant change in AFB1 at 0.5 mM concentration (vs. control; 28%, respectively, and decreased AFB2 by 14% at without any hand, at 0.25 mM increased AFB1 and AFB2 content by 17 and 52 and 135%, respectively (vs. control). Spd on the other

The cultures were grown in the dark for 8 days at 30°C. Data are Mean ± SE of three replicates (*P ≤ 0.05 and *P ≤ 0.10 between wild type (WT) and treatment, Student’s t-test); nd, not detected; Glu, glutamate; Orn, ornithine; Pro, proline; His, histidine; Arg+Thr, arginine-threonine; GABA, γ-aminobutyric acid; Ser, serine; Gly, glycine; Cys, cysteine; Lys, lysine; Ala, alanine; Asp, aspartate; Val, valine; Ile, isoleucine; Leu, leucine.

**TABLE 1 |** Cellular content of amino acids (AAs) in the control (CA14 pyrG-1) and Δspds Aspergillus flavus CA14 strains grown in Czapek’s liquid medium (shake culture) with or without exogenous spermidine (Spd).

| Treatment | Control (nmol/g dry wt) | Δspds (0.5 mM Spd) (nmol/g dry wt) | Control (0.5 mM Spd) (nmol/g dry wt) |
|-----------|------------------------|-----------------------------------|-----------------------------------|
| Glu       | 4712.40 ± 428.94       | 3163.80 ± 219.81**                | 2987.40 ± 666.34                 |
| Orn       | 7421.20 ± 465.23       | 7159.47 ± 491.46                 | 4428.33 ± 861.85*                |
| Pro       | 1103.80 ± 73.81        | 1186.07 ± 16.98                  | 840.33 ± 150.36                  |
| His       | n.d                    | 1240.40 ± 114.74**               | n.d                              |
| Arg+Thr   | 19818.07 ± 945.68      | 13079.27 ± 4183.29               | 16423.47 ± 2522.16               |
| GABA      | 5995.93 ± 257.44       | 5757.93 ± 88.34                  | 5858.00 ± 1159.22                |
| Ser       | 2125.80 ± 140.12       | 1651.00 ± 93.44*                 | 1848.53 ± 270.65                 |
| Gly       | 1859.33 ± 150.76       | 1491.27 ± 56.75                  | 1367.27 ± 239.29                 |
| Cys       | 85.40 ± 4.77           | n.d**                            | 23.13 ± 23.13                    |
| Lys       | 6284.72 ± 253.33       | 5185.80 ± 329.61*                | 4241.67 ± 892.49                 |
| Ala       | 10516.67 ± 453.23      | 12194.40 ± 339.64**              | 6727.73 ± 1135.51*               |
| Asp       | 4822.07 ± 386.00       | 2799.07 ± 304.30**               | 2858.40 ± 758.38                 |
| Val       | 596.40 ± 14.62         | 597.20 ± 18.45                   | 604.20 ± 114.76                  |
| Ile       | 429.20 ± 4.72          | 424.33 ± 6.90                    | 385.33 ± 81.91                   |
| Leu       | 425.90 ± 15.50         | 370.53 ± 20.16                   | 463.13 ± 94.44                   |

**Figure 4 |** Spermidine affects sclerotial formation. (A) Sclerotial morphology of A. flavus AF70 wild type (WT) strain grown on solid Czapek’s (CZ) medium supplemented with different concentrations of Spd and Spm along with control (no PAs); and (B) Mature (melanized) vs. total sclerotia counts for the AF70 strain grown on solid CZ medium supplemented with Spd and Spm along with control (no PAs). The cultures were grown in the dark for 14 days at 30°C. Data are Mean ± SE of four replicates (*P ≤ 0.05 between control and treatment, based on Student’s t-test).

92%, respectively (vs. control; **Figure 5B**), and AFB2 content by 52 and 135%, respectively (vs. control). Spd on the other hand, at 0.25 mM increased AFB1 and AFB2 content by 17 and 28%, respectively, and decreased AFB2 by 14% at without any significant change in AFB1 at 0.5 mM concentration (vs. control; **Figure 5B**).

As PAs in general, significantly increased SM production, we wanted to determine if this was due to up-regulation of SM pathway-specific and global regulatory genes. Expression of biosynthetic genes associated with production of aflavinine and aflatrem (atmC; AFLA_066460) as well as the global regulators of secondary metabolism, nsdC (AFLA_131330), laeA (AFLA_033290), and veA (AFLA_066460) was determined following 5 days growth of AF70 in CZ broth supplemented with Spd or Spm. The expression of atmC and atmM were both
up-regulated by Spm (Figure 5C), especially atmC up-regulated (10.7-fold) by 0.2 mM Spm compared to the control or Spd treated sample. Expression of laeA was increased by 2.6-fold in 0.5 mM Spd with a small but significant increase in response to Spm. Spermine (0.2 mM) down-regulated the expression of veA and nsdC by 2- to 3-fold whereas a small increase in the expression of nsdC and decrease in veA expression was observed by 0.5 mM Spd. No significant change in the expression of cpaA was observed in response to Spd or Spm treatments at 5 days of growth.

Analysis of Fungal Growth and Aflatoxin Production in Infected Maize Kernels

A maize seed infection assay using A. flavus WT CA14 and Δspds was performed to investigate if spds plays a role in pathogenicity and aflatoxin production during seed colonization. The Δspds mutant (a Spd auxotroph) was grown on CZ medium supplemented with ammonium sulfate and 0.5 mM Spd for 8 days along with WT grown on CZ medium (with or without 0.5 mM Spd) for the same time period prior to the harvest of spores for maize seed inoculation. In general, seeds infected with the WT strain (with or without Spd treatment) highly sporulated on the seed surface as opposed to the Δspds mutant strain that produced less spores (Figure 6A). Estimation of fungal load within the seeds showed a 140 to 144-fold higher growth in the WT A. flavus infected seeds as compared to Δspds mutant infected seeds (Figure 6B).

Aflatoxin analysis of maize seeds infected with WT A. flavus (with or without Spd) produced significantly higher amounts of aflatoxins (18–30 ng/mg FW AFB1 and 0.8–1.0 ng/mg FW AFB2; Figures 7A,B) than the Δspds infected seeds (7.74 ng/mg FW AFB1 and 0.26 ng/mg FW AFB2, respectively; Figures 7A,B).

Expression Analyses of Aflatoxin and Polyamine Metabolism and Transport Genes in A. flavus and Maize

During maize seed infection, the expression of putative PA transporter and pao genes was generally higher in
the WT *A. flavus* strain (control) in comparison to the expression of other PA biosynthetic genes (Figure 8A). Among the different putative PA uptake transporters (*dur3*, AFLA_029660; *pa*, AFLA_024200; *gap1*, AFLA_073560; and *agp2*, AFLA_113740) that were identified as yeast orthologs in *A. flavus*, expression of *dur3* was up-regulated (statistically significant) by 1.7-fold in the WT vs. Δ*spd* mutant strain. Among the PA biosynthetic genes, expression of *odc* (AFLA_011800) and *pao* (AFLA_118340) was down-regulated by 2- and 3-fold, respectively, in the Δ*spd* mutant compared to WT. Expression of *samdc* (AFLA_006490) gene was 2-fold higher in the Δ*spd* mutant and WT (0.5 mM Spd) vs. WT.

Different genes of the maize PA biosynthetic pathway as well as PA catabolism were analyzed to see if expression of genes associated with PA metabolism in the seeds respond differently when subjected to WT (virulent) vs. Δ*spd* mutant (less virulent) strains (Figure 8B). It is known that maize has multiple copies each of PA metabolism gene (Supplementary Table S2). Overall, the expression of ZmSamdc genes were highest followed by ZmAdc genes as compared to the other PA genes. Expression of ZmOdc genes, ZmOdc2 (XM_008672579) and ZmOdc3 (XM_008654778) did not vary much between samples and no expression of ZmOdc1 (NM_001148682) was detected in any of the samples. Among the different ZmAdc genes studied here, expression of both ZmAdc2 (NM_001138726) and ZmAdc3

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**FIGURE 6** The CA14 Δ*spd* mutant showing reduced pathogenicity during maize-*A. flavus* interaction. (A) Maize kernels at 8 days post-inoculation (dpi) were infected with either the CA14 WT or the Δ*spd* mutant (post-growth on CZ solid medium, which may or may not have been supplemented with Spd, for 1 week prior to the kernel inoculation); (B) Estimation of fungal load in maize kernels at 8 dpi, infected with WT and Δ*spd* CA14 strains. Fungal load was expressed as relative expression of the β-tubulin gene (AFLA_068620) to the maize ribosomal structural gene (used as housekeeping) GRMZM2G024838 (Shu et al., 2015).

Data are Mean ± SE of 3–4 replicates, each replicate consists of 4–5 seeds; (*P* ≤ 0.10 between WT and treatment, Student’s *t*-test).
expression of *ZmSamdc3* and *ZmAdc4* genes were up-regulated in the seeds infected with the WT strain (vs. WT mutant strain). Among the six *ZmPao* genes, expression of *ZmPao1* (NM_001111636) was highest and was slightly up-regulated in the seeds infected with the WT strain as compared to the Δspds mutant strain. No significant changes were observed in the expression of other *ZmPao* genes.

Among the aflatoxin biosynthetic genes in *A. flavus*, expression of *aflM* (AFLA_139300), *aflD* (AFLA_139390), *aflC* (AFLA_139410), and *aflR* (AFLA_139360) was significantly up-regulated by 110, 350, 100, and 3.5-fold, respectively, in the WT as compared to the Δspds mutant strain during seed infection (Figures 8C,D). The WT strain (0.5 mM Spd) had 1.4 to 2.8-fold higher expression of the aflatoxin biosynthetic genes in comparison to the WT strain grown on CZ agar medium without Spd (prior to the inoculation) during seed infection (Figure 8C).

**DISCUSSION**

**Ubiquitous Role of PAs in Fungal Growth, Development and Pathogenicity**

Polyamines are ubiquitous in living organisms and are involved in regulating several cellular processes including growth, differentiation, stress response, and pathogenesis (reviewed in Valdés-Santiago et al., 2012; Minocha et al., 2014; Miller-Fleming et al., 2015). Thus, the PA biosynthetic pathway has often been the target of novel strategies to control diseases associated with fungal and other microbial pathogens (Amano et al., 2015; Burger et al., 2015; Etiarte et al., 2017; Mounce et al., 2017). During the pathogenic interaction between the fungus *Colletotrichum truncatum* and soybean seeds, application of L-α-difluoromethylornithine (DFMO) and difluoromethylarginine (DFMA), inhibitors of Odc and Adc, respectively, reduced intracellular PAs in the fungi and significantly impacted fungal growth (Gaminrik et al., 1994). Exogenous supply of Put or Spd restored fungal growth, suggesting that intracellular contents of Spd might be critical for growth and pathogenesis. The current study, using a gene knock out approach, directly demonstrates that Spd is critical in *A. flavus* growth, development, pathogenesis, and the production of aflatoxins *in vitro*; especially during the maize-*A. flavus* interaction. The Δspds mutant was an auxotroph for Spd that required an exogenous supply of Spd to partially restore WT levels of growth and sporulation. The inability of Spd supplementation to fully reverse the Δspds mutation suggested a differential regulation of Spd uptake and distribution in the fungus as compared to its endogenous biosynthesis. Nevertheless, exogenous supply of Spd significantly increased sporulation in the control strain suggesting regulation of sporulation by Spd (Figure 2B). The observation of a 6- to 31-fold higher expression from day 1 to day 2 of *A. flavus* PA biosynthetic genes (Figure 3B) indicates that there is a high demand for PAs to support the early stages of fungal growth and development. The inability of the Δspds mutant strain to produce Spd resulted in a significant reduction in fungal growth and aflatoxin production during maize seed infection (Figures 6, 7). The results presented here are in line with earlier reports on the role PAs in fungal development and secondary metabolism. In *Fusarium graminearum* application of DFMO significantly reduced fungal growth (∼70%) and the production of DON by ∼53-fold, under *in vitro* conditions (Crespo-Sempere et al., 2015). A similar reduction in growth, sporulation, and SM production upon inhibition of Odc or spds knock out were also reported in *A. nidulans* and *A. parasiticus* (Khurana et al., 1996; Guzmán-de-Peña and Ruiz-Herrera, 1997; Guzmán-de-Peña et al., 1998; Jin et al., 2002; Khatri and Rajam, 2007). The data presented here demonstrate the role of Spd in *A. flavus* growth and development in relation to the expression of PA biosynthetic genes and cellular PA content *in vitro* and during maize seed infection.

**Inactivation of Spds Alters Nitrogen Metabolism in the Δspds Mutant**

Nitrogen metabolism plays a central role during normal fungal development as well as under stress conditions (Krappmann and Braus, 2005; Valdés-Santiago and Ruiz-Herrera, 2013). PAs can alter overall N metabolism as the PA biosynthetic pathway is intricately associated with AA biosynthesis (Mohapatra et al., 2010; Beckmann et al., 2013; Majumdar et al., 2013, 2016; Dalton et al., 2016; Wudineh et al., 2018). Inhibition of Spd biosynthesis
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FIGURE 8 | Aspergillus flavus CA14 Δspds mutant shows changes in PA and aflatoxin gene expression during maize-A. flavus interaction. Maize kernels were infected with WT and Δspds strains for 8 days, and gene expression was analyzed in the infected maize kernels at 8 days post-inoculation (dpi). (A) Expression of A. flavus PA biosynthetic genes (odc, spds, spms, sat1) and putative plasma membrane localized PA uptake transporters (dur3, PA, agp2, gap1) during host-pathogen interaction. (B) Expression of maize PA metabolism genes (Odc, Adc, Samdc, Spds, Spms, Pao) in the kernels during A. flavus infection; (C) Expression analyses of A. flavus aflatoxin biosynthetic genes (aflM, aflD, aflC); and (D) Expression of aflatoxin regulatory gene aflR in maize kernels infected with WT and Δspds CA14 strains. Data are Mean ± SE of 3–4 replicates, each replicate consists of 4–5 seeds (∗∗P ≤ 0.05 and ∗P ≤ 0.10 between WT and treatment, Student’s t-test).

in A. flavus resulted in a significantly higher accumulation of Put (≈2619%) than the control (Figure 3A). Putrescine overproduction is often accompanied by higher Put catabolism and rapid (within hours) turn-over of Put in plants (Bhatnagar et al., 2002; Shao et al., 2014). This might not be the case in A. flavus as a significant accumulation of Put was observed even after 8 days of culture. This could possibly be due to a greater half-life of Put in the fungus and previous observations that Put can be stored at a very high concentration (under certain conditions) in cells, where it might serve as a source of N during low N availability (Middlehoven et al., 1986). The accumulation of Put in the Δspds mutant of A. flavus could be a topic for future studies where radio-labeled PA substrates can be used to study Put turn-over rates and the possible back-conversion of Spd to Put by Sat and Pao enzymes. Other than PAs, intracellular AA content also has a substantial impact on overall N metabolism in fungi and AAs play an indispensable role in fungal development and pathogenesis. Thus, inhibitors of AA metabolism have been used as antifungal agents (Jastrzebowska and Gabriel, 2015). In the current study, supply of exogenous Spd to the Δspds mutant revealed that although A. flavus resumed growth and sporulation in vitro (albeit lower than the control), intracellular AA content in the mutant was still not on par with the WT strain (Table 1). Alterations in cellular PAs due to inactivation of PA biosynthetic genes (spds, odc) in A. nidulans were reported in earlier studies (Jin et al., 2002; Khatri and Rajam, 2007), but the current data show that alterations in Spd levels in A. flavus goes beyond the PA biosynthetic pathway and affects biosynthesis of AAs. Several of the AAs (such as Glu, Ser, Thr) that were decreased in the Δspds mutant (Table 1) are reported to be critical in fungal pathogenesis (Olivieri et al., 2002; de Sain and Rep, 2015; Muszewska et al., 2017; Zhou et al., 2017). Whether the reduction in pathogenicity in the Δspds mutant during maize seed infection is due to the decrease in Spd or possible alteration in relative AA content or both, will require further investigation.

Polyamines Modulate Secondary Metabolite (SM) Production

The PA-hypusine node is critical for cell survival and significantly affects pathogenicity in fungi and other organisms (reviewed in Park et al., 2010; Wolff and Park, 2015). The conversion of a specific Lys residue in the eIF5A to a rare AA hypusine (post-translational modification) and subsequent activation of eIF5A is absolutely required for cell proliferation. Spd in this regard serves as the only donor of a 4-aminobutyl moiety to the Lys residue of eIF5A carried out by the enzyme deoxyhypusine synthase. The overall effect of Spd depletion on growth and SM production in the Δspds mutant may be due to a combination of reduced activation of eIF5A leading to reduced fungal growth and direct regulation of SM biosynthesis by Spd (Martinez-Rocha et al., 2016). Supplementation of CZ minimal medium with Spd and to some extent Spm significantly increased the formation of sclerotia and production of aflavinines and aflatrems in AF70.
Polyamine metabolism plays a significant role in host defense and also in maintaining successful pathogenesis in fungi (and in other pathogens) during the host-pathogen interaction. The results presented here demonstrate the role of Spd in *A. flavus* growth, development, and SM production both *in vitro* and *in vivo*. Abrogation of intracellular Spd biosynthesis in *A. flavus* negatively affected fungal growth, expression of PA biosynthetic genes, and aflatoxin biosynthesis during maize seed infection. Increase in overall SM production in *A. flavus* by an exogenous supply of Spd (*in vitro*) also supports the role of Spd in SM production. Significant up-regulation of *A. flavus* plasma membrane-localized PA uptake transporters during maize seed infection suggests that dual targeting of PA uptake transporters and *spds* through an RNAi-based approach or application of biochemical inhibitors, might be an effective strategy to control *A. flavus* colonization and aflatoxin production in maize and other susceptible food crops.
AUTHOR CONTRIBUTIONS
RMa, JC, SM, and KR: conceived and designed the experiments. RMa, BM, and CS: performed the experiments. RMa, ML, RMi, SM, and CC-W: analyzed the data. RMa, ML, and RMi: wrote the paper. JC, RMi, SM, and KR: edited the draft manuscript. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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