Running Head:
Polyamines and *Botrytis* pathogenesis

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Polyamines Attenuate Ethylene-Mediated Defense Responses to Abrogate Resistance to *Botrytis cinerea* in Tomato

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ABSTRACT

Transgenic tomato (Solanum lycopersicum) lines over-expressing yeast spermidine synthase (ySpdSyn), an enzyme involved in polyamine (PA) biosynthesis, were developed. These transgenic lines accumulate higher levels of spermidine (Spd) than the wild type plants and were examined for responses to the fungal necrotrophs Botrytis cinerea and Alternaria solani, bacterial pathogen Pseudomonas syringae pv. tomato DC3000, and larvae of the chewing insect tobacco hornworm Manduca sexta. The Spd-accumulating transgenic tomato lines were more susceptible to B. cinerea than the wild type plants; however, responses to A. solani, P. syringae, or M. sexta were similar to the wild type plants. Exogenous application of ethylene precursors, S-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid, or PA biosynthesis inhibitors reversed the response of the transgenic plants to B. cinerea. The increased susceptibility of the ySpdSyn transgenic tomato to B. cinerea was associated with down-regulation of gene transcripts involved in ethylene biosynthesis and signaling. These data suggest that PA-mediated susceptibility to B. cinerea is linked to interference with the functions of ethylene in plant defense.
INTRODUCTION

A complex set of host and pathogen genetic factors determine the outcome of plant responses to pathogens. Necrotrophic fungi deploy a variety of virulence factors that assist in colonization of a wide range of host species (Groll et al., 2008). *Botrytis cinerea*, a necrotrophic fungal pathogen that infects over 200 plant species, is the causal agent of the gray mold disease, resulting in significant economic losses. Information on how plants combat necrotrophic pathogens such as *B. cinerea* and what signaling molecules are involved in such interactions is beginning to emerge (AbuQamar et al., 2008; Laluk et al., 2011). Host immune response to infection is mediated by diverse regulatory processes, of which, plant hormone functions have been studied extensively in relation to disease (Spoel and Dong, 2008; Bari and Jones, 2009; Pieterse et al., 2009). A number of reports suggest a significant role of ethylene (ET) in imparting resistance against *B. cinerea* and other necrotrophic fungi. These include: 1) requirement of the ET response pathway and its genetic components for efficient resistance to *B. cinerea* in different plant species (Thomma et al., 1999; Díaz et al., 2002); 2) enhanced resistance to *B. cinerea* by the expression of *Ethylene response factors1* (*ERF1*), a component of ET signaling in *Arabidopsis* (Berrocal-Lobo et al., 2002) and transgenic expression of *etr1* mutant allele in tobacco resulting in susceptibility to the necrotrophic oomycete Pythium spp (Knoester et al., 1998); 3) induction of over 30 ET-regulated transcription factors after infection of *Arabidopsis* with *B. cinerea* (AbuQamar et al., 2006), some of which directly contribute to resistance (Zheng et al., 2000); 4) increased resistance of *ctr1-1* (*CONSTITUTIVE TRIPLE RESPONSE 1*) and *eto2* (*ETHYLENE OVERPRODUCER 2*) plants to *B. cinerea* (Lloyd et al., 2011); 5) ET role in pathogen-associated molecular patterns (PAMP) triggered immunity to *B. cinerea* (Laluk et al., 2011); and 6) ET-regulated cell wall modifications involved in defense responses to *B. cinerea* (Lloyd et al., 2011). Additionally, a number of protein kinases have been shown to play a role in ET-dependent responses to *B. cinerea* infection. Thus, various genetic and biochemical data suggest a critical role of ET in *B. cinerea* resistance (Han et al., 2010; Lumbreras et al., 2010).
Polyamines (PAs) are polycationic, ubiquitous compounds that have essential functions in all organisms studied thus far involving regulation at both transcriptional and translational levels (Veress et al., 2000; Kasukabe et al., 2004; Yoshida et al., 2004; Alcázar et al., 2005; Igarashi and Kashiwagi, 2006; Srivastava et al., 2007; Mattoo and Handa, 2008; Handa and Mattoo, 2010). Putrescine (Put), spermidine (Spd) and spermine (Spm) are the three most prominent PAs in plants. Decarboxylation of ornithine by ornithine decarboxylase (ODC) or arginine by arginine decarboxylase (ADC) leads to the synthesis of Put, which is converted to Spd by Spd synthase (SpdSyn), and Spd, in turn, then converted to Spm by Spm synthase (SpmSyn; Nambeesan et al., 2008). In these reactions, both SpdSyn and SpmSyn enzymes use aminopropyl residues derived from decarboxylated S-adenosylmethionine (dcSAM) which is synthesized from S-adenosylmethionine (SAM) by SAM decarboxylase (SAMdc; Martin-Tanguy, 1997; Bouchereau et al., 1999; Mehta et al., 2002). SAM is also a substrate for 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, a reaction that generates ACC, the immediate precursor of ET (Fluhr and Mattoo, 1996). PAs are present in various cellular compartments such as vacuole, mitochondria, chloroplast as well as in cell wall fractions (Kaur-Sawhney et al., 2003). While both nuclear and cytoplasmic localization of ODC have been reported, the ADC pathway is predominantly localized to the chloroplast. Spd synthase has been reported to be localized both in the chloroplast and cytoplasm (Nambeesan et al., 2008).

The role of PAs in plant-pathogen interactions remains elusive. Nevertheless, dramatic changes in PA metabolism have been reported in both compatible and incompatible plant-microbial interactions (Walters, 2003a). Chinese cabbage leaves infected with turnip yellow mosaic virus and maize with fungal biotroph, *Ustilago maydis*, showed increase in Put and Spd compared to healthy leaves (Torget et al., 1979). Barley leaves infected with brown rust fungus *Puccinia hordei* exhibited over six-fold increase in Spd compared to control healthy leaves and resulted in the formation of green islands surrounding the site of infection due to chlorophyll retention (Greenland and Lewis, 1984). ADC, ODC and SAMDC activities increased in barley leaves infected with *Blumeria graminis* f. sp. *hordei* (Walter et al., 1985). Also, decreases in PAs have been
observed in plant-fungal and plant-viral infections (Bakanashvili et al., 1987; Edreva, 1997). PA conjugates, hydroxycinnamic acid amides, synthesized by the formation of an amide linkage with cinnamic acids, mainly p-coumaric, ferulic and caffeic acids, have been reported to enhance plant resistance to pathogens by forming an enzymatic hydrolysis-resistance phenolic barrier (Walters et al., 2001). PA catabolism by diamine and polyamine oxidases leading to the production of H$_2$O$_2$ has also been implicated in mediating programmed cell death and wall lignification (Walters, 2003b). A Spm-signaling pathway has been implicated in TMV-induced HR response that involves ZFT1 and ZFP1, two Cys$_2$/His$_2$-type transcription factors (Uehara et al., 2005; Mitsuya et al., 2009). PAs may also induce nitric oxide (NO), an important signaling molecule involved in disease resistance in Arabidopsis (Delledonne et al., 1998; Tun et al., 2006), although its involvement as a trigger for disease tolerance was ruled out in field-grown tomato (Kumar et al., 2004). There have been few studies suggesting the effect of transgenic manipulation of PA-biosynthesis genes during pathogen response. Transgenic tobacco plants expressing SAMdc under the control of CaMV35S promoter exhibited tolerance to Verticillium dahliae and Fusarium oxysporum (Waie and Rajam, 2003). Arabidopsis plants engineered to over-express SpmSyn under the CaMV35S promoter also showed resistance to Pseudomonas viridiflava (Gonzales et al., 2011).

We have demonstrated a role of PAs in plant growth and development using high Spd producing transgenic plants generated by expressing yeast SpdSyn in tomato (Nambeesan et al., 2010). Here, we investigate the response of these plants to the fungal pathogens B. cinerea and A. solani, bacterial pathogen P. syringae and the chewing insect tobacco hornworm Manduca sexta. These transgenic lines that accumulated higher Spd levels were more susceptible to B. cinerea while showing no differences in their response to A. solani, P. syringae and M. sexta as compared to the wild type plants. The increased susceptibility of the high-Spd lines to B. cinerea was found interrelated to the altered functions of the plant hormone ET.

**RESULTS**

**Over-Expression of the ySpdSyn Increases Spd Levels in Tomato Leaves**
Tomato cultivar Ohio 8245 was used to generate transgenic lines expressing the \textit{ySpdSyn} gene under the control of the CaMV35S promoter using \textit{Agrobacterium}-mediated transformation (Fig. 1A). Several independent T\textsubscript{0} plants transformed with this chimeric construct were selected for kanamycin resistance and screened for the presence of the \textit{ySpdSyn} gene using PCR. Kanamycin-resistant and PCR-positive plants were then screened for transgene and immunoreactive protein expression using RNA-blot and immunoblot analyses, respectively, as described in the methods section. Based on these analyses of the T\textsubscript{0} transgenic lines, two independent transgenic lines C4 and C15 were selected for studies described in this manuscript (Fig. 1B). T\textsubscript{1} seedlings were grown and homozygous C4 and C15 plants were generated. Spd levels in the leaf tissue were 2.1- and 1.3-fold higher in C4 and C15, respectively, compared to the wild type (WT) cultivar (Fig. 1C; 0 hours post infection). However, the pattern of Put levels was inconsistent, being 1.2-fold higher in C4 and 1.3-fold lower in C15 compared to WT plants (Fig. 1C). Spm levels were lower in leaves of both the transgenic lines compared to WT, but C4 plants had greatly reduced Spm content than the C15 plants. These data suggest that \textit{ySpdSyn} was functional in tomato and led to higher Spd levels.

\textbf{Leaf Polyamine Levels Increase during \textit{Botrytis} Infection}

To determine the relationship between polyamine (PA) content and disease response, changes in PA levels were quantified after inoculation with \textit{B. cinerea} (Fig. 1C). In WT plants, Put levels increased by 1.4-fold within 24 hours post-infection (hpi) and thereafter remained elevated. Leaves of C15 plants had a similar increase in Put levels at 24 hpi which increased to about 2-fold at 48 hpi, while that of C4 remained unaltered during \textit{B. cinerea} infection. Also, Spd levels in WT leaves continued to increase after \textit{B. cinerea} infection, increasing over 2-fold at 48 hpi. Prior to infection, Spd levels were higher in C4 (>2-fold) and C15 leaves than in the WT plants. Spd levels increased further (by 1.5-fold) at 48 hpi in \textit{B. cinerea}-infected C15 but not C4 leaves. Spm levels decreased 24 hpi in WT and C15 lines upon infection with \textit{B. cinerea} but were similar in C4 leaves (Fig. 1C). Collectively, these results indicate that an increase in
Spd and Put levels occurs with a concomitant decrease in Spm levels during tomato infection with *B. cinerea*.

**Increased Spd levels in Tomato Leaves Enhance Susceptibility to *Botrytis cinerea***

Leaves from C4, C15 and WT plants were inoculated with *B. cinerea* spores and the development of lesions on the leaf was followed. Visible disease symptoms were apparent within 24 hpi in WT leaves but the size of the lesion was similar to the size of the drop-inoculum indicating limited progression of the disease. At 24 hpi, the lesion size in transgenic C4 and C15 plants was larger than that in WT leaves (Fig. 2A). At 48 hpi, average lesion diameter in WT plants was 5.4 cm while it was significantly higher, by 100% and 72%, respectively, in C4 and C15 plants (Fig. 2B). Tissue maceration and extensive necrosis in the transgenic plants occurred within 72 hpi (data not shown). In each independent experiment, the C4 line was found to be more susceptible than the C15 line. Susceptibility of C4 and C15 plants to *B. cinerea* was associated with higher accumulation of fungal biomass as was indicated by higher steady state levels of *B. cinerea* ActinA transcripts at 24 and 48 hpi in γSpdSyn leaves compared to the WT (Fig. 2C). Steady state levels of γSpdSyn transcripts in *B. cinerea* and mock-inoculated C4 and C15 plants confirmed continued expression of this gene during infection (Fig. 2D). Since increase in Spd levels were noted in WT plants during infection, its level at the time of infection may be important for the progression of the disease.

**Inhibitors of Polyamine Biosynthesis Reverse Susceptibility of Transgenic Plants to *B. cinerea* Infection***

Leaves of WT, C4 and C15 plants were pre-treated for 24 h in the absence and presence of 1 mM Spd, and 1 mM of difluoromethylornithine (DFMO), or 1 mM of cyclohexylamine (CHA), compounds that inhibit ornithine decarboxylase (Rajam and Galston, 1985) and SpdSyn (Saftner et al., 1997), respectively. Then, these plants were challenged with *B. cinerea*, and the lesion development was monitored. Pre-treatment with DFMO or CHA did not alter the response of WT plants to *B. cinerea* but eliminated the enhanced susceptibility of the transgenic leaves to this pathogen (Fig. 3A-C). At 72
hpi, the lesion sizes in WT, C4 and C15 leaves treated with DFMO or CHA were of the same extent (Fig. 3E) suggesting that inhibitors of PA biosynthesis decrease susceptibility of transgenic leaves to *B. cinerea*. Treatment with Spd significantly increased lesion diameter in WT leaves at 72 hpi which was similar to that seen in C4 and C15 plants (Fig. 3F-H). Thus, these data support above results that elevated levels of Spd increase susceptibility of tomato to *B. cinerea*.

**Spd-Induced Susceptibility of Tomato to *Botrytis cinerea* is Mediated by Down-Regulation of *SlACS1* and *SlERF1B***

Different defense pathways mediated by plant hormones interact in plant responses to infection. ET and salicylic acid (SA) have antagonistic roles in determining plant responses to *B. cinerea* infection in *Arabidopsis* (Glazebrook, 2005). *B. cinerea* exploits the antagonistic interactions between jasmonic acid (JA) and SA mediated defense pathways to establish disease in tomato. The production of exopolysaccharide by *B. cinerea*, which elicits the SA pathway and suppresses the JA signaling pathway, has been implicated in enhancing disease caused by this pathogen in tomato (El Oirdi et al., 2011). To understand the involvement of these signaling pathways in Spd-mediated susceptibility of tomato to *B. cinerea*, we analyzed the transcript levels of tomato genes involved in ET biosynthesis (*ACC synthase; SlACS*) and that of SA (pathogenesis-related 1; *SlPR-1*) mediated defense responses (Fig. 4). Indeed, the expression of *SlACS* increased by 15-fold in response to *B. cinerea* infection in WT leaves at 24 hpi, suggesting an activation of the ET pathway. However, in the transgenic C4 leaves only a 2-fold increase in *SlACS* expression was observed while C15 registered a 10.6-fold increase in its expression, suggesting attenuation of ET biosynthesis (Fig. 4A). This pattern of gene expression is interesting since PAs and ET are synthesized from common upstream substrates and over-expression of *ySpdSyn* may have interfered with the expression of *SlACS*. In contrast, the expression of the SA response marker gene, *SlPR1*, showed a minor increase in *B. cinerea*-inoculated WT leaves but a significantly higher expression in both the transgenic lines at 24 hpi (Fig. 4B). These data indicate that Spd levels in transgenic leaves modulate ET biosynthesis pathway, and thereby may affect
ET-regulated processes during *B. cinerea* infection. The elevated levels of *SlPR1* expression may be a function of higher fungal growth in transgenic lines or its positive regulation, and possibly of the SA signaling pathway, by PAs in tomato.

The role of ET signaling in regulating Spd-mediated disease symptoms was further examined by following the expression of genes in the ET response pathway. The gene transcripts examined included the tomato transcription factors, *SlEIL1* and *SlEIL2* (*EIN3-like 1 and 2*), which are positive regulators of ET responses. EIN3, EIL1 and EIL2 like transcription factors are members of a redundant gene family in tomato with complex functions throughout plant development (Tieman et al., 2001), and in turn regulate *ERF1*, a transcription factor required for activation of defense response genes (Solano et al., 1998; Guo and Ecker, 2004). As shown in Fig. 4C, a 6- and 4-fold increase in the expression of *SlEIL1* occurred in C4 and C15 transgenic plants, respectively, by 24 dpi, which was not observed in WT. The expression pattern of *SlEIL2* was variable: in the WT and C4 these increased similarly, 2.5-fold, upon infection but in C15 leaves they were suppressed by 1.3-fold during infection (Fig. 4D). The expression of *SlERF1B* increased ~ 59-fold in WT compared to a relatively moderate, 14- and 4-fold increase in C4 and C15 leaves, respectively, at 24 hpi, which is consistent with the disease susceptibility phenotypes of the transgenic lines (Fig. 4E). These results support our contention that impaired or altered expression of components of ET biosynthesis and signaling pathways account for enhanced susceptibility of high-Spd tomato plants to *B. cinerea*. Interestingly, transcripts of *β-1,3-glucanase*, another *PR* gene, increased in both the C4 and C15 transgenic plants upon *B. cinerea* infection but this did not induce resistance (Fig. 4F). It is therefore likely that higher PA levels may contribute to general perturbations of cellular homeostasis that could subsequently cause a general upregulation of PR proteins and disease susceptibility.

**Treatment with Ethylene Precursors Reverses Spd-Mediated Susceptibility of Tomato to *B. cinerea***

Another approach used to establish ET involvement in *B. cinerea-γ*SpdSyn plant interaction was to treat the plants with precursors of ET biosynthesis and then challenge
them with *B. cinerea*. Accordingly, C4 and C15 leaves were pre-treated with 100 μM ACC or 200 μM SAM, and then inoculated with *B. cinerea*. Results indicated that treatment with either ACC or SAM effectively mitigated the enhanced susceptibility of C4 and C15 leaves to *B. cinerea* (Fig. 3A, 3D-E; Fig. 5A-C). These data confirm that ET is an important factor in this interaction. With respect to plant growth responses, ET is known to produce a characteristic seedling growth response called the triple response (Guzmán and Ecker, 1990). In the presence of exogenous ACC such a response was, however, not observed with the transgenic C4 and C15 seedlings compared to WT (Supplemental Fig. S1). This observation suggests that PA-mediated changes in plant defense are separate from the function of ET in plant development.

Plant responses to *B. cinerea* also involve other plant hormones such as abscisic acid (ABA) and JA (Anderson et al., 2004; Mauch-Mani and Mauch, 2005). Tomato and Arabidopsis mutants deficient in ABA were resistant to *B. cinerea* (Audenaert et al., 2002) and *P. syringae* (Thaler and Bostock, 2004; Torres-Zabala et al., 2007) whereas inhibition of JA responses enhances susceptibility to *B. cinerea* (Kunkel and Brooks, 2002). We, therefore, tested WT and transgenic tomato seed germination for altered hormone sensitivity. WT, C4 and C15 seeds were plated on medium containing methyl-JA (MeJA) or ABA, and their germination and/or root elongation were analyzed (Supplemental Fig. S2). Neither C4 nor C15 transgenic plants showed germination defects or differences in root elongation as compared to WT in medium containing either hormone, suggesting that increased Spd does not interfere with responses to ABA or MeJA.

**Spd-Accumulating Transgenic Lines are Similar to WT in Responses to *Alternaria solani*, *Pseudomonas syringae* and Tobacco Hornworm**

The C4 and C15 transgenic plants were also tested for responses to other virulent pathogens such as *A. solani* (the causal agent of early blight), *P. syringae* (the bacterial speck disease), as well as tobacco hornworm *M. sexta*. Inoculation with *A. solani* did not result in enhanced lesion development in transgenic C4 and C15 leaves compared to WT (Fig. 6A). Infiltration of leaves with *P. syringae* and subsequent analysis of bacterial
growth revealed no significant changes in bacterial titer 3 days after infiltration (Fig. 6B). In plants exposed to tobacco-hornworm larvae, increased defoliation was not observed (data not shown) but slight, though insignificant, differences in larval weight between the WT and the transgenic leaves were apparent. The leaves of C15 transgenic line were relatively more inhibitory to larval growth (Fig. 6C).

**Increase in Spd Levels does not Alter Responses to Oxidative Stress**

The response of WT, C4 and C15 seedlings to oxidative stress was measured by determining seedling growth in the presence and absence of hydrogen peroxide (H₂O₂). As shown in Supplemental Fig. S2, seedling growth of all the three genotypes was lower in the presence of H₂O₂. However, no differences were apparent in root or shoot growth between the WT, C4 and C15 seedlings (Supplemental Fig. S2). Additionally, treatment of leaves with methyl viologen did not exhibit any difference in response amongst the three genotypes (Supplemental Fig. S3). We interpret these data to suggest that increased Spd in transgenic leaves does not alleviate oxidative stress caused by H₂O₂ or methyl viologen.

**DISCUSSION**

We present molecular and pharmacological evidence suggesting that Spd plays a significant and specific role in tomato response to the necrotrophic fungal pathogen *B. cinerea*. We further show that the Spd-mediated enhanced susceptibility of transgenic tomato plants to *B. cinerea* is associated with reduced expression of *SlACS*, a key regulatory gene in ET biosynthesis. The suppression of ET biosynthesis gene is associated with the attenuated expression of ET-response gene *SlERF1B* in the two transgenic plants upon challenge with *B. cinerea*.

Two transgenic tomato lines engineered to accumulate Spd showed enhanced susceptibility to *B. cinerea*, C4 line being more prone to this pathogen than the C15 line. Although increases in the Spd levels in the transgenic lines were moderate (1.3 to 2-fold higher), yet the susceptibility to *B. cinerea* was greatly increased. Moderate changes in PA levels, especially increases in Spd, have been shown to cause significant
physiological changes in transgenic *Arabidopsis*, rice, and pear plants over-expressing *SpdSyn* and *SAMdc* genes (Kasukabe et al., 2004; Wen et al., 2008; Peremarti et al., 2009). Unsuccessful attempts to obtain stable transgenic potato plants expressing a potato *SAMdc* under the CaMV 35S promoter while *SAMdc* antisense lines led to a range of stunted phenotypes also support this contention (Kumar et al., 1996). PAs play important roles during meiosis, sporulation and cell division, and thus have been suggested to modulate fungal development. Depletion of PAs is lethal for fungi (Tabor, 1981; Rajam and Galston, 1985; Walters, 1995). Use of DFMO and CHA, inhibitors of ODC and *SpdSyn* proteins, respectively, were effective in mitigating the growth of various fungal species such as *B. cinerea* (Rajam and Galston, 1985; Saftner et al., 1997), *Tilletia spp* (Trione et al., 1988), *Penicillium expansum* (Saftner et al., 1997), *Gaeumannomyces graminis* (West and Walters, 1989) and powdery mildew (Mackintosh and Walters, 1998). In our study, treatment of plants with the two inhibitors of PA biosynthesis, DFMO and CHA, eliminated enhanced susceptibility of transgenic leaves to *B. cinerea* while treatment with exogenous Spd increased the susceptibility of WT leaves, lending support to the hypothesis that Spd modulates host responses to infection. The application of PA biosynthesis inhibitors did not cause any significant alteration in lesion diameter in the WT leaves suggesting that the concentrations of DFMO and CHA used did not influence PA levels enough to directly affect growth and/or survival of *B. cinerea*. However, the altered PA homeostasis in transgenic leaves treated with PA biosynthesis inhibitors was enough to influence host physiology and reduce lesion due to infection.

*Spd*-mediated enhanced susceptibility of transgenic tomato leaves suggests an impaired host-signaling network that leads to weakened immune responses against *B. cinerea* infection. Depending on the plant-pathogen interaction, treatment with ET may enhance resistance (Esquerré-Tugayé et al., 1979; El-Kazzaz et al., 1983b; Marte et al., 1993), induce susceptibility, or have no effect (El-Kazzaz et al., 1983a; Brown and Lee, 1993; Thomma et al., 1999). Our data indicate a crosstalk between PA and ET in modulating responses to *B. cinerea*. *SlACS* expression was induced in WT leaves upon *B. cinerea* infection but attenuated in transgenic plants. Consistent with this observation was the reversion of host susceptibility by ACC, ET biosynthesis precursor. Taken together,
these results support the interpretation that Spd-induced susceptibility to *B. cinerea* is due to its interference with ET biosynthesis (*SlACS*) and response (*SlERF1B*) pathways.

The effect of Spd on downstream ET signaling was investigated to understand Spd-mediated alteration of ET-dependent resistance to *B. cinerea*. ET is sensed and bound by a family of membrane receptor proteins that activate *CTR1*, a negative regulator of the ET-signaling cascade. Downstream, this signaling cascade is facilitated by the transcription factors EIN2, EIN3 and paralogs, the EIN3-like proteins (EILs; Solano et al., 1998; Stepanova and Ecker, 2000; Guo and Ecker, 2004). Three functionally redundant homologs of *Arabidopsis EIN3, SIEIL1–3*, have been identified in tomato (Tieman et al., 2001). *ERF1* is an early response gene that has been implicated in several necrotrophic pathogen responses (Lorenzo et al., 2003). In line with Spd and ET interaction being competitive in *B. cinerea*-tomato interactions, we found that ET-responsive pathway genes such as *SlERF1B*, but not *SIEIL1* and *SIEIL2*, displayed reduced expression in both the transgenic lines compared to the WT (Fig. 4).

Genetic data in *Arabidopsis* and tomato have established the role of signaling molecules such as SA and JA in addition to ET, and their interactions, in mediating responses to pathogen infection (Thomma et al., 1998; 1999; Alonso et al., 2003; Ferrari et al., 2003). *ERF1* integrates ET/JA-dependent responses during pathogen infection in *Arabidopsis* (Lorenzo et al., 2003), while JA-ET mediated induction of PR genes was shown to play a role in *B. cinerea* infection (Thomma et al., 1999). However, in tomato, JA acts independent of ET in inducing resistance to *B. cinerea* (Díaz et al., 2002). The influence of JA on *SlERF1B* expression in *ySpdSyn* over-expressing lines remains to be established.

The role of SA-dependent signaling pathway against biotrophs is well documented (Glazebrook, 2005). Although the role of SA in *Arabidopsis-B. cinerea* interaction is controversial (Thomma et al., 1998; Zimmerli et al., 2001), SA has been reported to enhance tolerance to *B. cinerea* in tomato (Audenaert et al. 2002), tobacco (Murphy et al., 2000) and french beans (De Meyer and Höfte, 1997). *PRI* is induced by SA during defense responses (Thomma et al., 1998; Glazebrook, 2005; AbuQamar et al., 2009). We observed an increase in the expression of SA marker *SlPRI* in C4 and C15
transgenic (Fig. 4B). Increased expression of PR1 was also observed in BOTRYTIS-INDUCED KINASE1 (BIK1) Arabidopsis mutant plants that showed enhanced susceptibility to Botrytis (Veronese et al., 2006). The role of BIK1 in SA signaling is still unclear and the increased growth of the fungus in the bik1 mutant may have caused the enhanced expression of PR1.

SA signaling has been implicated in resistance of hydroponically-grown tomatoes to another necrotroph, A. solani. Exogenously applied SA induced systemic acquired resistance (SAR) in these tomatoes, which led to effective resistance against A. solani (Spletzer and Enyedi, 1999). Likewise, application of a chemical inducer of SAR such as benzothiadiazole conferred resistance to potato against A. solani (Bokshi et al., 2003). In this context, it is interesting that the transgenic C4 and C15 tomato plants were similar to the WT in their response to inoculation with the hemibiotroph, P. syringae or with A. solani. Previously, studies with Arabidopsis have suggested that the ET-mediated disease resistance may be pathogen-specific based on the type of the necrotroph since ein2-1 mutants were more susceptible to B. cinerea but not A. brassicicola (Thomma et al., 1999). Our studies are consistent with these findings. Therefore, observed expression of PR1 in ySpdSyn transgenic plants might be a reflection of increased susceptibility to Botrytis rather than being a marker of enhanced SA response. It is noted here that SA or polyamines (Spd or Spm) when applied to tomato slices leads to suppression of SlACS transcripts and ET biosynthesis (Li et al. 1992), as depicted for SlACS here. Our work also differentiates Spd involvement from that of Spm in B. cinerea pathogenesis (Gonzales et al., 2011) since the ySpdSyn transgenic plants are relatively deficient in Spm compared to WT and, notably, during B. cinerea infection Spm levels actually decreased in the WT. Collectively, these data suggest a negative effect of increased Spd levels on ethylene synthesis and/or signaling, which leads to higher susceptibility of tomato leaves to B. cinerea (summarized in Fig.7).

MATERIALS AND METHODS
Plant Growth
Tomato (Solanum lycopersicum cv. Ohio 8245) plants were grown in plastic pots containing compost soil mix in a greenhouse with a photoperiod extended to 15 h under fluorescent lights (160 W mol\(^{-1}\) m\(^{-2}\) s\(^{-1}\)) at a temperature of 24 ± 4°C.

Generation of Transgenic Plants
Transgenic lines expressing \(\gamma\text{SpdSyn}\) gene driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter were generated. The \(\gamma\text{SpdSyn}\) was amplified from a yeast genomic library using the forward primer: ScSpe3XhoF (5’GCCGCTCGAGATGGCACAAGAAATCACTCACCCAA3’) and reverse primer: ScSpe3XbaR (5’GCCGTCTAGACTAATTTAATTCCTTGGCTGCCCAG3’), and cloned into the pGEM-T Easy vector system (Promega). The insert was excised using restriction endonucleases, \(XhoI\) and \(XbaI\), and cloned in the sense orientation between a CaMV35S promoter and the 3’ end of a pea rbcS-E9 gene in pKYLX71 (Scharld et al., 1987). This construct was introduced into the disarmed Agrobacterium tumefaciens LBA4404 by chemical transformation. Agrobacterium strains harboring the chimeric constructs were used to transform cotyledons of tomato cv. Ohio 8245 (Tieman et al., 1992). Fifteen independent transgenic plants expressing \(\gamma\text{SpdSyn}\) under the CaMV35S promoter were generated (Nambeesan et al., 2010). Based on the transcript and protein expression analysis of T\(_0\) transgenic plants, two independent transgenic plants, C4 and C15, were selected and used for further studies. Seeds from T\(_1\) seedlings were analyzed for selecting homozygous plants using PCR. Lines homozygous for the transgenes were selected after PCR analyses of seedlings from T\(_2\) seeds and used in studies presented here.

Fungal and Bacterial Disease Assay
The \(B.\ cinerea\) strain BOS-10 was used for disease assays. Fungal culture and preparation of conidial spore suspension were as described previously (AbuQamar et al., 2006). \(B.\ cinerea\) disease assays were done on detached leaves by drop inoculation of a conidial suspension on tomato leaves (3x10\(^5\) spores/ml; AbuQamar et al., 2008) and lesion
diameter was determined (n=60). WT and transgenic leaves were inoculated with 300 mg/ml *A. solani* cultures and lesion diameter was calculated 7 days after inoculation. Bacterial disease assays were done essentially as described (Mengiste et al., 2003). Fully expanded leaves of 6-week-old tomato plants were infiltrated with suspensions of the bacterial strain *P. syringae* (OD₆₀₀ = 0.001, ~5x10⁵ CFU/ml in 10 mM MgCl₂). Bacterial growth was determined using leaf discs from infected leaves at 0 and 3 days after infection (DAI) as described (AbuQamar et al., 2008). Bacterial titer per leaf area was determined in uniform leaf discs using a hole-punch. Each experiment was performed in triplicate and two leaf discs were collected from C4, C15 and WT plants for each replicate.

**Tobacco Hornworm Feeding Trials**

Tobacco hornworm *M. sexta* eggs and an artificial diet for the larvae were purchased from Carolina Biological Supply Company (Burlington, North Carolina). As recommended by the supplier, eggs were hatched by incubation at 26°C. The artificial diet for the hatched larvae was continued for 3 days before transfer to detached leaves or whole tomato plants. For whole plant assay, four larvae weighing 9-11 mg each were placed on each of six 8-week old WT, C4 and C15 plants grown in the green house. The average larval weight at the beginning of the feeding trial was 7-9 mg for detached leaf assay and 9-11 mg for whole plant assay. The insects were left to feed for one and two weeks for the detached leaf and whole plant assays, respectively, after which the larval weight was determined (Abu-Qamar et al., 2008).

**RNA-Blot, and Quantitative and Semi-quantitative RT-PCR Analyses**

Total RNA was extracted from frozen (in liquid nitrogen) tomato leaf tissues as described (Lagrimini et al., 1987). For RNA-blot analyses, RNA was separated on 1.2% formaldehyde agarose gels and blotted onto Hybond N⁺ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The probes were labeled with ³²P by random priming using a commercial kit (Sigma). Probe hybridization was performed as described (Church and Gilbert, 1984).
For semi-quantitative RT-PCR analysis, cDNA synthesis was performed using 2 μg of total RNA from control and *B. cinerea*-treated leaves. Reverse transcription was performed using AMV reverse transcriptase (Promega, Madison, WI) and oligo (dT15) primers according to manufacturer’s instructions. PCR was performed using gene-specific primers and 2.5 μl cDNA as a template for 28 cycles. PCR conditions were 94°C-30 sec; 55°C-30 sec; 72°C-1 min. The amplified products were subjected to electrophoresis on 1.5% agarose gels and visualized under UV light after ethidium bromide staining. The primers used are shown in Supplemental Table 1. The tomato translation initiation factor (eIF4A) gene was used as a control for equal cDNA loading between samples.

For real time PCR analysis RNA extraction, cDNA synthesis and quantitative RT-PCR were performed as previously described (Dhawan et al., 2009). Quantitative RT-PCR was performed using gene-specific primers (Supplemental Table 1), with tomato actin used as an endogenous reference for normalization.

**Inhibitor Treatments**

WT and transgenic tomato plants were grown for 6 weeks in the greenhouse using compost soil mix with a photoperiod of 16h/8h (day/night) at a day/night temperature of 23/18°C. For growth regulator treatments, leaves were clipped and the petiole was immersed either in 1 mM DFMO (a gift from Dr. Patrick M. Woster, Wayne State University, MI), 1 mM CHA (Sigma), 100 μM ACC, 1 mM Spd (Sigma), 200 μM SAM (Sigma), or water (control). After 24 h of treatment, the leaves were placed on plates and inoculated with *B. cinerea* as described above.

**Polyamine Quantification**

Tomato leaf tissue (200 mg) infected with *Botrytis* was sampled at 0, 24 and 48 dpi, ground in liquid N2 and re-suspended in 800 μl of 5% (w/v) cold perchloric acid (PCA) per 0.2 g of ground tissue (Minocha et al., 1994). The samples were mixed and centrifuged at 20,000 g for 30 min. To each 100 μl of the supernatant, 20 μl of 0.1 mM of an internal standard, 1, 7 diaminohexane, was added. The samples were dansylated at
60°C for 1 h using 100 μl of 20 mg/ml dansyl chloride (in acetone) with 100 μl saturated sodium carbonate. L-asparagine (50 μl of 20 mg/ml) was added to terminate the reaction at 60°C for 30 min. The samples were placed in a SpeedVac for 30 min to evaporate acetone after which 400 μl of toluene was added to the sample, which was mixed and centrifuged. This was followed by removing 200 μl of toluene layer and air-drying it. The sample was dissolved in methanol (1 ml) by mixing for 1 min, and 50 μl of the filtered sample (0.45 μm filter; National Scientific) was injected into a Waters HPLC system consisting of two model 510 pumps and a model 715 WISP autosampler. A reverse phase Xterra C18 (3.8x100mm) column was used to separate dansylated PAs. Detection was performed using a Hewlett Packard model 1046A fluorescence detector (excitation and emission wavelengths were 340 and 510 nm, respectively). A binary gradient composed of solvent A was used: 100% acetonitrile and solvent B: heptanesulfonate (10 mM, pH 3.4):acetonitrile (90:10) and a 1.1 mL/min flow rate was maintained. A gradient elution was used where initial conditions were set at 50:50 (A:B) followed by a linear gradient as follows: 80:20 (A:B) at 2 min, 100:0 (A:B) at 9 min, 80:20 (A:B) at 12 min, and back to 50:50 (A:B) at 20 min. Authentic standards of PAs (Sigma) were similarly extracted and analyzed as described above. For quantification, the peaks were integrated. Quantification of PA was performed in triplicate with each replicate consisting of at least 3 leaves.

**Ethylene Response Assays**

Seeds were surface sterilized with 70% ethanol for 2 min followed by a treatment with 35% commercial bleach (5.25% [w/v] sodium hypochlorite) plus 0.1% Tween 20 for 30 min in solution, and finally thoroughly rinsed with water to remove the bleach. For the triple response assay, tomato seeds were cultured on MS medium and 0.8% agar with or without ACC and incubated in the dark for 6 days (Abu-Qamar et al., 2008).

**Hormone Treatments**

Seeds prepared as above were plated *in vitro* on the medium containing MS with 0.8% agar with or without 10 μM methyl-jasmonate (MeJA), or 2 μM ABA. Seed germination
was recorded after 7 days of growth. For ABA transfer assay, seeds were initially germinated on MS media for 3 days and then transferred to MS media supplemented with or without 10 µM ABA and seedling growth was studied. Solutions of MJ and ABA used in the experiments reported here were prepared and used as described previously (Anderson et al., 2004)

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Figure Legends

**Figure 1.** Changes in polyamine levels in fully expanded leaves of WT and transgenic (T$_2$ homozygous C4 and C15) tomato plants expressing yeast spermidine synthase gene. A, The chimeric construct containing ySpdSyn cloned between the CaMV35S promoter and the 3’ end of a pea rbcS-E9 gene in pKYLX71 vector was used to develop transgenic tomato plants. B, Presence (+) or absence (-) of introduced ySpdSyn transgene as determined by PCR; ySpdSyn transcript (RNA-blot) and protein levels (Western-blot) in leaves of WT, T$_0$ C4 and C15 plants. C, The levels of Put, Spd and Spm quantified in leaves at 0, 24 and 48 h post-infection with *B. cinerea*. The fully expanded leaves of 6-week-old plants were drop inoculated with a conidial suspension of *B. cinerea* containing $3 \times 10^5$ spores/ml. The samples were collected at the indicated post-inoculation time periods and the levels of Put, Spd and Spm quantified using HPLC as described in the methods. Error bars represent standard error of means ($n=3$) and asterisks indicate significant difference from the wild type ($\alpha=0.05$).

**Figure 2.** Expression of ySpdSyn increases tomato leaf susceptibility to *B. cinerea*. A, Response of WT and T$_2$ homozygous C4 and C15 leaves to *B. cinerea* inoculation. Leaves were spot inoculated with 5 µL of $3 \times 10^5$ spores/mL of *B. cinerea*; hpi, h post inoculation. B, Mean lesion size at 48 hpi in WT, C4 and C15 leaves. Error bars represent standard error of means ($n=60$) and asterisks indicate significant difference from the WT ($\alpha=0.05$). C, RT-PCR showing the expression of *B. cinerea* actinA (*BcActA*) and tomato eukaryotic initiation factor4A (*eIF4A*) in *B. cinerea* infected WT, C4 and C15 leaf tissues. D, RNA-blot showing the expression of ySpdSyn transgene in water treated and *B. cinerea* inoculated leaves with increasing time period. Ribosomal RNA (rRNA) bands are shown to indicate total RNA loading.

**Figure 3.** Spermidine, PA biosynthesis inhibitors and ethylene precursor alter susceptibility of WT and T$_2$ homozygous ySpdSyn transgenic tomato leaves to *B. cinerea*
infection. A-D and F-G. Detached leaves were pretreated for 24 h with: A, water (mock control); B, 1 mM difluromethylornithine (DFMO); C, 1 mM cyclohexylamine (CHA); D, 100 μM aminocyclopropanecarboxylate (ACC); F, water (control); G, 1 mM of spermidine (Spd) and spot inoculated with of 3x10^5 spores/mL of B. cinerea. E and H, Disease lesion diameter determined 72 h after inoculation. Error bars indicate standard error of the means (n≥50). Statistical significance was determined using analysis of variance and Tukey’s HSD (Honestly Significant Difference) test for the separation of mean disease lesion size. Letters on top of the bars indicate significantly different values from each other at α=0.05.

**Figure 4.** Expression of ySpdSyn gene alters expression of ethylene biosynthesis gene ACC synthase 2 – (SlACS2; A), SA response marker gene SlPR1 (B), ethylene-signaling genes SlEIL1 (C), SlEIL2 (D) and SlERF1B (E), and ethylene-dependent pathogenesis related gene β-1, 3- glucanase (F) in B. cinerea-inoculated and mock (water treated) WT and T2 homozygous C4 and C15 leaves at 0 and 24 h post infection. Quantitative RT-PCR analysis was used to quantify transcript levels of tomato. Relative expression was calculated by the ΔΔCt method using actin as the reference gene. Error bars represent standard error of the means (n=3).

**Figure 5.** Pretreatment with S-adenosylmethionine (SAM) decreases susceptibility of T2 homozygous ySpdSyn transgenic tomato leaves to B. cinerea infection. A-B, Detached leaves were pretreated for 24 h with water (mock control; A) or 200 μM SAM (B) and spot inoculated with 3x10^5 spores/mL of B. cinerea. Leaves were photographed 72 h after inoculation. C, Disease lesion diameter determined 72 h after inoculation. Error bars indicate standard error of the means (n≥50). Statistical analysis was performed using analysis of variance and Tukey’s HSD test for the separation of mean disease lesion size. Letters on top of the bars indicate significantly different values from each other at α=0.05.
Figure 6. Responses of ySpdSyn-expressing tomato transgenic plants to Alternaria solani, Pseudomonas syringae pv. tomato DC3000 and Manduca sexta. A, WT and T₂ homozygous transgenic C4 and C15 leaves were drop inoculated with 300mg/ml A. Solani cultures and lesion diameter was determined 7 days after inoculation and photographed. B, WT, C4 and C15 leaves were infiltrated with P. syringae suspension (OD₆₀₀ = 0.001). Bacterial colony forming units (cfu) were determined 0 and 3 days after infiltration. Data represent average values ± SE from (n=3). C, Four newly hatched tobacco hornworm larvae weighing 9-11 mg each were placed on each of six 8-week old WT, C4 and C15 plants growing in a greenhouse. The larval weight was determined 14 days after the start of the feeding experiment. Data represent average values ± SE from (n=24).

Figure 7. A model depicting interaction between PAs and ethylene biosynthesis and signaling during B. cinerea infection. Solid up arrow indicates increased Spd levels in C4 and C15 transgenic plants which attenuates ethylene biosynthesis gene ACS and may affect ethylene levels (dotted down-arrow). Ethylene response gene, ERF1 when induced enhances resistance in WT leaves or when impaired leads to susceptibility in transgenic C4 and C15 leaves.

Supplemental Data
Supplemental Table 1: Sequences of forward (F) and reverse (R) primers used for gene expression studies shown in Figure 2C and Figure 4

Supplemental Figure S1. Expression of ySpdSyn does not alter the triple response of WT and T₂ homozygous C4 and C15 transgenic seedlings in the presence of exogenous ACC. Seeds were germinated on MS medium with or without 1 µM ACC and incubated in the dark for 6 days before responses of seedling growth were recorded.

Supplemental Figure S2. Expression of ySpdSyn did not alter germination in the presence of exogenous hydrogen peroxide (H₂O₂), methyl-JA (MeJA) or ABA. T₂
homozygous C4 and C15 transgenic lines showed germination response similar to WT in MS medium (control; A) and MS medium supplemented with 3 mM H$_2$O$_2$ (B), 10 µM MeJA (C), and 2 µM ABA (D).

**Supplemental Figure S3.** Response of wild type (WT) and T$_2$ homozygous C4 and C15 transgenic leaves were similar after treatment with methyl viologen (MV). Leaves were treated with 7 µl of water (control) or water containing 50 µM or 100 µM MV.
A. *Alternaria solani*

B. *Pseudomonas syringae*

C. *Manduca sexta*
