Apoplastic Polyamine Oxidation Plays Different Roles in Local Responses of Tobacco to Infection by the Necrotrophic Fungus Sclerotinia sclerotiorum and the Biotrophic Bacterium Pseudomonas viridiflava\textsuperscript{1}[W]

María Marina, Santiago Javier Maiale, Franco Rubén Rossi, Matías Fernando Romero, Elisa Isabel Rivas, Andrés Gárriz\textsuperscript{2}, Oscar Adolfo Ruiz, and Fernando Luis Pieckenstain\textsuperscript{*}

Unidad de Biotecnología 1, Instituto Tecnológico de Chascomús/Universidad Nacional de General San Martín-Consejo Nacional de Investigaciones Científicas y Técnicas, B7130IWA Chascomus, Argentina

The role of polyamine (PA) metabolism in tobacco (Nicotiana tabacum) defense against pathogens with contrasting pathogenic strategies was evaluated. Infection by the necrotrophic fungus Sclerotinia sclerotiorum resulted in increased arginine decarboxylase expression and activity in host tissues, as well as putrescine and spermine accumulation in leaf apoplast. Enhancement of leaf PA levels, either by using transgenic plants or infiltration with exogenous PAs, led to increased necrosis due to infection by S. sclerotiorum. Specific inhibition of diamine and PA oxidases attenuated the PA-induced enhancement of leaf necrosis during fungal infection. When tobacco responses to infection by the biotrophic bacterium Pseudomonas viridiflava were investigated, an increase of apoplastic spermine levels was detected. Enhancement of host PA levels by the above-described experimental approaches strongly decreased in planta bacterial growth, an effect that was blocked by a PA oxidase inhibitor. It can be concluded that accumulation and further oxidation of free PAs in the leaf apoplast of tobacco plants occurs in a similar, although not identical way during tobacco defense against infection by microorganisms with contrasting pathogenesis strategies. This response affects the pathogen’s ability to colonize host tissues and results are detrimental for plant defense against necrotrophic pathogens that feed on necrotic tissue; on the contrary, this response plays a beneficial role in defense against biotrophic pathogens that depend on living tissue for successful host colonization. Thus, apoplastic PAs play important roles in plant-pathogen interactions, and modulation of host PA levels, particularly in the leaf apoplast, may lead to significant changes in host susceptibility to different kinds of pathogens.

Polyamines (PAs) are natural aliphatic polycations ubiquitous in prokaryotic and eukaryotic cells and are essential for cell growth, proliferation, and differentiation (Cohen, 1998). Although the mechanism of PA action in many of these processes is not completely clear, it is well known that they are positively charged at cellular pH, and therefore bind to negatively charged molecules such as nucleic acids, acidic phospholipids, and proteins (Igarashi et al., 1982). In this way, PAs modulate DNA-protein (Shah et al., 1999) interactions, protein-protein (Thomas et al., 1999) interactions, and RNA structure (Igarashi and Kashiwagi, 2000). In addition to their free forms, PAs also exist as conjugates, covalently bound to small molecules and proteins. The most abundant PAs, namely the diamine putrescine, the triamine spermidine, and the tetraamine spermine are synthesized from the amino acids Orn and Arg by two alternative pathways. One of these pathways is shared by almost all organisms and involves the decarboxylation of Orn by the enzyme Orn decarboxylase (ODC; EC 4.1.1.17) to form putrescine. This diamine is then successively aminopropylated to produce spermidine and spermine by spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22), respectively. The aminopropyl groups are donated by decarboxylated S-adenosyl-Met, a compound synthesized by S-adenosyl-Met decarboxylase (AdoMetDC; EC 4.1.1.50) from S-adenosyl-Met. An alternative pathway occurs in plants and bacteria, where Arg is decarboxylated to agmatine by Arg decarboxylase (ADC; EC 4.1.1.19), and agmatine is then transformed into putrescine via N-carbamoyl-putrescine.

In addition to the above-mentioned biosynthetic pathways, oxidative deamination mediated by amine oxidases plays an important role in the regulation of cellular PA levels (Tiburcio et al., 1997). Amine oxidases include the copper-containing diamine oxidases...
(DAOs) and the FAD-containing PA oxidases (PAOs). DAOs (EC 1.4.3.6) from plants oxidize the diamines putrescine and cadaverine at the primary amino groups. PAOs (EC 1.5.3.11) use FAD as a cofactor and catalyze the oxidation of spermidine, spermine, and/or their acetylated derivatives at the secondary amino groups (Cohen, 1998). The action of DAO on putrescine yields pyrroline, hydrogen peroxide, and ammonia, while PAO action on spermidine and spermine yields pyrroline and 1-(3-aminopropyl)pyrroline, respectively, as well as 1,3-diaminopropane and hydrogen peroxide. 1-(3-Aminopropyl)pyrroline, which exists as the iminium cation, is converted to the bicyclic compound 1,5-diazabicyclononane (Cohen, 1998). There is evidence that DAO and PAO are predominantly located in the cell wall (Angelini 1998). There is evidence that DAOs (EC 1.4.3.6) from plants oxidize the diamines putrescine and cadaverine at the primary amino groups. PAOs (EC 1.5.3.11) use FAD as a cofactor and catalyze the oxidation of spermidine, spermine, and/or their acetylated derivatives at the secondary amino groups (Cohen, 1998). The action of DAO on putrescine yields pyrroline, hydrogen peroxide, and ammonia, while PAO action on spermidine and spermine yields pyrroline and 1-(3-aminopropyl)pyrroline, respectively, as well as 1,3-diaminopropane and hydrogen peroxide. 1-(3-Aminopropyl)pyrroline, which exists as the iminium cation, is converted to the bicyclic compound 1,5-diazabicyclononane (Cohen, 1998). There is evidence that DAO and PAO are predominantly located in the cell wall (Angelini et al., 1993; Sebela et al., 2001), and the production of hydrogen peroxide resulting from PA has been correlated with developmental cell wall maturation and lignification in several plant species (Cona et al., 2003; Paschalidis and Roubelakis Angelakis, 2005; Cona et al., 2006a).

PA metabolism undergoes significant modifications during plant responses to various abiotic and biotic stresses (for review, see Flores, 1990; Walters, 2003). The levels of free and conjugated PAs, as well as the activities of PA biosynthetic and catabolic enzymes have been shown to increase during the hypersensitive response (HR) of barley (Hordeum vulgare) to the powdery mildew fungus Blumeria graminis f. sp. hordei (Cowley and Walters, 2002). A similar picture was found during the HR of a tobacco (Nicotiana tabacum) cultivar resistant to the Tobacco mosaic virus (TMV). In this case, ODC, ADC, and AdoMetDC activities were found to increase, thus leading to elevated concentrations of free and conjugated PAs, mainly in necrotic lesions (Torrigiani et al., 1997). Moreover, it was later demonstrated that both PA biosynthesis and catabolism are up-regulated during the HR of tobacco plants to TMV (Marini et al., 2001). Thus, the above-mentioned works indicate that PA oxidation plays a role in cell death programs associated to the HR. Moreover, hydrogen peroxide derived from PA oxidation has been shown to contribute to wound healing (Angelini et al., 2008) and cell wall reinforcement in response to invasion of chickpea (Cicer arietinum) tissues by the fungal pathogen Ascochyta rabiei (Rea et al., 2002).

The bulk of the work performed in relation with plant PAs during the response to pathogen attack has pointed to metabolic changes occurring at the organ or tissue level, without focusing on intercellular spaces. Most phytopathogenic bacteria multiply in the intercellular space, also known as the apoplast, and frequently, this is the first site of pathogenic fungi invasion (Glazebrook, 2005). Moreover, pathogenesis-related proteins are accumulated in the intercellular space (Van Loon, 1997), and it has been shown that some hormones such as salicylic acid or its glucoside, which inhibit the multiplication of certain pathogens, increase their concentration in apoplastic spaces during plant-pathogen interaction (Beckers and Spoel, 2006; Grant and Lamb, 2006). Thus, it is of great interest that Yamakawa et al. (1998) found TMV infection to induce spermine accumulation in apoplastic fluids of tobacco plants, this tetraamine acting as an inducer of acidic pathogenesis-related gene expression in a salicylic acid-independent manner. In addition, these authors demonstrated that changes in PA levels elicited by TMV infection at the whole leaf level were not correlated with those occurring specifically in intercellular spaces, thus suggesting that apoplastic PA metabolism deserves further attention in relation with plant responses to pathogen attack. Later works demonstrated that spermine causes mitochondrial dysfunction via a signaling pathway that stimulates the activity of mitogen-activated protein kinases (Takahashi et al., 2003), which in turn activates a subset of HR-specific genes (Takahashi et al., 2004). This observation stresses the relevance of spermine accumulation in the intercellular space of pathogen-infected leaves as a potential mechanism of plant defense activation.

To our knowledge, information about the role of apoplastic PAs in plant defense against pathogens is restricted to the tobacco-TMV pathosystem, the consequences of fungal or bacterial infection on apoplastic PA metabolism having not been studied so far. Moreover, a comparison of the changes in PA metabolism elicited in a given plant host by pathogenic organisms with contrasting (necrotrophic and biotrophic) pathogenic strategies was not performed so far. Therefore, this work aimed to determine the role of plant PAs as part of local defense responses to plant infection by pathogenic microorganisms with contrasting pathogenesis strategies, with emphasis on the consequences of apoplastic PA oxidation on the ability of plant pathogens to colonize host tissues. For this purpose, experiments were carried out using tobacco as a plant host, and two different pathogens. One of them was the cosmopolitan and polifagous ascomycete Sclerotinia sclerotiorum, which infects more than 400 plant species. This fungus is considered a necrotroph because it kills host cells during the infection process and causes extensive tissue damage (Bolton et al., 2006). The other pathogen employed in this study was Pseudomonas viridiflava, a bacterium that causes tomato (Solanum lycopersicum) pith and basal stem rot (Malathrakis and Goumas, 1987), and is also pathogenic on other hosts, including tobacco (Alippi et al., 2003). Similarly to other Pseudomonas species (Glazebrook, 2005), P. viridiflava multiplies asymptomatically in the intercellular spaces of the host cell early during the infection process, chlorosis and necrosis being evident only at later stages. Therefore, this bacterium can be considered as a biotroph, at least during the initial phase of infection. This work contributes to the understanding of the relevance of apoplastic PA accumulation and oxidation in relation with plant tolerance to necrotrophic and biotrophic pathogens.
RESULTS

Accumulation of Free PAs in Leaf Extracts and Apoplastic Fluids of Tobacco Plants Infected by *S. sclerotiorum*

Tobacco 'Xanthi-nc' leaves developed typical brownish necrotic lesions 24 h after inoculation (HAI) with *S. sclerotiorum* mycelium, and lesion size further increased as a function of time. Samples for free PA determination in leaf extracts and apoplastic fluids were taken from noninfected tissues adjacent to the lesions 48 HAI, and were found to be free of fungal mycelium by observation under a binocular microscope. In leaf extracts, putrescine and spermidine were the most abundant free PAs, their levels being not affected by fungal infection (Fig. 1A). A small but significant decrease of spermine level was detected in leaf extracts of inoculated plants, as compared with controls (Fig. 1A). When apoplastic PA levels were evaluated, an increase of free putrescine, spermidine, and spermine was detected in leaves infected by *S. sclerotiorum*, although the increase in spermidine levels lacked statistical significance (Fig. 1B). Thus, the levels of free putrescine in intercellular washing fluids (IWFs) of infected leaves increased by 7-fold, as compared with noninoculated controls. Apoplastic spermine level was below the detection limit in control leaves, and reached 0.013 nmol mL⁻¹ in infected ones (Fig. 1B).

Leaf Infection by *S. sclerotiorum* Increases ADC Activity and Induces ADC Transcript Accumulation

To determine the biochemical basis of the above-described changes in PA levels elicited by *S. sclerotiorum* infection, the activities of several enzymes involved in PA biosynthesis were determined. For this purpose, leaf samples were taken from noninfected tissues adjacent to the lesions 48 HAI, as described in the preceding section. ADC activity was 4-fold higher in extracts obtained from leaves infected by *S. sclerotiorum* than in controls (mock-inoculated) 48 HAI (Fig. 2A). On the contrary, ODC and AdoMetDc activities were not affected by fungal infection (Fig. 2A). ADC, ODC, and AdoMetDc activities were also evaluated in IWFs and found to be extremely low, being <0.5% of the activities detected in leaf extracts (data not shown), thus suggesting that the apoplastic compartment makes no significant contribution to the activity of these enzymes detected in leaf extracts.

Given that ADC was the sole enzymatic activity found to be modified as a consequence of fungal infection, the steady-state levels of ADC mRNA were analyzed by semiquantitative reverse transcription (RT)-PCR and found to be higher in infected than in mock-inoculated leaves (Fig. 2B).

PA Accumulation Increases Disease Severity in Leaves Infected by *S. sclerotiorum*

As demonstrated in previous sections, infection of tobacco leaves by *S. sclerotiorum* resulted in the induction of changes in free PA levels, both in leaf extracts and apoplastic fluids. To understand the consequences of changes in PA levels on the local response to fungal infection and further disease development, tobacco leaf discs with enhanced PA levels were used as a target for inoculation with *S. sclerotiorum* and subsequent determination of the percentage of necrotic tissue, as an indicator of disease severity. For this purpose, two alternative approaches were employed. The first approach aimed to modify cellular PA levels, and was based in the use of transgenic tobacco 'Wisconsin W38' TetR/Oat ADC plants that express an oat (*Avena sativa*) ADC gene under the control of a tetracycline-inducible promoter (Masgrau et al., 1997). In this way, ADC activity and putrescine and spermidine levels were, respectively, 3-, 2-, and 3-fold higher in extracts obtained from discs induced by floating in a tetracycline solution during 24 h than in extracts obtained from noninduced discs to which no tetracycline-inducible promoter (Masgrau et al., 1997).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Free PA levels in tobacco leaves infected by *S. sclerotiorum*. Leaves were inoculated on the adaxial surface by placing discs of *S. sclerotiorum* mycelium cut from the edge of an actively growing colony. A and B. Samples for free PA determination in leaf extracts (A) and IWFs (B) were taken from noninfected tissues adjacent to the lesions 48 HAI. Putrescine (Put), spermidine (Spd), and spermine (Spm) concentrations were determined in mock-inoculated (white bars) and *S. sclerotiorum*-inoculated (black bars) plants using HPLC after derivatization with dansyl chloride. The values are reported in nanomoles relative to leaf fresh weight (FW) in A and to the volume of IWF in B. Statistical differences in the level of each PA between mock-inoculated and infected plants according to Student's *t* test are shown as: *, *P* < 0.05; ***, *P* < 0.001.


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The second approach for the modification of PA levels prior to inoculation with *S. sclerotiorum* aimed toward the modification of apoplastic PAs, and consisted of vacuum infiltration of leaf discs of wild-type Xanthi-nc tobacco plants with 5 μM solutions of putrescine, spermidine, and spermine in water. Infiltration with these PAs increased lesion size, respectively, by 12-, 18-, and 15-fold, as compared with control discs infiltrated with water (Fig. 4).

**Activity and Inhibition of Amine-Oxidizing Enzymes in Tobacco Leaf Apoplast**

DAO and PAO activities were detected in vitro using IWFs as a source of enzyme and PAs as substrates (Fig. 5, A and B), these activities being not affected by *S. sclerotiorum* infection (Supplemental Fig. S1A). A temporal analysis of DAO and PAO activities throughout the life cycle of tobacco plants was also performed, both activities being found to decline as plant age increased (Supplemental Fig. S2). To evaluate the participation of DAO and PAO in lesion development as a consequence of PA accumulation triggered by *S. sclerotiorum* infection, the ability of specific inhibitors to interfere with the activity of these enzymes was tested. Two of the inhibitors used were \( \text{N,N}'\)-diaminoguanidine, a compound reported to act as a competitive inhibitor of soybean (*Glycine max*) DAO (Nikolov et al., 1990; Xing et al., 2007) and guazatine, which competitively inhibits maize (*Zea mays*; Cona et al., 2004) and tobacco (*Nicotiana tabacum*) PAOs. In addition, the PA analog 1,19-bis(ethylamino)-5,10,15-triazanonadecane (Bacchi et al., 2002), which has been found by our group to inhibit oat and maize

![Graph](image.png)

**Figure 2.** A, ADC, ODC, and AdoMetDC activities in tobacco leaves infected by *S. sclerotiorum*. Leaves were inoculated with *S. sclerotiorum* and samples for the determination of enzyme activities were taken from mock-inoculated tissues adjacent to the lesions 48 HAI. ADC, ODC, and AdoMetDC activities were determined in mock-inoculated (white bars) and *S. sclerotiorum*-inoculated plants as described in “Materials and Methods.” Statistical differences in each enzymatic activity between mock-inoculated and infected plants according to Student’s t test are shown as **, \( P \leq 0.01 \). B, Steady-state levels of ADC mRNA in tobacco leaves infected by *S. sclerotiorum*. Samples were taken as described above. Semiquantitative RT-PCR analysis of ADC mRNA was performed with RNA extracted from mock-inoculated and *S. sclerotiorum*-inoculated plants. ADC and actin cDNA bands were visualized by ethidium bromide staining in agarose gels.

![Image](image.png)

**Figure 3.** Necrosis caused by *S. sclerotiorum* infection on leaf discs of transgenic tobacco plants that overexpress an oat ADC gene. Leaf discs of transgenic tobacco V38 TetR/Oat ADC and V38 TetR plants were incubated in 5 mM, pH 6.5, MES buffer amended with tetracycline (2 mg mL\(^{-1}\); +tet) or without amendments (−tet) for 24 h, prior to inoculation with *S. sclerotiorum*. The size of the necrotic area around the inoculation site was determined 48 HAI, using the Image-ProPlus V 4.1 software (Media Cybernetics) and was expressed as a percentage of the whole disc area. Statistical differences in the percentage of leaf necrosis between discs induced with tetracycline and noninduced controls of the same transgenic line according to Student’s t test are shown as ***, \( P \leq 0.001 \).
PAOs in vitro (S. Maiale, unpublished data) was also employed. The ability of the above-mentioned compounds to inhibit amine-oxidizing enzymes under the experimental conditions employed in this work was tested in vitro, using putrescine and spermine as substrates. In this way, N,N′-diaminoguanidine was found to inhibit DAO activity by 58%, while the inhibition caused by 1,19-bis(ethylamino)-5,10,15-triazanonadecane and guazatine reached 17% and 30%, respectively (Fig. 5A). 1,19-Bis(ethylamino)-5,10,15-triazanonadecane and guazatine also inhibited PAO activity by 64% and 75%, respectively (Fig. 5B).

Given that no information was available regarding the ability of 1,19-bis(ethylamino)-5,10,15-triazanonadecane to inhibit PA oxidation in vivo, the effect of this compound on spermine oxidation was also evaluated in vivo, by infiltration of leaf discs and subsequent determination of apoplastic spermine levels, as well as those of 1,3-diaminopropane, one of the products of spermine oxidation. As expected, infiltration of leaf discs with spermine led to a significant increase in the apoplastic concentration of this tetraamine 4 h after infiltration, as compared with controls, in which spermine was undetectable (Fig. 6A). Disc infiltration with 1,19-bis(ethylamino)-5,10,15-triazanonadecane and guazatine also inhibited PAO activity by 64% and 75%, respectively (Fig. 5B).

Severity of Tobacco Leaf Rot Caused by S. sclerotiorum Is Reduced by Inhibition of Apoplastic PA Oxidation

Results presented in the previous section suggested that the use of amine oxidase inhibitors could contrib-
ute to determine whether apoplastic PA oxidation plays a role during plant defense against *S. sclerotiorum* infection. Taking into account that PA accumulation in the leaf apoplast led to enhanced severity of leaf rot caused by *S. sclerotiorum*, it was decided to evaluate whether this effect is mediated by PA oxidation. For this purpose, the compounds demonstrated in previous sections to act as tobacco amine oxidase inhibitors were used to evaluate their effect on leaf rot severity. Previously, the effect of these compounds on mycelial growth of *S. sclerotiorum* was tested in vitro. Concentrations up to 100 μM of *N,N*-diaminoguanidine (data not shown) and 1,19-bis(ethylamino)-5,10,15-triazanonadecane (S. Maiale, unpublished data) proved to exert no effect on mycelial growth when added to the growth medium. Guazatine, on the contrary, strongly inhibited mycelial growth in concentrations as low as 5 μM (S. Maiale, unpublished data), which is in good agreement with the previously reported fungicidal effect of this compound (Mackintosh and Walters, 2003; Dreassi et al., 2007). As a consequence, guazatine was not included in the experiments aimed to evaluate the effect of amine oxidase inhibitors on leaf rot severity; in that lesion development could be restricted because of a direct effect of this compound on fungal growth. Thus, lesion size was determined in leaf discs inoculated with *S. sclerotiorum* after infiltration with *N,N*-diaminoguanidine and 1,19-bis(ethylamino)-5,10,15-triazanonadecane, either separately or in combination with PAs.

As demonstrated in previous sections, infiltration with putrescine, spermidine, and spermine led to an increase in the percentage of leaf necrosis, as compared with controls infiltrated with no PAs (Fig. 7, A and B). In this way, infiltration with putrescine increased leaf necrosis by 2-fold. However, when putrescine was coinfiltrated with the DAO inhibitor *N,N*-diaminoguanidine, the percentage of leaf necro-

![Figure 6](image-url) **Figure 6.** Inhibition of spermine oxidation by 1,19-bis(ethylamino)-5,10,15-triazanonadecane (SL-11061) in tobacco leaf discs. Discs were infiltrated with 5 μM spermine (Spm) and 1,19-bis(ethylamino)-5,10,15-triazanonadecane (SL-11061), either separately or in combination. Discs infiltrated with water were used as controls. Intercellular washing fluids were collected 4 (A) and 24 (B) h after incubation. Apoplastic spermine levels, as well as those of the product of spermine oxidation 1,3-diaminopropane (Dap) were determined using HPLC after derivatization with dansyl chloride. 1,3-Diaminopropane and spermine were undetectable 4 and 24 h after infiltration, respectively. Different letters indicate statistically significant differences (*P* ≤ 0.01) according to one-way ANOVA and Bonferroni’s test.

![Figure 7](image-url) **Figure 7.** A and B, Effects of DAO (A) and PAO (B) inhibition on necrosis of tobacco leaf discs infected by *S. sclerotiorum*. A, Leaf discs were vacuum infiltrated with 5 μM putrescine (Put) and *N,N*-diaminoguanidine monohydrochloride (AG), either separately or in combination. B, Discs were vacuum infiltrated with 5 μM spermidine (Spd), 5 μM spermine (Spm), and 5 μM 1,19-bis(ethylamino)-5,10,15-triazanonadecane (SL-11061), according to the combinations indicated in the figure. In both panels, leaf discs infiltrated with water were used as controls. After infiltration, discs were inoculated with *S. sclerotiorum* and incubated for 48 h. The size of the necrotic area around the inoculation site was determined using the Image-ProPlus V 4.1 software (Media Cybernetics) and was expressed as a percentage of the whole disc area. Different letters indicate statistically significant differences (*P* ≤ 0.05), according to one-way ANOVA and Bonferroni’s test.
Oxidation of Apoplastic PAs and Its Relation with the Generation of ROS

Besides 1,3-diaminopropane, PA oxidation also leads to hydrogen peroxide formation. Taking into account the diversity and importance of the roles played by hydrogen peroxide and other reactive oxygen species (ROS) in plant defense against pathogen attack, it was decided to evaluate whether oxidation of apoplastic PAs contributes to ROS accumulation in the apoplastic compartment of tobacco leaves. In this way, hydrogen peroxide production by leaf discs infiltrated with 5 \( \mu M \) putrescine, spermidine, and spermine was evaluated using 3,3'-diaminobenzidine, no accumulation of this active oxygen species being detected (data not shown).

In addition, superoxide generation in leaf discs infiltrated with putrescine and spermine was evaluated, using nitroblue tetrazolium (NBT). Infiltration with either 5 \( \mu M \) putrescine or spermine led to superoxide accumulation in the intercellular spaces, as evidenced by the formation of a blue formazan precipitate. In this way, superoxide levels in discs infiltrated with these PAs were 2-fold higher than in water-infiltrated controls, in which superoxide levels were very low (Fig. 8). Coinfiltration of discs with 5 \( \mu M \) putrescine and 20 \( \mu M \) \( N,N' \)-diaminoguanidine reduced superoxide formation to levels similar to those of controls (Fig. 8). Similarly, coinfiltration of 5 \( \mu M \) spermine with 20 \( \mu M \) 1,19-bis(ethylamino)-5,10,15-triazanonadecane or 20 \( \mu M \) guazatine also reduced superoxide generation to levels similar to those of controls (Fig. 8).

Changes in Apoplastic PA Levels and Amine-Oxidizing Enzymes in Response to Infection by the Biotrophic Bacterial Pathogen P. viridiflava

As a whole, results obtained in previous sections demonstrated that infection of tobacco leaves by the necrotrophic fungus S. sclerotiorum induces PA accumulation in the apoplastic compartment, which results in PA oxidation and thus increases leaf rot severity. This prompted us to evaluate whether similar responses operate during infection of tobacco leaves by biotrophic pathogens. For this purpose, tobacco plants were infiltrated with the bacterium P. viridiflava. Samples for the determination of free PAs and PAO and DAO activities in leaf extracts and apoplastic fluids were taken from tissues adjacent to the infiltration zone, 20 and 48 HAI. Prior to using IWFs for the above-mentioned determinations, bacteria were eliminated by centrifugation to minimize contamination of this fraction with metabolites from bacterial origin. The IWFs obtained in this way were free of bacterial cells, as demonstrated by the absence of bacterial colonies after inoculation of 20 \( \mu L \) aliquots onto solid King’s B medium. No changes in PA levels were evident in leaf extracts of inoculated plants at both HAI times, as compared with controls (data not shown). On the contrary, changes in apoplastic spermine levels were evident in leaves infected by P. viridiflava, the levels of this tetraamine being 4- and 10-fold higher in leaves infected by P. viridiflava than in controls, when evaluated 20 (Fig. 9A) and 48 (Fig. 9B) HAI, respectively. As occurred after leaf infection by S. sclerotiorum, DAO and PAO activities were not affected by P. viridiflava infection (Supplemental Fig. S1B).

PA Accumulation and Oxidation Decreases Leaf Colonization by P. viridiflava

The effect of increased PA levels on P. viridiflava multiplication in planta was evaluated by two alter-
native approaches similar to those previously described for the determination of leaf rot severity caused by S. sclerotiorum. The first approach consisted of tetracycline-mediated induction of heterologous ADC gene expression in leaf discs of transgenic tobacco W38 Tet/Oat ADC plants, which results in 2- and 3-fold increases in putrescine and spermidine levels, respectively, as described in previous sections. Bacterial growth was strongly reduced in tetracycline-induced discs of W38 Tet/Oat ADC plants, as compared with noninduced discs of the same transgenic line, in which 3.3 \times 10^9 colony-forming units (CFU) mL$^{-1}$ were detected (Fig. 10). Incubation in tetracycline of leaf discs obtained from the transgenic W38 TetR line, which expresses no heterologous ADC gene, had no effect on bacterial growth, as compared with discs of the same line incubated in the absence of the antibiotic (Fig. 10). In this way, the transgenic W38 TetR line served to verify that tetracycline itself does not inhibit bacterial growth in planta.

The second approach for the modification of tobacco PA levels prior to inoculation with P. viridiflava was based on vacuum infiltration of leaf discs obtained from wild-type Xanthi-nc plants with a 5 \mu M solution of spermine in water. Infiltration with this tetraamine strongly decreased bacterial growth in inoculated discs, as compared with controls infiltrated with water (Fig. 11). Inclusion of both 1,19-bis(ethylamino)-5,10,15-triazanonadecane and spermine in the infiltration solution partly reverted the inhibitory effect of spermine on bacterial growth in planta, this parameter reaching an intermediate value between that of discs infiltrated only with spermine and controls (Fig. 11). Moreover, infiltration of discs with 1,19-bis(ethylamino)-5,10,15-triazanonadecane and no spermine resulted in higher bacterial growth than in controls infiltrated with water, this treatment leading to the highest bacterial growth in planta, as compared to all the treatments evaluated (Fig. 11).

**DISCUSSION**

Infection by pathogenic microorganisms has been reported to induce modifications in PA metabolism in different plant hosts (for review, see Walters, 2003), which in many cases consisted of PA accumulation, either in their free or conjugated forms. Although the understanding of the physiological significance of pathogen-induced changes in PA metabolism of plants is far from being complete, PAs have been shown to play several roles in plant defense against pathogen attack. Conjugated PAs exert antimicrobial effects (Peng and Kuc, 1992; Walters et al., 2001), and the
oxidation of free PAs has been proposed to contribute to cell-death processes occurring during the HR of tobacco plants to TMV (Marini et al., 2001). In addition, the tetraamine spermine acts as an inducer of pathogenesis-related proteins (Yamakawa et al., 1998) and also participates in signaling cascades that activate HR-specific genes in tobacco (Takahashi et al., 2003, 2004; Kusano et al., 2007). In this work, the consequences of apoplastic PA oxidation on host tissue colonization by microorganisms with contrasting pathogenic strategies, and its relation with plant tolerance to these kinds of pathogens, were investigated.

Changes in PA Metabolism of Tobacco Plants Infected by the Necrotrophic Fungus S. sclerotiorum

Studies conducted so far in relation with the role of PAs in plant-pathogen interactions have focused mainly on viruses (Negrel et al., 1984; Bellés et al., 1991; Torrigiani et al., 1997; Yamakawa et al., 1998; Hiraga et al., 2000; Marini et al., 2001; Yoda et al., 2003; Yoo et al., 2004) and biotrophic fungi causing rusts and mildews on several different hosts (Walters et al., 1985; Walters and Wylie, 1986; Cowley and Walters, 2002). However, the attention previously devoted to the effects of necrotrophic plant pathogens on host PA metabolism has been very scant (Angelini et al., 1993; Rea et al., 2002). In this work, tobacco plants infected by the ascomycete S. sclerotiorum were used as a model for the study of perturbations induced by necrotrophic pathogens on host PA metabolism. Healthy tissues adjacent to necrotic lesions, which were proved to be free of fungal mycelium, were used as a target for the study of changes in free PA levels and PA biosynthesis enzymes. When PA levels were evaluated in tissue extracts, a slight decrease in free spermine levels was the sole change induced by fungal infection. A very different picture was found when apoplastic PA levels were evaluated, free putrescine and spermine levels being significantly higher in infected plants than in noninfected controls. Similarly, Yamakawa et al. (1998) found that apoplastic spermine levels of tobacco plants undergo distinctive changes in response to infection by the TMV, which are not evidenced by the evaluation of PA levels in leaf extracts. Thus, it is of interest that pathogens as different as S. sclerotiorum and TMV both induce accumulation of apoplastic spermine. PA accumulation in apoplastic fluids of tobacco leaves infected by S. sclerotiorum could be due to an increase in the activity of enzymes involved in PA biosynthesis. In support of this view, both ADC activity and mRNA levels were found to be increased in leaves infected by S. sclerotiorum. As opposed to what was reported for the interaction between tobacco and TMV, PA biosynthetic enzymes other than ADC were not induced by S. sclerotiorum infection. However, it should be kept in mind that Arg decarboxylation mediated by ADC is considered to be a rate-limiting step in PA biosynthesis, an increase in the activity of this enzyme being enough to enhance the levels of all the PAs in the biosynthetic pathway. Thus, the increase in host ADC activity probably contributed in a significant degree to apoplastic PA accumulation in infected tobacco plants. In addition, PAs released from conjugated and bound pools could also contribute to the raise in apoplastic free PA levels. The intracellular location of ADC and other PA biosynthetic enzymes has been demonstrated by other authors (Kaur-Sawhney et al., 2003), and in this work, the activities of these enzymes were found to be extremely low in IWFs. Therefore, mechanisms of PA export to the apoplastic compartment are expected to participate in the raise of apoplastic PA leaves in response to fungal infection, a subject that deserves further investigation. It is also worth pointing out that S. sclerotiorum, like the majority of fungi studied so far, lacks ADC activity (Pieckenstain et al., 2001). In addition, spermine levels in S. sclerotiorum cells are very low, both in mycelium and ascospores (Garriz et al., 2003, 2004). Thus, the increase in ADC activity and apoplastic spermine occurring in leaves infected by S. sclerotiorum is expected to be derived from the host plant and not to result from contamination with fungal metabolites during sample preparation. To our knowledge, this is the first report about changes in PA levels and biosynthetic enzymes in response to plant infection by a necrotrophic pathogen.

The Consequences of Alterations in PA Metabolism and Inhibition of Amine Oxidase Activities on Severity of S. sclerotiorum Infection on Tobacco Plants

To evaluate the consequences of changes in host PA levels on tolerance to pathogen infection, tobacco leaf
discs with enhanced PA levels were infected with S. sclerotiorum. Accumulation of free putrescine and spermidine in leaf discs of transgenic tobacco plants as a consequence of tetracycline-induced overexpression of an oat ADC gene, led to a strong increment in the necrosis caused by S. sclerotiorum infection. Similarly, enhancement of apoplastic putrescine, spermidine, or spermine levels by means of infiltration also caused a strong increase of leaf disc necrosis caused by this fungus. Thus, the two above-mentioned alternative approaches demonstrated that enhanced PA levels increase the severity of S. sclerotiorum infection. The question then rose about the mechanism by which PA accumulation in plant tissues increases disease severity. Yoda et al. (2003) demonstrated that PA infiltration into tobacco tissues induces hypersensitive cell death as the result of hydrogen peroxide formation due to PA oxidation, and proposed this mechanism to be involved in the HR of tobacco plants to TMV. Although effective against plant pathogens that depend on living host tissues, such as viruses and biotrophic fungi and bacteria, the HR is far from being beneficial as a plant response to infection by necrotrophic microorganisms, in that host-cell death favors tissue colonization by this kind of pathogens (Govrin and Levine, 2000; Mayer et al., 2001; Glazebrook, 2005). Therefore, the contribution of PA oxidation to the development of necrotic lesions in tobacco leaves infected by S. sclerotiorum was investigated. As an initial approach, the activity of amine oxidases involved in PA catabolism was evaluated in IWFs obtained from leaf discs. Taking into account that amine oxidases involved in PA catabolism are predominantly located in cell walls (Angelini et al., 1993; Sebela et al., 2001), IWFs were used as a source for the evaluation of these enzyme activities in vitro. Both DAO and PAO activities were detected and, although they were not affected by S. sclerotiorum infection, basal DAO and PAO activities could contribute to the development of necrotic lesions as a consequence of PA accumulation in the apoplast after infection. Therefore, it was decided to study the consequences of in vivo tobacco amine oxidase inhibition on the development of necrotic lesions during leaf infection by S. sclerotiorum. For this purpose, compounds capable of inhibiting amine oxidase activities of the plant host without exerting fungicidal effects would be useful. In this regard, N,N′-diaminoguanidine, a compound reported to inhibit soybean DAO (Nikolov et al., 1990; Xing et al., 2007), was found in this work to inhibit tobacco DAO, and exerted no effect on mycelial growth of S. sclerotiorum in vitro. Guazatine has been demonstrated to inhibit tobacco PAO activity (Yoda et al., 2006), but is a powerful fungicidal agent (Mackintosh and Walters, 2003; Dreassi et al., 2007), which was also found to cause a strong inhibition of mycelial growth of S. sclerotiorum in vitro (S. Maiale, unpublished data). As a consequence, a PAO inhibitor other than guazatine was necessary for in vivo studies about the role of PAO in the response of tobacco leaves to infection by S. sclerotiorum. Therefore, the utility of the synthetic PA analog 1,19-bis(ethylamino)-5,10,15-triazanonadecane (Bacchi et al., 2002), which was found in our laboratory to inhibit oat and maize PAOs in vitro (S. Maiale, unpublished data), was evaluated. Thus, this compound was found to inhibit tobacco PAO activity in vitro in a similar degree to guazatine. Taking into account the scarcity of information about the effects of 1,19-bis(ethylamino)-5,10,15-triazanonadecane on plant PA metabolism, its ability to inhibit PAO activity in vivo was also evaluated. In this way, 1,19-bis(ethylamino)-5,10,15-triazanonadecane was found to inhibit tobacco PAO activity in vivo. Even though endogenous spermine levels in control (noninfiltrated) plants were not high enough to be used as an indicator of PAO inhibition by 1,19-bis(ethylamino)-5,10,15-triazanonadecane, this compound led to an increase in apoplastic spermine levels when coinfiltinated with this tetraamine into leaf discs, as detected shortly (4 h) after infiltration. Moreover, 1,19-bis(ethylamino)-5,10,15-triazanonadecane decreased the accumulation of the product of spermine oxidation, 1,3-diaminopropane in discs infiltrated with spermine, as evaluated 24 h after infiltration. Thus, these results demonstrate that 1,19-bis(ethylamino)-5,10,15-triazanonadecane acts as a PAO inhibitor in tobacco leaves. Interestingly, and as opposed to guazatine, 1,19-bis(ethylamino)-5,10,15-triazanonadecane exerted no inhibition of mycelial growth of S. sclerotiorum in vitro. On the basis of the results obtained, N,N′-diaminoguanidine and 1,19-bis(ethylamino)-5,10,15-triazanonadecane were selected for further experiments aimed to inhibit tobacco DAO and PAO activities during the response to S. sclerotiorum infection. Moreover, results obtained in this work demonstrate that the PA analog 1,19-bis(ethylamino)-5,10,15-triazanonadecane is a powerful tool for the study of the physiological roles of PAOs, similarly to other inhibitors such as N-prenylagmatine (Cona et al., 2006b).

The DAO inhibitor N,N′-diaminoguanidine was found to attenuate the development of necrotic lesions caused by S. sclerotiorum infection, both in leaf discs infiltrated with putrescine and in control discs infiltrated with water. Similarly, the PAO inhibitor 1,19-bis(ethylamino)-5,10,15-triazanonadecane diminished S. sclerotiorum rot in leaf discs infiltrated with spermidine and spermine, as well as in control discs infiltrated with water. As a whole, these results demonstrate that DAO and PAO-mediated PA oxidation occurs during infection of tobacco tissues by S. sclerotiorum and, far from being beneficial to the host, contributes to disease development. This observation is in good agreement with previous works that found oxidative responses to increase the severity of infections caused by necrotrophic pathogens in plant hosts (Govrin and Levine, 2000; Mayer et al., 2001). On the other hand, results of this work are in apparent contradiction with those of Rea et al. (2002), who found DAO to participate in cell wall strengthening as part of chickpea (Cicer arietinum) local responses to Ascochyta rabiei, and proposed that this enzyme contributes to...
plant tolerance to infection by this necrotrophic fungus. However, these authors based their conclusions on experiments performed by infiltrating a DAO inhibitor into chickpea tissues once infection was already established, as opposed to this work, in which infiltrations with the inhibitors were done prior to infection. In this way, differences in the methodological approaches employed in both works, as well as in the pathogenic strategies of both organisms and defense mechanisms of both hosts probably explain these seemingly contradictory findings.

**PA Oxidation and the Generation of Active Oxygen Species**

Hydrogen peroxide is known to play several roles in plant defense against pathogen attack. In addition to the proposed direct antimicrobial effect (Mehdy et al., 1996; Rokjind et al., 2002), hydrogen peroxide acts via signal transduction pathways that lead to the expression of defense genes (Orozco-Cárdenas et al., 2001; Torres et al., 2006). Moreover, hydrogen peroxide formed through PA oxidation significantly contributes to hypersensitive cell death in tobacco (Yoda et al., 2003). In this work, infiltration of leaf discs with PA concentrations capable of leading to an increase in leaf necrosis caused by *S. sclerotiorum* infection, were not found to result in the accumulation of detectable hydrogen peroxide levels. In this regard, it should be noticed that PA concentrations used for infiltration experiments were in the range of those concentrations detected in IWFs after leaf infection by *S. sclerotiorum*. In this way, hydrogen peroxide formed through oxidation of PA quantities used in this work is expected to reach very low levels, which probably are not detected by the 3,3′-diaminobenzidine method employed.

As opposed to hydrogen peroxide, superoxide radical was found to be accumulated when leaf discs were infiltrated with PA concentrations capable of increasing leaf necrosis caused by *S. sclerotiorum*. PA-induced accumulation of superoxide was decreased by amine oxidase inhibitors, thus demonstrating that production of this active oxygen species was the result of PA oxidation. In this way, superoxide produced after PA accumulation and oxidation in the apoplast probably contributes to the development of leaf necrosis during infection by *S. sclerotiorum*, as a consequence of the toxicity of this active oxygen species on host tissues.

The way in which superoxide was produced after PA oxidation remains to be explained. A possible explanation is that hydrogen peroxide is converted into superoxide by the nonenzymatic reactions of Fenton and Haber-Weiss (Fenton, 1894; Haber-Weiss, 1934). The fact that hydrogen peroxide was not detected in this work does not rule out this hypothesis, given that this active oxygen species could be produced in low amounts and be converted rapidly into other active oxygen species by the above-proposed mechanism.
growth in tobacco leaves. However, it cannot be ruled out that spermine plays additional roles during the response of tobacco plants to *P. viridiflava* infection, as well as in response to *S. sclerotiorum*. Spermine has been shown to act as a systemic inducer of PR gene expression (Yamakawa et al., 1998) and participates in signaling cascades that activate HR-specific genes in tobacco (Takahashi et al., 2004). Therefore, additional work will be necessary to determine whether spermine also plays such roles in tobacco plants infected by the two pathogens studied in this work.

CONCLUSION

PA metabolism of tobacco plants undergoes similar, although not identical, changes in response to infection by two pathogens with opposing pathogenic strategies. These changes lead to the accumulation of apoplastic free putrescine and spermine in response to infection by the necrotrophic fungus *S. sclerotiorum*, and free spermine in response to the biotrophic bacterium *P. viridiflava*, these PAs acting as substrates of amine oxidases. PA accumulation and oxidation play a role that is beneficial for the host during local responses to infection by the biotrophic bacterium *P. viridiflava*, by restricting bacterial growth in planta. On the contrary these responses are detrimental in local defense to infection by the necrotrophic fungus *S. sclerotiorum*, and lead to increased disease severity. It still remains to be determined whether reactions similar to those found in this work are of general occurrence in other plant hosts infected by necrotrophic and biotrophic pathogens. However, the findings of this work are in good agreement with the fact that host responses leading to cell death contribute to restricting infection by biotrophic pathogens, but play a negative role in local responses to necrotrophic organisms. As a summary, it can be concluded that apoplastic free PAs play an important role in local responses to pathogen infection by affecting the ability of pathogenic microorganisms to colonize host tissues. This work also demonstrates that modification of plant PA metabolism by means of a transgenic approach based on the use of PA biosynthetic genes under the control of an inducible promoter can contribute to modifying plant responses to pathogen infection.

MATERIALS AND METHODS

**Fungal and Bacterial Strains**

An isolate of *Sclerotinia sclerotiorum* from the IIB-INTECH Fungal Culture Collection (IFCC 458/02), originally isolated from sunflower (*Helianthus annuus*) capitala with head rot symptoms, was used for all the experiments. The fungus was routinely maintained in potato-dextrose agar slants at 4°C.

Prior to inoculation on tobacco (*Nicotiana tabacum*), mycelium was grown in solid Czapek-Dox medium (50 g L⁻¹ CaCl₂, 2 g L⁻¹ NaNO₃, 1 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ MgSO₄ ∙ 7H₂O, 0.5 g L⁻¹ KCl, 0.05 g L⁻¹ FeSO₄ ∙ 7H₂O, 20 g L⁻¹ agar, pH 5.5–6.0) at 24°C. *Pseudomonas viridiflava* strain Pva1B8 (Alippi et al., 2003) was maintained at ~80°C in King's B medium (King et al., 1954) amended with 20% glycerol.

**Plant Material and Growth Conditions**

Tobacco (Wisconsin W38 and Xanthi-nc) seeds were disinfected with 70% (v/v) ethanol for 2 min, 5% (w/v) sodium hypochlorite, and 0.1% (w/v) SDS for 15 min and thoroughly rinsed with sterile distilled water. Disinfected seeds were dispensed in petri dishes containing Murashige and Skoog agar medium (Murashige and Skoog, 1962) and incubated in a plant growth chamber. After 2 weeks, plantlets were transferred to pots filled with a mixture of soil, sand, and perlite (1:1:1), and were irrigated with half-strength Hoagland’s nutrient solution (Hoagland and Arnon, 1950). Plants were grown for 6 to 8 weeks in a growth chamber with a 16-h/8-h photoperiod at 24/21 ± 2°C and 55/75 ± 5% relative humidity (day/night) and a photon flux density of 200 μmol m⁻² s⁻¹ provided by cool-white fluorescent and incandescent lamps. Transgenic tobacco Wisconsin W38 TeⅢ-Oat ADC and tobacco Wisconsin W38 TeⅢ (Masgrau et al., 1997) were cultivated under similar conditions.

**Plant Inoculation and Disease Analysis**

**Inoculation with *S. sclerotiorum***

Tobacco plants were inoculated with *S. sclerotiorum* by placing discs of mycelium on the adaxial face of leaves of the third or fourth pair. Mycelium discs were cut from the edge of a colony actively growing on Czapek-Dox medium. Two discs of mycelium were placed per leaf, each one on different sides of the mid-vein, and leaves were covered with a translucent film to maintain high relative humidity. A similar procedure was used for the inoculation of leaf discs. In this case, a disc of mycelium was placed in the center of a 18-mm-diameter leaf disc, which were subsequently dispersed on petri dishes containing water-agar (0.8% w/v). Inoculated plants and leaf discs were incubated in the plant growth chamber for 48 to 72 h, until development of necrotic lesions around the inoculation site. Leaves and leaf discs inoculated with a disc of Czapek-Dox medium not inoculated with *S. sclerotiorum* were used as controls (mock-inoculated). The size of the necrotic area around the inoculation site was determined using the Image-ProPlus V 4.1 software (Media Cybernetics) and was expressed as a percentage of the whole disc area.

**Inoculation with *P. viridiflava***

Inoculums of *P. viridiflava* were prepared by streaking bacteria from ~80°C onto King’s B agar-medium plates, incubating plates at 28°C for 24 h and scraping bacterial cells off plates in 10 mM MgCl₂, pH 7.0, to yield 5 × 10⁶ CFU per milliliter. Bacterial suspensions (0.5 mL) were infiltrated into tobacco leaves using a 1-mL hypodermic syringe without a needle as described by Wei et al. (1992). Leaves infiltrated with 10 mM MgCl₂, pH 7.0, were used as controls. A similar procedure was used for the inoculation of leaf discs, which were immersed for 30 min in a bacterial suspension obtained as described above, subsequently covered with a translucent film and incubated under continuous light. After 2 h, discs were rinsed with sterile 10 mM MgCl₂, pH 7.0, dispensed onto petri dishes containing water-agar, and incubated for 48 h in a plant growth chamber. For the evaluation of bacterial growth, leaf discs were ground in 250 μL of 10 mM MgCl₂, pH 7.0, and the extracts thus obtained were plated on King’s B agar medium. The number of CFU mL⁻¹ was determined after 24-h incubation at 28°C. Leaf discs infiltrated with 10 mM MgCl₂, pH 7.0, were used as controls.

**Chemicals**

Standard chemicals and guazatine acetate of the highest purity available were purchased from Sigma Chemical. L-19-(Bis(ethylamino)-5,10,15-triazazanoradacene was kindly gifted by Dr. Benjamin Frydman (SLIB, Biomedical Corporation). N,N’-Diaminouanidined monohydrochloride was purchased from ICN Biomedicals. L-1-[¹⁴C]-Orn was purchased from NEN Life Sciences Products. L-[¹⁴C]-Arg and 5-adenosyl-L-[carboxyl-¹⁴C]-Met were purchased from Amersham Pharmacia Biotech. Inhibitors and PAs were dissolved in distilled water and stored at ~20°C until used.

**In Vivo O₂⁻ Production Assays**

Superoxide (O₂⁻) accumulation in tobacco tissues was determined with NBT, which reacts with O₂⁻ producing a blue formazan precipitate. Leaf discs were infiltrated with 5 μM putrescine and 5 μM N,N’-diaminouanidined monohydrochloride, either separately or in combination. Another set of leaf
Discs were incubated with 5 μM spermine, either separately or in combination with 5 μM each of the amine oxidase inhibitors, 1,19-bis(ethylamino)-5,10,15-triazanonadecane and 5 μM guazatine. Discs infiltrated with water were used as controls of O$_2$~ accumulation in the absence of chemical treatments. NBT was added to all the infiltration solutions at a final concentration of 0.01% (w/v). After infiltration, discs were incubated in the infiltration solution for 3 h in the dark at 30°C with gentle shaking. Stained leaf discs were mounted on glass slides and scanned. Images thus obtained were inverted to render a negative prior to transformation into black and white 8-bit images. Formazan color intensity (in a negative corresponding to the lighter tones of gray) was determined by using the Image-ProPlus V.4.1 software (Media Cybernetics). Discs infiltrated with 10 μM MnCl$_2$ showed a highly effective O$_2$~-dismutating catalyst, developed no blue formazan precipitate, thus confirming the specificity of the method for the detection of O$_2$~.

**Preparation of IWFs**

IWFs were collected from tobacco leaves as described by De Wit and Spikman (1982) with some modifications. For the determination of apoplastic PA concentrations, leaf discs (~50) were vacuum infiltrated (two cycles, 667 mbar, 20 min) with distilled water, while 0.1 M, pH 8.0, Tris-HCl buffer with 0.5 mM phenylmethylsulfonyl fluoride was used for the infiltration of discs to be used for the determination of DAO and PAO. After gently drying on filter paper, leaf discs were introduced into a 10-mL syringe and IWFs were recovered by centrifugation for 20 min at 2,000 g. Nalgene centrifuge tube. Chlorophyll and cytosolic nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase activity were determined in IWFs as described by Lichtenhainer (1987) and Bustos and Iglesias (2002), respectively, to verify that the infiltration procedure caused no significant cellular disruption. The activity of these enzymes and chlorophyll levels were only 1.0% to 2.0% of those obtained by grinding the whole leaf discs, thus demonstrating that the degree of cellular disruption was extremely low, and the IWFs obtained are not expected to be contaminated with cellular contents.

**Determination of Free PA Concentration in Leaf Extracts and IWFs**

To determine free PA levels in leaf extracts, plant material (300 mg) was ground in liquid nitrogen, extracted in 600 μL 5% (v/v) perchloric acid, and incubated overnight at 4°C. After centrifugation at 10,000 g for 15 min, 10 μL of 100 μM 1,7-heptanediameine (ICN Biomedicals) was added as internal standard to 200 μL aliquots of leaf extracts. For PA determinations in the apoplastic compartment, IWFs obtained as described in the previous section were mixed with perchloric acid to a final concentration of 5% (v/v). Then, 200 μL of saturated Na$_2$CO$_3$ and 400 μL of dansyl chloride (10 mg mL$^{-1}$) were added and the mixture was incubated in the dark at room temperature. Reaction was stopped by adding 100 μL of Pro (100 mg mL$^{-1}$) and dansylated PAs were extracted in 500 μL of toluene. Organic phase was vacuum evaporated and dansylated PAs were dissolved in 200 μL of acetonitrile and analyzed by reversed phase HPLC as described previously (Maré et al., 1995).

**Enzyme Activity Assays**

ADC, ODC, and AdoMetDC were extracted by homogenization of leaf tissue (500 mg fresh weight) in 2 volumes of 100 mM HEPES, 0.1 M Tris-HCl, 10 mM MgCl$_2$, 0.5% (v/v) perchloric acid, and incubated overnight at 4°C. Protein concentration in the supernatants was determined as described by Bradford (1976), using bovine serum albumin as the standard. Enzyme activities were determined by mixing 190 μL of the extract with 10 μL of the substrate solution in a glass tube fitted with a rubber stopper and a filter paper disc soaked in 2N KOH. Substrate solutions for the determination of ADC, ODC, and AdoMetDC activities contained 1 mM nonradioactive spermine and 5 mM C, 1,4-1C putrescine. A similar procedure was used for the determination of PAO activity, by using a substrate solution consisting of 10 mM nonradioactive spermine and 5 mM C, 1,4-1C putrescine. For inhibition of amine oxidases, guazatine acetate, 1,19-bis(ethylamino)-5,10,15-triazanonadecane, and N,N',N'-diaminoguanidine monohydrochloride were added to the reaction mixture in a final concentration of 0.5 mM.

**Analysis of ADC Gene Expression by RT-PCR**

Total RNA was extracted from frozen leaf tissue using TRI reagent (Sigma) according to the manufacturer’s instructions. First strand of cDNA was obtained by using the following mixture: 2 μg of total RNA, 0.5 mM dNTPs, 1 μL of moloney murine leukemia virus RT (200 units μL$^{-1}$; Promega), 5 μL 5× reaction buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl$_2$, 50 mM DTT, pH 8.3), 10 pmol of oligo(dT) primer (Biodynamics SRL), and distilled water up to a total volume of 25 μL. The reaction mixture was incubated at 37°C for 1 h. PCR amplification was done with 2 μL of RT reaction as template, 4 μL of 10× Taq polymerase buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0), 0.05 mM dNTPs, 0.2 μL Taq polymerase (5 μM L$^{-1}$; Promega), 12.5 pmol of primers 5'-TGGCCTTCAGGAGTATGCATC-3' (sense), and 5'CCA- CGAATACGAGCAGCAGCA-3' (antisense) corresponding to NtADC1 (GenBank accession no. AF217240), or 5'-GGATTCTGGTGATGGTGTTAG-3' (sense) and 5'ACITCTCCTACGTTGAGCTAC-3' (antisense) corresponding to tobacco actin (GenBank accession no. X63603), in a total volume of 40 μL. Amplification conditions for both genes were 20, 24, 27, and 30 cycles (94°C, 1 min; 58°C, 1 min; and 72°C, 1 min) and a final elongation at 72°C for 7 min. Amplification products were visualized by staining gels with ethidium bromide and equal loading of RNAs was confirmed by monitoring the levels of actin gene. Bands were analyzed by densitometry using the Gel Pro Analyzer 3.0 software (Media Cybernetics).

**Pharmacological Treatments**

PAs (putrescine, spermidine, and spermine), guazatine acetate, 1,19-bis(ethylamino)-5,10,15-triazanonadecane, and N,N'-diaminoguanidine monohydrochloride were infiltrated into the apoplastic space of leaf discs (18-mm diameter) using a vacuum pump (2 cycles, 667 mbar, 20 min). All these chemicals were employed at a concentration of 5 μM. After infiltration, discs were placed in petri dishes containing water-agar and incubated in the plant growth chamber during the time periods indicated for each experiment.

**Induction of ADC Expression in Transgenic Plants**

Leaf discs obtained from transgenic tobacco Wisconsin W38 TetrOat plants, which express an oat (Avena sativa) ADC gene under the control of the tetracycline-inducible promoter TetR (Masgrau et al., 1997), were incubated in a tetracycline solution (2 mg L$^{-1}$ in 5 mM MES, pH 6.5) for 24 h. Discs obtained from transgenic tobacco Wisconsin W38 TetR plants, which harbor the tetracycline-inducible promoter but no heterologous ADC gene, were treated similarly.

**Statistics**

Treatments consisted of three to five replicates, and each experiment was conducted at least two times with similar results. Results from representative experiments are shown as means ± SD. Data were analyzed by appropriate Student’s t test or ANOVA followed by posthoc comparisons by Bonferroni or Dunnet’s test.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** In vitro determination of apoplastic DAO and PAO in tobacco plants infected by S. sclerotiorum and P. viridiflava.

**Supplemental Figure S2.** In vitro DAO and PAO activities of tobacco as a function of plant age.
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