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e-Xtra*

Oxalic Acid Is an Elicitor of Plant Programmed Cell Death during *Sclerotinia sclerotiorum* Disease Development

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Accumulating evidence supports the idea that necrotrophic plant pathogens interact with their hosts by controlling cell death. *Sclerotinia sclerotiorum* is a necrotrophic ascomycete fungus with a broad host range (>400 species). Previously, we established that oxalic acid (OA) is an important pathogenicity determinant of this fungus. In this report, we describe a mechanism by which oxalate contributes to the pathogenic success of this fungus; namely, that OA induces a programmed cell death (PCD) response in plant tissue that is required for disease development. This response exhibits features associated with mammalian apoptosis, including DNA laddering and TUNEL reactive cells. Fungal mutants deficient in OA production are nonpathogenic, and apoptotic-like characteristics are not observed following plant inoculation. The induction of PCD by OA is independent of the pH-reducing abilities of this organic acid, which is required for sclerotial development. Moreover, oxalate also induces increased reactive oxygen species (ROS) levels in the plant, which correlate to PCD. When ROS induction is inhibited, apoptotic-like cell death induced by OA does not occur. Taken together, we show that *Sclerotinia* spp.-secreted OA is an elicitor of PCD in plants and is responsible for induction of apoptotic-like features in the plant during disease development. This PCD is essential for fungal pathogenicity and involves ROS. Thus, OA appears to function by triggering in the plant pathways responsible for PCD. Further, OA secretion by *Sclerotinia* spp. is not directly toxic but, more subtly, may function as a signaling molecule.

The induction of programmed cell death (PCD) in plants can be beneficial or harmful. As in animals, a programmed type of cell death occurs in plants as part of normal growth and development, including reproduction, seed germination, aerenchyma formation, tracheary element differentiation, and senescence (Filonova et al. 2002; Fukuda 2000; Lim et al. 2007; Pennell and Lamb 1997; Turner et al. 2007). Regulation of cell death pathways also occurs in response to environmental abiotic stresses (Ryerson and Heath 1996). Moreover, cell suicide programs are activated during pathogen attack that in some cases are correlated with resistance, as exemplified by the hypersensitive response (HR) which, by definition, is a form of PCD (Dangl and Jones 2001; Goodman and Novacky 1994; Heath 2000). Emerging evidence has indicated that, in necrotrophic fungus–plant interactions, host cell death is beneficial for the pathogen as opposed to the plant, suggesting active involvement of the pathogen in influencing host pathways that direct cell death (Navarre and Wolpert 1999; Van Baarlen et al. 2004; Wang et al. 1996).

We have been studying pathogenic development in the broad host range necrotrophic fungus, *Sclerotinia sclerotiorum* (de Bary 1886). This ascomycete causes diseases in over 400 species of plants (Boland and Hall 1994). Such diseases are difficult to control genetically where breeding programs have had limited success. Management strategies, including crop rotation, are hampered due to the broad fungal host range (virtually all dicots), and spray regimes have been largely ineffective, particularly when climactic conditions are favorable to the fungus. The development of fungal sclerotia also contributes to the pathogenic success of *Sclerotinia* spp. Sclerotia are highly melanized and environmentally durable structures, which can overwinter and remain viable for many years in the soil. Sclerotia serve as a major source of inoculum, capable of infecting hosts through direct germination, or they can produce ascospores through apothecial development (Steadman 1979; Tourneau 1979).

Sclerotinia spp. secrete a wide array of substances to facilitate their necrotrophic life style (Annis and Goodwin 1997; Fraissinet-Tachet et al. 1995; Marciano et al. 1983; Riou et al. 1991). In addition to cell-wall-degrading enzymes, oxalic acid (OA) is known to play a key role in pathogenesis and fungal development (Godoy et al. 1990; Lumsden 1979). Oxalate-deficient mutants of *S. sclerotiorum* are nonpathogenic on all host plants tested and also are unable to develop sclerotia (Godoy et al. 1990). Oxalate secretion might enhance *Sclerotinia* virulence in several ways (Dutton and Evans 1996). First, many fungal enzymes secreted during invasion of plant tissues (e.g., polygalacturonase) have maximal activities at low pH (Bateman and Beer 1965). Consistent with the potential importance of pH, a *Sclerotinia* mitogen-activated protein kinase (MAPK) was cloned, characterized, and shown to be required for sclerotial formation in *S. sclerotiorum*. Gene expression of this Erk-like MAPK (SMK1) is activated by acidic pH mediated via OA. If the pH is not reduced, pathogenic development does not occur (Chen et al. 2004; Rollins and Dickman 2001). Second, OA also can degrade or weaken the plant cell wall via acidity or chelation of cell wall Ca^{2+} (Bateman and Beer 1965). Third, OA crystals have been found in plants and can cause vascular plugging. OA is directly toxic, functioning as a nonspecific phytotoxin. We have shown that OA also is able to suppress the host plant oxidative burst, which is one of the earliest and most universal of plant defense responses (Cessna et al. 2000). Recently, OA has been reported to interfere with the function of guard cells, resulting in abnormal stomatal opening and inhibition of

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abscisic acid-induced stomatal closure during infection (Guimarães and Stotz 2004). Thus, it is evident that this "simple" dicarboxylic acid is used by the fungus in a number of ways when interacting with host plants. The importance of the multifunctional OA for *Sclerotinia* pathogenic success is underscored by the fact that OA mutants are nonpathogenic even though the fungus still maintains its full arsenal of cell-wall-degrading enzymes (Godoy et al. 1990).

Previous studies have shown that injection of exogenous oxalate or application of fungal culture filtrates containing secreted oxalate mimics disease symptoms of the actual fungal infection (Bateman and Beer 1965; Noyes and Hancock 1981). However, the manner by which host cell death occurs has not been established. We have shown that transgenic tobacco expressing mammalian anti-apoptotic genes were resistant to *Sclerotinia* challenge (Dickman et al. 2001), suggesting that pathogenicity/cell death is an active gene-directed process. In accordance with this premise, *Sclerotinia*-induced disease in tobacco shares features of apoptotic-like cells in animals such as DNA fragmentation and chromatin condensation. This suggests that some components of fungal origin induce or direct PCD in plants (Dickman et al. 2001). Studies involving other host-necrotrophic pathogen systems have reported similar observations (Govrin and Levine 2000; Navarre and Wolpert 1999). Taken together, necrotrophic fungi may co-opt plant host cell death pathways to establish compatibility.

In this report, we show that *Sclerotinia*-secreted OA is an elicitor of PCD in plants and is responsible for induction of apoptotic-like features in the plant during disease development. This PCD is essential for fungal pathogenicity and involves reactive oxygen species (ROS). We suggest that OA functions by triggering pathways in the plant responsible for PCD. Thus, via OA, *S. sclerotiorum* can subvert host cell death regulatory machinery as a means to pathogenic success. Further, OA secretion by *S. sclerotiorum* is not directly toxic but may function as a signaling molecule.

RESULTS

Apoptosis-like cell death in tobacco is induced by oxalate.

Our previous study showed that inoculation of tobacco leaves with *S. sclerotiorum* resulted in disease and lesion formation that were preceded at the cellular level by DNA fragmentation and cleavage (DNA ladders and TUNEL positively reacting cells), features associated with apoptotic-like PCD (Dickman et al. 2001). To determine whether *Sclerotinia* cell extracts harbored a component or components responsible for induction of plant PCD, tobacco leaf disks and seedlings were treated with extracts from the wild-type 1980 strain or the nonpathogenic OA-deficient mutant (A-1). Both strains were cultured in 100 ml of potato dextrose broth, pH 7.0, and grew at equivalent rates. Significant but expected changes in pH in the media were noted; pH 3.35 ± 0.13 or 5.47 ± 0.16 after the 6 days of culturing 1980 or A1, respectively. OA concentrations were 4.35 ± 0.12 or 0.16 ± 0.05 mM for wild-type and mutant cultures, respectively. When plant tissue was treated with culture filtrates, DNA laddering was observed only in wild-type filtrates. When the wild-type filtrate was autoclaved, DNA laddering and disease symptoms still were evident (Fig. 1C; data not shown). These data argue against a proteinaceous compo-

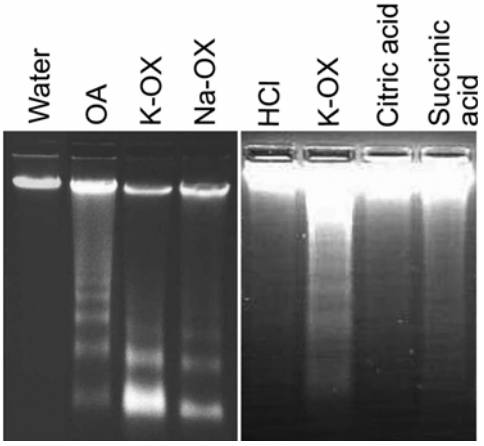

Fig. 2. Effects of oxalate formulations and other acids on DNA laddering. Tobacco leaf disks were treated with different formulations of oxalate (OX) and selected acids. These include salt-free oxalic acid (OA), sodium OX (Na-OX), potassium OX (K-OX), HCl, citric acid, and succinic acid. Solutions used were 20 mM, except for 10 mM of OA, and all adjusted to pH 5.0 with either HCl or NaOH. Genomic DNA was extracted 36 h after treatment.

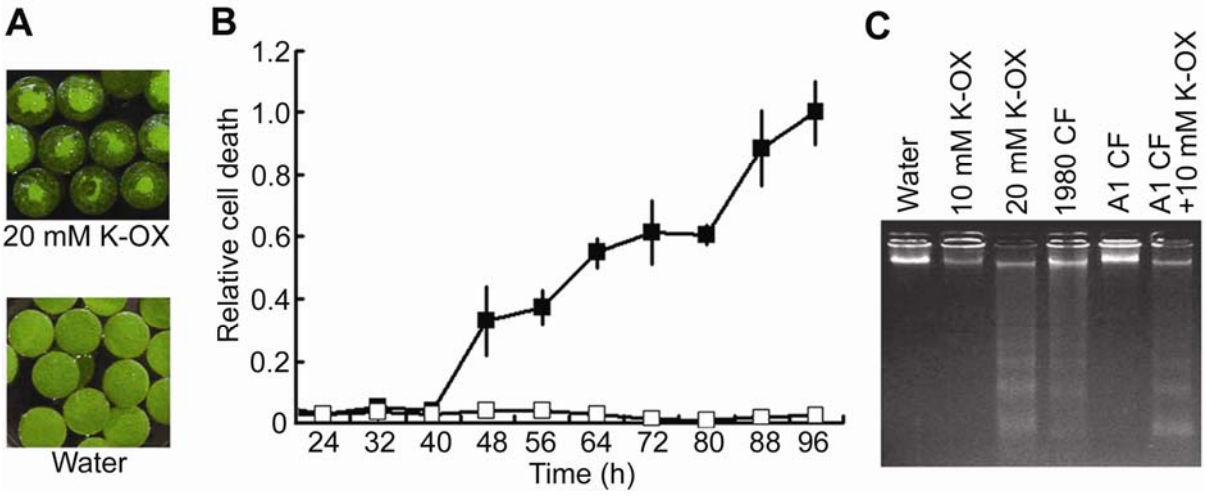

Fig. 1. Oxalate-induced cell death and DNA laddering in tobacco leaf disks. **A,** Macroscopic symptoms of tobacco leaf disks treated with 20 mM potassium oxalate (K-OX, pH 7.0) or water (as a control) for 72 h. **B,** Time course of cell death following K-oxalate (20 mM, pH 7.0) treatment. Cell death in tobacco leaf disks was determined using Evans blue staining. Dye released from dead cells was measured at absorbance at 600 nm. Measurements were expressed as relative values with 1 corresponding to the maximum of a sample. Data are presented as means ± standard deviation from three independent experiments. **C,** DNA fragmentation in tobacco leaf disks 36 h after treatment with K-OX (10 or 20 mM, pH 7.0) and autoclaved culture filtrates (CF) from a wild-type (1980) or an oxalate-defective mutant (A1) of *Sclerotinia sclerotiorum.*

nent from the filtrate being responsible for cell-death-inducing activity. Therefore, we focused our attention on an obvious candidate, OA.

Based on our preliminary data and previous research (Cessna et al. 2000), tobacco leaf disks were treated with potassium oxalate (K-OX), pH 7.0. Water-soaked lesions developed in leaf disks treated with 20 mM K-OX after 72 h (Fig. 1A). In addition, seedling treatment showed a similar response with respect to DNA laddering and disease symptoms at 5 mM K-OX (data not shown). Quantitative and time-course analysis of cell death in tobacco leaf disks was measured using Evans blue staining (Fig. 1B). No significant difference in cell viability was observed in the treatment of K-OX and water until 48 h after treatment was initiated. However, from 48 through 96 h, substantial increases in cell death were observed in oxalate-treated leaf disks. By 96 h, extensive cell death and tissue collapse occurred. Lower concentrations of oxalate (e.g., 10 mM) yielded similar but delayed results (data not shown). Thus, oxalate induced plant cell death in a dose- and time-dependent manner. Based on the kinetics of the cell death in response to oxalate, tobacco DNA was isolated at several time points. DNA cleavage resulting in a characteristic DNA laddering pattern associated with apoptotic cell death was observed (Fig. 1C). DNA laddering was detected in the oxalate treatment before macroscopic symptoms were observed. DNA fragmentation appears to be specific to OA because other acids such as citric acid, succinic acid, and hydrochloric acid did not yield DNA ladders (Fig. 2). DNA cleavage also was independent of oxalate formulation because OA, potassium oxalate, and sodium oxalate all caused DNA laddering (Fig. 2), thus suggesting that PCD induction is not due to the acidic nature of oxalate but rather to a property of oxalate itself. Further, the addition of 10 mM K-OX, pH 7.0, to the A1 culture filtrate induced DNA laddering (Fig. 1C). When tomato leaves were pretreated with 20 mM K-OX, the A-1 mutant showed a partial restoration of the disease phenotype (data not shown). Taken together, fungal-secreted oxalate is responsible for the induction apoptotic-like PCD in tobacco.

Effects of pH and light on oxalate-induced cell death in tobacco.

pH is an important factor in *Sclerotinia* pathogenesis (Chen et al. 2004; Dickman 2007). To further evaluate the effect of pH on oxalate-induced cell death, we compared the effects of various pHs with respect to the level of oxalate-mediated cell death (Fig. 3). Leaf disks were treated with K-OX adjusted to pHs 3 to 6 with HCl. pH clearly had an effect on symptoms (Fig. 3A) because pH 3 conditions caused extensive yellowing and water soaking of the plant tissue; pH 5 and 6 showed milder symptoms but tissue death was evident. Importantly, DNA laddering was observed only at pH 5 and 6, not at pH 3 or 4 (Fig. 3B). Na-citrate, which is similar to oxalate in its ability to modulate pH and chelate calcium and inhibit the plant oxidative burst (Cessna et al. 2000), also was used as a control. No DNA laddering was observed at any of the pH conditions used. These observations are consistent with a specific induction of plant PCD by oxalate that is independent of its acidic pH (≤4.0) regulatory properties.

It has been reported that light often is required for PCD (Chen and Dickman 2004; Samuilov et al. 2002, 2003). To determine whether or not light is necessary for oxalate-induced cell death, oxalate-treated leaf disks were incubated under fluorescent light at room temperature or in the dark for 48 h (Fig. 3C). Cell death under both light and dark conditions was induced significantly compared with the control. However, a greater proportion of cells died under dark conditions than under lighted conditions. These results suggest that dark conditions may augment oxalate-induced cell death of tobacco plants or that light has an inhibitory effect.

ROS are involved in oxalate-induced cell death.

The generation of ROS (e.g., hydrogen peroxide and superoxide) is well documented as one the earliest and most universal responses of plants to biotic stress. A frequent outcome of this controlled response is a PCD that often is correlated with plant resistance, commonly known as the HR. Oxidative stress is also a trigger of PCD in mammals (Nakazato et al. 2007). In plants, oxidative stress has been shown to induce apoptotic-like features, including DNA laddering, chromatin condensation, and cell shrinkage (Heath 2000; Houot et al. 2001). ROS regulation also been implicated as a developmental signal and is involved with regulating pathogenicity in several fungi (Egan et al. 2007; Leuthner et al. 2005; Tanaka et al. 2006). Therefore, we were interested in determining whether ROS was involved in Sclerotinia disease development and, more specifically, as it relates to PCD. Leaf disks were treated with K-OX for 24 h, then stained for with 3′3-diaminobenzidine (DAB), which commonly is used to detect H_2O_2 (Thordal-Christensen et al. 1997). All leaf disks had a ring of lightly stained material at the periphery, presumably as a result of wounding (Fig. 4A). However, K-OX-treated leaf disks were

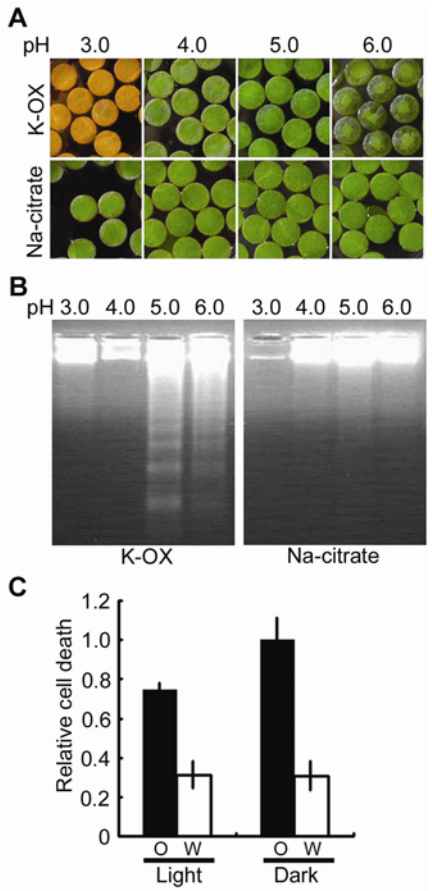

Fig. 3. Effect of pH and light on potassium oxalate (K-OX)-induced cell death in tobacco leaf disks. **A,** Macroscopic symptoms of tobacco leaf disks incubated with different pHs of K-OX (20 mM) and Na-citrate (5 mM). HCl was used to adjust the pH. **B,** pH effects on K-OX-induced DNA laddering in leaf disks. DNA was extracted from tested disks in A. **C,** Light and dark effects on K-OX-induced plant cell death. Leaf disks were incubated at room temperature either under fluorescent light or dark condition with K-OX (O, 20 mM, pH 5.0) or water (W, pH 5.0). Cell death was measured using Evans blue staining 40 h after treatment. Data are presented as means ± standard deviation from three independent experiments.

heavily stained throughout and clearly distinct from the control disk. The intensity of staining also was dose and pH dependent (Fig. 4A and B). Tobacco leaf disks were treated with K-OX adjusted to pH 3 to 6 for 24 h. Leaf disks treated at pH 5 and 6 were positively stained by DAB; disks at pH 3 and 4 were not (Fig. 4B). Oxalate-induced PCD, as we have shown, occurs at relatively higher pH (5 to 6), and was not observed at acidic pH (3 to 4). Thus, the observation that there was no ROS detected at acidic pH coincided with the lack of PCD. At the higher pHs where DNA laddering occurred, ROS increases were correspondingly evident. Thus, there was a very good relationship between the ROS levels and the ability of oxalate to induce PCD.

We examined the ROS detoxification enzymes superoxide dismutase (SOD), which dismutates superoxide to H_2O_2, and catalase, which converts H_2O_2 to water, for effects on OA-induced PCD. Leaf disks were treated simultaneously with catalase or SOD with K-OX, pH 5.5, and stained with DAB 24 h post treatment, which was prior to the appearance of water-soaked lesions. H_2O_2 generation was inhibited by catalase (40 units for 10 mM and 200 units for 20 mM of K-OX) (Fig. 4C). However, SOD did not reduce H_2O_2 generation in co-treatment with K-OX and actually enhanced H_2O_2 levels in the higher SOD treatment (Fig. 4D). Because SOD increases H_2O_2 levels, this result was not entirely unexpected. Consistent with these observations, the induction of DNA ladders in these treatments showed that catalase inhibited DNA laddering and SOD did not (Fig. 4C and D). Thus, K-OX generates H_2O_2 that is catalase but not SOD inhibitable. H_2O_2 generation also is required for DNA ladder formation indicative of PCD.

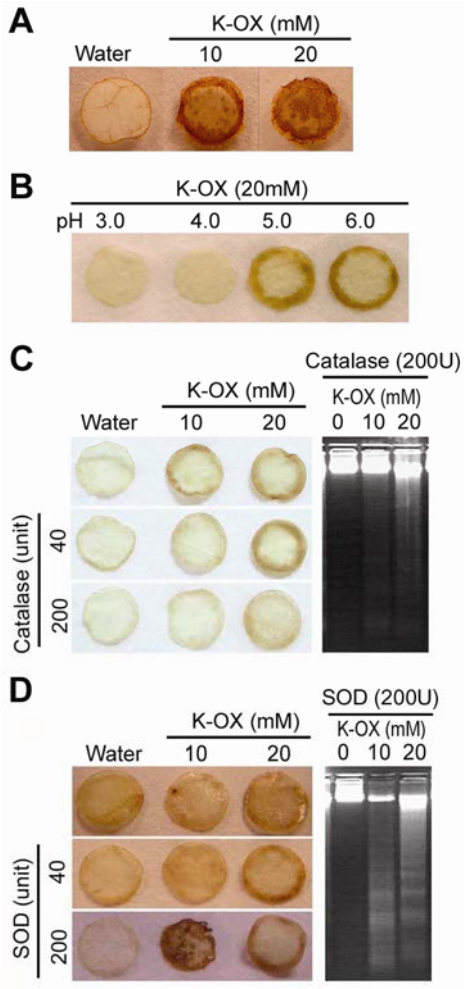

Fig. 4. Involvement of reactive oxygen species (ROS) in potassium oxalate (K-OX)-induced cell death in tobacco leaf disks. **A,** In situ detection of H_2O_2 in leaf disks treated with K-OX (pH 7.0). H_2O_2 production was visualized by 3,3′-diaminobenzidine (DAB) staining. H_2O_2 production (revealed by reddish-brown stain) was detected 24 h after K-OX treatment. **B,** Effect of pH of K-OX on ROS generation in leaf disks. Disks were treated in a different pH of K-OX (20 mM) adjusted with HCl. Tested disks were stained after 24 h. **C,** Effect of catalase on H_2O_2 production and programmed cell death (PCD). Leaf disks were co-treated for 24 h in a combination of K-OX (10 or 20 mM, pH 7.0) with catalase (40 or 200 units), after which disks were stained with DAB or used for DNA extraction. **D,** Effect of superoxide dismutase (SOD) on H_2O_2 production and PCD. Leaf disks were co-treated for 24 h in a combination of K-OX (10 or 20 mM, pH 7.0) with SOD (40 or 200 units), after which disks either were stained with DAB or used for DNA extraction.

Application of antioxidants or expression of CED-9 prevents OA-induced PCD in tobacco.

Our data suggested that *S. sclerotiorum*–induced plant disease involved the activation of pathways culminating in PCD. To gain insight into the pathways or pathway components mediating *S. sclerotiorum* (OA)-induced plant PCD, various reagents, including the protease inhibitor E-64, antioxidants *N*-acetyl cysteine (NAC) and diphenyleneiodonium (DPI), calcium influx blockers lanthanum chloride ($LaCl_3$) and verapamil, the catalase inhibitor 3-aminotriazole (3-AT), the de novo protein synthesis inhibitor cycloheximde, and the serine/threonine protein kinase inhibitor K-252a, were used to treat tobacco leaf disks with or without K-oxalate treatment to evaluate effects on oxalate-induced PCD. DNA ladder formation was used as a readout because there was a consistent correlation with DNA laddering, cell death, and lesion formation. At the concentrations used, none of the chemicals alone induced cell death. DPI, an inhibitor of NADPH oxidase (which generates superoxide), prevented DNA laddering induced by both 10 and 20 mM K-OX (Fig. 5A). NAC was less effective, as was E-64, a cysteine protease inhibitor. 3-AT had no effect on inhibition of oxalate-induced DNA laddering, as expected. (The difference in laddering observed in the 10 mM K-OX treatments between Figure 1C and Figure 5A is attributed to increased DNA concentration in Figure 5A.) Measurement of oxalate-induced cell death using Evans blue staining was consistent with the DPI results; DPI significantly prevented plant cell death (Fig. 5B), suggesting the involvement of an NADPH oxidase in the induction of PCD. Co-treatment with verapamil, cycloheximide, or K-252a and, to a lesser extent, $LaCl_3$ had little effects on oxalate-induced cell death, suggesting that oxalate-induced cell death may not require calcium ion channel activity, de novo protein synthesis, or general serine/threonine protein kinase activity.

Previous studies described the generation of transgenic tobacco plants expressing negative regulators of mammalian apoptosis and resistance to *Sclerotinia* spp. challenge (Dickman et al. 2001). Because oxalate is an elicitor of cell death in tobacco plants, we tested the response of CED-9 expressing transgenic tobacco to oxalate. CED-9 is a Bcl-2 family member from the nematode *Caenorhabditis elegans,* and is a well-characterized inhibitor of developmental PCD in the worm (Hengartner and Horvitz 1994). In transgenic tobacco expressing CED-9, cell death was reduced dramatically (Fig. 5B), consistent with our previous data showing increased disease resistance.

DISCUSSION

PCD is a major process by which eukaryotes regulate normal growth and development as well as stress responses (Vaux and Strasser 1996). Accumulating evidence shows that inap-

propriate regulation of PCD can have dire consequences for an organism. In humans, cancer can be promoted when PCD does not occur when it normally should. Alternatively, Parkinson's and neurological disorders are observed when cell death occurs when it should not. Thus, the potential benefits of a given PCD regime are clearly context dependent. In plant–pathogen interactions, PCD historically has been associated with plant resistance via the HR. The HR and subsequent cell death have been correlated with the accumulation of ROS during the oxidative burst (Jabs 1999). By definition, the HR is a programmed death and shares characteristics of mammalian apoptosis (Dickman and Reed 2003; Heath 2000).

In this report, we show that OA, a key determinant of pathogenicity for the necrotrophic fungal phytopathogen *S. sclerotiorum*, uses oxalate to elicit an apoptosis-like PCD in tobacco, and the induction of this fungal-mediated plant cell death process is required for pathogenic success. Thus, OA, a nonspecific phytotoxin at levels found in plant tissue, does not appear to be directly toxic but rather functions as a signaling molecule or elicitor to subvert and redirect host pathways toward cell death. Our data also indicate that ROS are involved in the *S. sclerotiorum* interaction with host plants. ROS also have been considered detrimental to cells because they can be toxic, causing damage to proteins, lipids (membranes), and nucleic acids. However, it is now evident that low concentrations of ROS have a beneficial function as regulatory molecules in cell-signaling pathways. By virtue of their diffusibility, controlled production, and regulation, ROS, particularly H_2O_2, can function as second messengers, suggesting that intra- and intercellular signaling within the pathogen and during host–pathogen crosstalk are key components dictating the eventual outcome of this interaction. Moreover, our data are consistent with the premise that necrotrophic plant pathogens interact with their hosts in a manner much more subtly than perhaps originally considered, and that the success of necrotrophs as pathogens may not be simply attributed to "brute force" via degradative enzymes or toxins. Consistent with this idea is the observation that the *Cochliobolus victoriae* host-selective toxin victorin does not directly kill plant cells but induces an apoptotic-like cell death in oat as well as inducing "defense" responses (Navarre and Wolpert 1999; Tada et al. 2001; Yao et al. 2001).

Additionally, ROS has a role in plant–fungus mutualistic associations. *Epichloe festucae* requires fungal-generated ROS to regulate its mutualistic symbiosis with perennial ryegrass (Tanaka et al. 2006). In a REMI screen to identify genes needed for symbiosis, a fungal NADPH oxidase (*noxA*) knockout was found that not only resulted in the loss of mutualism but also, interestingly, pathogenicity ensued. Deletion of the second fungal *E. festucae nox* gene had no such effect. ROS accumulation was detected at the interface between the two partners in wild-type but not mutant associations, indicating that fungal ROS production is necessary for maintenance of mutualism and, possibly, prevention of runaway cell death (Jabs et al. 1996).

The broad host range necrotrophic fungus *Botrytis cinerea* also modulates the redox environment during pathogenesis (Unger et al. 2005; Van Baarlen et al. 2004). Work in the laboratory of Tudzynski and colleagues has shown that a deletion mutant of a *B. cinerea* Cu/Zn SOD resulted in a significant reduction in lesion formation on bean leaves. A similar knockout mutant of a glucose oxidase gene showed no effect on virulence. Because both enzymes can generate H_2O_2, the specific importance of the SOD was demonstrated (Rolke et al. 2004). These data also suggest the importance of ROS generation for necrotrophic fungal virulence. The same group also characterized a transcription factor (CPTF1) from *Claviceps pupurea,* which is responsive to and responsible for cellular redox status. Knockouts of CPTF1 were reduced in virulence and no longer responsive to oxidative stress in the knockout mutant strain (Nathues et al. 2004). Of relevance to our work was the observation that mutant inoculated plants had significant amounts of H_2O_2, which was not observed in wild-type inoculations. Thus, *Claviceps* redox regulation in the context of host–fungus interactions may have a dual function: inducing ROS for disease development and regulating the plant oxidative burst defense response. This dual regulation of both the fungal and host redox environment by the pathogen is consistent with observations we have made in *S. sclerotiorum*.

Oxalate suppresses the host plant oxidative burst.

The oxidative burst, the controlled release of O_2^- and H_2O_2, is one of the earliest and most universal responses observed in plants following pathogen challenge (Apel and Hirt 2004; Bolwell 1999; Torres et al. 1998). Such responses occur in

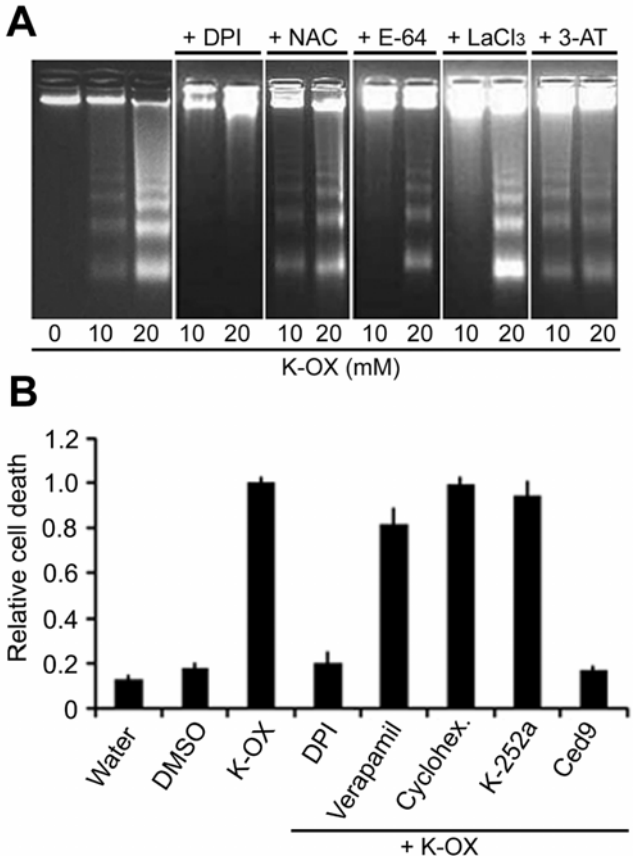

Fig. 5. Effect of selected reagents on oxalate-induced programmed cell death (PCD). **A,** Effects of various reagents on potassium oxalate (K-OX)-induced PCD in tobacco leaf disks. DNA was extracted from tissues co-incubated in a solution of K-OX (10 or 20 mM, pH 7.0) with or without reagents, including diphenyleneiodonium (DPI) (100 μM), N-acetyl cysteine (NAC) (100 μM), E-64 (100 μM), lanthanum chloride ($LaCl_3$) (10 nM), or 3-aminotriazole (3-AT) (2 nM). DNA was isolated from tissues after treatment for 36 h. **B,** Effect of various reagents on K-OX-treated wild-type and *ced9*-transgenic tobacco leaf disks. Wild-type tobacco disks were incubated with K-OX (20 mM, pH 7.0) with or without inhibitors, including DPI (100 μM), verapamil (200 μM), cycloheximide (2 μM), or K-252a (2 μM). Transgenic tobacco disks expressing CED-9 were treated with K-OX (20 mM). Solutions of 2% dimethyl sulfoxide (DMSO) or water alone served as controls. Cell death was determined by Evans blue staining after 48 h of treatment. Spectrophotometric measurement at absorbance at 600 nm was expressed as relative values, with 1 corresponding to the maximum of a sample. Data are presented as means ± standard deviation from three independent experiments, each of which contained pooled disks.

both compatible and incompatible responses, although the timing and magnitude may differ. The oxidative burst also occurs during plant development (Foreman et al. 2003) and is expressed in most if not all plant species (Bolwell 1999). The oxidative burst also is known to be suppressed at low pH (Legendre et al. 1993). Because release of oxalate could lower the pH, we hypothesized that OA might enhance fungal pathogenicity by inhibiting or dampening the oxidative burst of the host plant. In biochemical studies, we showed this to be the case both in vitro and in vivo (Cessna et al. 2000). The nonpathogenic OA^- mutants were unable to inhibit plant ROS induction. Importantly, oxalate blocked the oxidative burst even at the optimal pH of the pathway. These data revealed a previously unrecognized function of oxalate secretion by *Sclerotinia* spp.; namely, to suppress ROS generation and, thereby, compromise plant defense responses. We show that OA and *Sclerotinia* spp. increase ROS levels in the plant and induce DNA laddering and TUNEL positively reacting cells, all of which are hallmarks of apoptotic-like cell death. Thus, OA appears to be central to ROS signaling, causing a programmed-like cell death of the host, which is consistent with necrotrophy.

Oxalate-induced ROS production and PCD activities also correlate with pH. Oxalate induces PCD together with positive DAB staining at higher pH (5 to 6); these features are not observed at relatively acidic conditions (pH 3 to 4), although cell death occurs. Moreover, when cells are co-treated with OA and DPI, not only are ROS diminished but DNA laddering also is abolished and lesion formation is not observed. Thus, there is an apparent dual ROS regulatory scheme occurring: *S. sclerotiorum* downregulates the plant oxidative burst via OA, possibly in a pH-independent fashion (Cessna et al. 2000); while also generating ROS in a pH-dependent manner, which is necessary for inducing PCD pathways. A key question is: how does the fungus suppress the ROS production involved in the plant defense response on the one hand while similarly inducing ROS to promote compatibility? Possibly, there are temporal differences in OA and consequent pH changes that occur during pathogen ingress into host tissue; in the early stages, ROS and PCD are triggered by low levels of OA at relatively high pH (>5). As OA accumulates, the pH is lowered and the interaction becomes more necrotic in nature, which is accompanied by suppression of ROS and PCD, enabling the pathogen further ingress into plant tissue.

We used chemical treatments as a first pass to gain insight into the players and pathways mediating PCD. E-64, a cysteine protease inhibitor, partially blocked PCD, as did $LaCl_3$, a calcium channel blocker. DPI effectively inhibited DNA laddering and cell death in oxalate-treated leaf disks, suggesting the involvement of an NADPH oxidase-mediated ROS during the fungus-induced PCD. Conversely, 3-AT, a catalase inhibitor, exhibited no inhibitory abilities of this type, as expected (Fig. 5). Similar observations also were noted in *B. elliptica*-induced PCD (Van Baarlen et al. 2004).

OA is multifunctional and an elicitor of plant PCD.

For many years, OA has been known as a key determinant in *Sclerotinia* pathogenicity, initially because OA was found in high concentrations (>10 mM) in diseased plant tissue. Accordingly, this correlation led to speculation as to how OA secretion might enhance *Sclerotinia* virulence (Dutton and Evans 1996). We have added to this expanding list of functions that this relatively simple dicarboxylic acid can perform. The ability of OA to downregulate the plant oxidative burst has been discussed. We also have established OA involvement in pathogenic development during sclerotium formation. OA^- mutants do not form sclerotia (Godoy et al. 1990). The *Sclerotinia* Erk-like MAPK (encoded by *smk-1*) is activated by OA via reduction in pH (Chen et al. 2004). If the pH is buffered and acidification does not occur, sclerotia do not form (Rollins and Dickman 2001).

Previously, we showed that expression of selected mammalian inhibitors of apoptosis protected tobacco plants from infection by *Sclerotinia* spp., suggesting that a PCD might be induced in the plant by the fungus. Consistent with this idea was the additional observation that, during fungal infection of wild-type susceptible tobacco, DNA laddering and TUNEL positive-reacting plant cells were observed. Our conclusion was that *S. sclerotiorum* was hijacking plant machinery to induce PCD. The initial basis for the work presented here was to identify the molecules responsible for cell death induction. Fungal extracts induced DNA ladders, lesion formation, and cell death when tobacco leaf tissue was treated with these extracts. Boiled or autoclaved extracts gave identical results, thus indicating that the responsible party was not likely to be a protein. Although we cannot exclude unknown heat-stable nonproteinaceous factors being responsible for PCD activity, several lines of evidence were consistent with OA being the specific elicitor of plant PCD: i) addition of OA in several formulations induced DNA laddering and lesion formation in a time- and dose-dependent manner; ii) OA-deficient nonpathogenic mutant culture filtrates did not exhibit laddering; however, laddering was restored when OA was exogenously supplied; and iii) other organic acids at similar concentrations were unable to induce ladders. Consistent with these observations is the fact that OA-induced PCD is not induced at acidic pH (3 to 4) but rather at pH 5 and 6. Low pH can cause cell death but it does not appear to be programmed and, presumably, is a necrotic type of death. Thus, OA-induced PCD is not due to pH signaling as we have shown to be operative during sclerotial development in *Sclerotinia* spp. via OA-activated MAPK (Chen et al. 2004). Thus, some other property of oxalate is responsible for its ability to induce PCD.

Oxidative stress signaling is a key component of plant and fungal development as well as plant stress responses and PCD, and appears to be induced by oxalate. When tobacco leaf disks were treated with K-OX under conditions that cause PCD (as evidenced by tissue death and ladder formation) followed by DAB staining for H_2O_2, plant tissue was positively stained, indicative for ROS production. Consistent with the presence of H_2O_2 is the observation that catalase significantly blocked staining and disease whereas SOD treatment of plant tissues had negligible and, in some cases, enhanced effects. Moreover, DNA laddering was inhibited by catalase but not by SOD. When K-OX-treated tissues were treated with DPI not only were ROS diminished but DNA laddering (indicative of cell death) also was abolished. Oxalate is a substrate for the germin family of plant proteins, which are found in many plant species (Lane et al. 1993). Germins have oxalate oxidase activity resulting in the conversion of oxalate to H_2O_2. The production of H_2O_2 by oxalate oxidase has been proposed to play a role in plant defenses against biotic and abiotic stresses (Berna and Bernier 1999; Hurkman et al. 1994). Moreover, transgenic dicot plants (soybean and sunflower) expressing a monocot oxalate oxidase gene showed increased resistance to *Sclerotinia* spp. This could explain why *S. sclerotiorum* is restricted to dicot hosts, which do not appear to have oxalate oxidase activity (Cober et al. 2003; Hu et al. 2003). We are currently exploring this possibility.

We conclude that *S. sclerotiorum*-secreted oxalate induces ROS in the plant host that appears to trigger a PCD pathway, resulting in plant cell death and the generation of a suitable environmental niche for fungal pathogenic development, nutrient acquisition, and the establishment of a necrotrophic relationship. Thus, for a number of plant–pathogen interactions,

the outcome of disease versus resistance is dependent on which partner is in control of the cell death process.

MATERIALS AND METHODS

Plant and fungal materials.

S. sclerotiorum isolate 1980 and an oxalate-deficient mutant (A-1) of this strain were maintained at 24°C on potato dextrose agar as previously described (Godoy et al. 1990). The culture filtrates of *Sclerotinia* isolates were obtained from 6-day-old growth of mycelia in 100 ml of potato dextrose broth at 24°C. The cultures were filtered through miracloth (Calbiochem, San Diego, CA, U.S.A.).

Wild-type and transgenic tobacco (*Nicotiana tabacum* cv. Glurk) expressing the nematode *CED-9* gene were grown from seed in a growth room at 25°C with 16-h light periods as previously described (Dickman et al. 2001). Leaf disks were obtained from 7-week-old plants using a 10-mm-diameter cork borer (Boekel Scientific, Feasterville, PA, U.S.A.). Leaf disks were vacuum infiltrated for 3 min in oxalate solutions and placed under fluorescent lights at room temperature. For dark growth conditions and inhibitor assays, plates containing tested disks were wrapped with foil and placed at room temperature.

OA measurement.

Culture filtrates of strains 1980 or A-1 were centrifuged at 1,500 × *g* for 10 min, discarding the pellet. The concentration of OA was determined using an oxalate detection kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis) and a standard curve using potassium oxalate. Each experiment was performed twice in duplicate. Values were presented as means ± standard deviation of experiments.

Chemicals.

All reagents were purchased from Sigma-Aldrich or Calbiochem. Chemicals were solubilized in water or dimethyl sulfoxide. An equivalent amount of dimethyl sulfoxide was used as a control.

Measurement of cell death.

Cell death was determined and quantified by using Evans blue. Cell membranes are permeable and, thus, stained when cell death occurs (Baker and Mock 1994). Briefly, treated leaf disks were submerged in 0.25% (wt/vol) Evans blue solution (MP Biomedicals, Santa Anna, CA, U.S.A.) for 20 min. Excess and unbound dye was removed with deionized water. The disks were placed in liquid nitrogen, homogenized, placed in 1% (wt/vol) sodium dodecyl sulfate (SDS) solution, and incubated for 10 min at 37°C. Solutions were centrifuged at 13,000 × *g* for 5 min. The quantity of remaining dye was spectrophotometrically measured at 600 nm. Measurements were expressed as relative values, with 1 corresponding to a maximum of a sample tested.

For the detection of DNA laddering, total plant DNA was extracted using a modified method (Ryerson and Heath 1996). Leaf disks were homogenized using liquid nitrogen. Samples were incubated for 30 min at room temperature in DNA extraction buffer (0.1 M glycine, 50 mM NaCl, 10 mM EDTA, 2% SDS, and 1% sodium lauryl sarcosine) and mixed with an equal volume of Tris-saturated phenol. The mixture was centrifuged for 15 min at 10,000 × *g*. The supernatant was treated with chloroform/isoamyl alcohol (24:1, vol/vol). After centrifugation as above, DNA was precipitated with a twofold volume of 100% ethanol, washed with 70% ethanol, and dissolved in Tris-EDTA buffer containing RN*ase* A (40 μg/ml). DNA was recovered after phenol extraction and ethanol precipitation. DNA samples were separated on a 1.5% agarose gel in 0.5× Tris-borate-EDTA (1× 89 mM Tris, 89 mM H_3BO_3, and 2 mM EDTA), stained with ethidium bromide, and visualized under UV.

Detection of H_2O_2.

In situ hydrogen peroxide (H_2O_2) was detected by DAB staining as previously described (Thordal-Christensen et al. 1997). H_2O_2 reacts with DAB to form a reddish-brown stain. Inoculated leaf disks were incubated in DAB solution, pH 7.5, at 1 mg/ml. After incubation in the dark at room temperature for 20 h, samples were boiled in a solution containing alcohol and lactophenol (2:1) for 5 min and rinsed twice with 50% ethanol.

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