Role of Lipid Composition and Lipid Peroxidation in the Sensitivity of Fungal Plant Pathogens to Aluminum Chloride and Sodium Metabisulfite[▽]

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Aluminum chloride and sodium metabisulfite have shown high efficacy at low doses in controlling postharvest pathogens on potato tubers. Direct effects of these two salts included the loss of cell membrane integrity in exposed pathogens. In this work, four fungal potato pathogens were studied in order to elucidate the role of membrane lipids and lipid peroxidation in the relative sensitivity of microorganisms exposed to these salts. Inhibition of mycelial growth in these fungi varied considerably and revealed sensitivity groups within the tested fungi. Analysis of fatty acids in these fungi demonstrated that sensitivity was related to high intrinsic fatty acid unsaturation. When exposed to the antifungal salts, sensitive fungi demonstrated a loss of fatty acid unsaturation, which was accompanied by an elevation in malondialdehyde content (a biochemical marker of lipid peroxidation). Our data suggest that aluminum chloride and sodium metabisulfite could induce lipid peroxidation in sensitive fungi, which may promote the ensuing loss of integrity in the plasma membrane. This direct effect on fungal membranes may contribute, at least in part, to the observed antimicrobial effects of these two salts.

Organic and inorganic salts are widely used in the food industry as preservatives and antimicrobials (26). These salts have a broad spectrum of antimicrobial activity with low mammalian toxicity (22), possess biocompatibility (13), and are generally recognized as safe. Several studies suggest that salts represent an interesting alternative to synthetic fungicides and could find application for the control of plant pathogens (13, 20, 22), particularly in the case of postharvest diseases that directly affect foodstuffs that will be consumed by the public (12, 21). Recently, organic and inorganic salts have revealed efficacy against postharvest pathogens affecting potato tubers (12, 20, 21, 22, 32). Of the tested salts, aluminum chloride and sodium metabisulfite were among the most efficient in controlling potato dry rot (*Fusarium sambucinum* [20]), soft rot (*Erwinia carotovora* [32]), and silver scurf (*Helminthosporium solani* [12]).

Recent microscopic work with different potato pathogens, including the gram-negative bacterium *E. carotovora* (32) and the fungus *F. sambucinum* (T. J. Avis, M. Michaud, and R. J. Tweddell, unpublished data), has demonstrated that aluminum chloride and sodium metabisulfite produce changes in both bacterial and fungal cells, leading to loss of membrane integrity. These results are in agreement with other studies that have postulated that, under certain conditions, aluminum chloride and sodium metabisulfite may act through cell disruption, compromised membrane permeability, and lipid peroxidation (1, 3, 6, 15, 17, 34).

In an effort to gain better insight into the mode of action of

these salts and in an attempt to ascertain biochemical determinants in the implicated mechanisms, four fungal potato pathogens were assayed with regard to (i) sensitivity toward aluminum chloride and sodium metabisulfite, (ii) intrinsic fatty acid unsaturation, (iii) changes in unsaturation following sublethal concentrations of the two salts, and (iv) the implication of lipid peroxidation in mycelial inhibition in sensitive fungi.

MATERIALS AND METHODS

Fungal material. *Fusarium sambucinum* and *Phytophthora infestans* were obtained from the Laboratoire de Diagnostic en Phytoprotection (MAPAQ, Québec, Canada). *Helminthosporium solani* (DAOM 233452) and *Rhizoctonia solani* (ATCC 10183) were isolated from infected potato tubers.

Fusarium sambucinum, *H. solani*, and *R. solani* were maintained on potato dextrose agar (Becton Dickinson, Sparks, MD), and *P. infestans* was maintained on clarified V8 agar containing 200 ml liter⁻¹ of clarified V8 Juice (Campbell's Soup Company, Toronto, Ontario), 3 g liter⁻¹ of CaCO₃, and 15 g liter⁻¹ of Bacto agar (Becton Dickinson) at pH 7.2. The fungi were stored at 4°C and served as stock cultures. Prior to inoculation in liquid medium, all fungi were repeatedly cultured on clarified V8 agar.

Chemicals. Aluminum chloride (AlCl₃), sodium metabisulfite (Na₂S₂O₅), BF₃-methanol (14%), fatty acid methyl ester (FAME) standards, 1,1,3,3-tetramethoxypropane (TMP), and pentafluorophenylhydrazine (PFPH) were obtained from Sigma (Mississauga, Canada).

Effects of salts on mycelial growth. Fungi were cultured in 500-ml flasks containing 0 to 5 mM concentrations of either aluminum chloride or sodium metabisulfite in 100 ml of clarified V8 broth (200 ml liter⁻¹ of clarified V8 juice and 3 g liter⁻¹ of CaCO₃) at pH 6.5. Fungi were cultured for 4 days at 23°C on a rotary shaker (120 rpm). Mycelia were then recovered by filtration on Whatman paper (no. 1), lyophilized, and stored at -80°C until use. Dry weight of the samples was recorded, and inhibition by salt treatments was calculated as follows: [dry weight (control) - dry weight (salt treatment)]/dry weight (control) × 100. For each salt-fungus combination, the MIC was defined as the lowest concentration of each salt at which no macroscopic evidence of growth was observed. In order to determine the dose of each salt that reduced mycelial growth of each fungus by 50% (50% effective concentration [EC₅₀]), Probit analysis was performed with the PROC Probit procedure in the SAS System (SAS Institute, Cary, NC).

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FIG. 1. Effects of aluminum chloride (AlCl_3) (A) and sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) (B) on mycelial growth of four fungal potato pathogens in liquid culture. Symbols: $\blacktriangle$, *Fusarium sambucinum*; $\square$, *Helminthosporium solani*; $\bullet$, *Phytophthora infestans*; $\diamond$, *Rhizoctonia solani*. Error bars show standard errors from three independent repetitions.

Effects of salts on total fatty acid unsaturation. Lyophilized fungal mycelia were ground to a powder and transferred to 15-ml screw-cap tubes. FAME were directly prepared by adding 4 ml of BF_3 -methanol to the powdered mycelium. Methylation of total fatty acids was performed under reflux at 90°C for 90 min, with shaking every 15 min. Following cooling on ice, water (4 ml) and hexane (4 ml) were added to the tubes, which were shaken and centrifuged ($500 \times g$) for 10 min at room temperature. The hexane fraction, containing the FAME, was recovered, and the extraction was repeated twice with 4 ml of hexane. The pooled fractions were evaporated to dryness under a stream of nitrogen, resuspended in 4 ml of hexane, and directly submitted to analysis by gas chromatography-mass spectrometry (GC-MS).

FAME were analyzed by use of a gas chromatograph (Hewlett Packard model 5890) coupled to a mass spectrum detector (Hewlett Packard model 5972), with a 30-m DB225 column (J&W Scientific, Folsom, CA). Injector and detector temperatures were 200°C and 250°C , respectively. The oven temperature program was as follows: 100°C for 2 min; $40^\circ\text{C}/\text{min}$ to 195°C , held for 7 min; $40^\circ\text{C}/\text{min}$ to 220°C , held for 18 min. Helium was used as the carrier gas. FAME were identified by comparing retention times and mass spectrum data with those of authentic methylated standards. Unsaturation of total fatty acids was expressed as the percentage of unsaturated and polyunsaturated fatty acids, the ratio of unsaturated to saturated fatty acid (U/S), the ratio of polyunsaturated to monounsaturated fatty acid (P/M), and the average unsaturation of fatty acids (Δ/mol), calculated according to the formula of Avis and Bélanger (2) as follows: [% monoenoic fatty acids + 2(% dienoic fatty acids) + 3(% trienoic fatty acids) + 4(% tetraenoic fatty acids)]/100.

Effects of salts on lipid peroxidation. Mycelium from the treated fungi was assayed for malondialdehyde (MDA), a biochemical marker of lipid peroxidation. Preparation of the MDA standard by acid hydrolysis of TMP was performed according to the method of Li et al. (18). Total MDA was obtained from the samples by acid hydrolysis (to extract bound MDA) as previously described (33). MDA standard and extracted samples were derivatized with PFPH as previously described (33). MDA-PFPH adduct was analyzed by use of a gas chromatograph coupled to a mass spectrum detector, with a 30-m DB225 column. Injector and detector temperatures were 250°C and 280°C , respectively. The oven temperature program was as follows: 50°C for 1 min; $20^\circ\text{C}/\text{min}$ to 280°C , held for 1 min. Helium was used as the carrier gas. MDA-PFPH adduct was identified by comparing retention time and mass spectrum data with those of the prepared

standard. MDA-PFPH present in the samples was quantified by using calibration curves of the prepared standard. MDA was expressed as nanomoles per milligram (dry weight).

Statistical analysis. All experiments were conducted according to a completely randomized design with each experiment repeated three times. Analysis of variance was performed with the SAS System (SAS Institute, Cary, NC). When significant, mean comparisons were performed with Fisher's protected least significant difference (LSD) test at P of 0.05.

RESULTS

Effects of salts on mycelial growth. Each of the four tested fungi was inhibited by both aluminum chloride and sodium metabisulfite, albeit at different concentrations. In general, the fungi could be broadly classified into two sensitivity groups based on inhibition curves (Fig. 1) and MICs (Table 1), with *F. sambucinum* and *R. solani* being less sensitive and *H. solani* and *P. infestans* being more sensitive to the two salts. Among the less sensitive fungi, *F. sambucinum* was less sensitive to both aluminum chloride and sodium metabisulfite than was *R. solani*, based on EC_{50} analysis (Table 1). Within the more sensitive fungal group, *H. solani* was less sensitive to sodium metabisulfite than was *P. infestans* (Table 1).

Effects of salts on total fatty acid unsaturation. In order to determine the effects of salt treatments on fatty acid unsaturation, the intrinsic fatty acid composition of the fungi as well as the fatty acid compositions of selected salts concentration treatments were analyzed by GC-MS. When intrinsic lipid unsaturation was analyzed, *F. sambucinum* revealed a generally lower unsaturation of total fatty acids than any of the other fungi (Table 2). Indeed, all five measured unsaturation parameters revealed the lowest unsaturation levels in *F. sambucinum*, with the percentage of polyunsaturated fatty acids and average unsaturation (Δ/mol) being the lowest of all tested fungi. *R. solani* unsaturation measures were generally higher than those of *F. sambucinum* though lower than *H. solani* and *P. infestans*. In *H. solani*, unsaturation was generally higher than that of either *F. sambucinum* or *R. solani* and typically lower than *P. infestans* intrinsic unsaturation. *P. infestans* had the highest values of unsaturation of all measured parameters, with the exception of U/S ratio. Of particular interest, *P. infestans* was the only fungus tested containing arachidonic acid (a long-chained fatty acid containing four double bonds) in any appreciable amount. Indeed, *P. infestans* contained ca. 9% of this highly unsaturated fatty acid, whereas the other fungi all contained less than 0.1%.

When the fungi were treated with sublethal doses of alumi-

TABLE 1. MICs and EC_{50} s of aluminum chloride and sodium metabisulfite for four potato pathogens^a

Pathogen	Aluminum chloride		Sodium metabisulfite	
	MIC (mM)	EC_{50} (mM)	MIC (mM)	EC_{50} (mM)
<i>Phytophthora infestans</i>	0.5*	0.20*	0.2*	0.08*
<i>Helminthosporium solani</i>	0.5*	0.25*	0.5†	0.25†
<i>Rhizoctonia solani</i>	3†	2.2†	3‡	1.6‡
<i>Fusarium sambucinum</i>	3†	2.5‡	3‡	2.2§

^a Values within a column followed by the same symbol are not significantly different from each other, according to Fisher's protected LSD test, at a P value of 0.05.

TABLE 2. Intrinsic lipid unsaturation in *Phytophthora infestans*, *Helminthosporium solani*, *Rhizoctonia solani*, and *Fusarium sambucinum*^a

Pathogen	% Fatty acids		U/S ratio	P/M ratio	Δ /mol
	Unsaturated	Polyunsaturated			
<i>P. infestans</i>	73.2*	57.1*	2.73†	3.55*	1.56*
<i>H. solani</i>	79.3*	51.8†	3.89*	1.88†‡	1.42†
<i>R. solani</i>	60.4†	41.7‡	1.54‡	2.31†	1.14‡
<i>F. sambucinum</i>	56.2†	30.9§	1.28‡	1.22‡	0.94§

^a Values within a column followed by the same symbol are not significantly different from each other, according to Fisher's protected LSD test, at a *P* value of 0.05.

num chloride and sodium metabisulfite, various changes in unsaturation levels were detected in all fungi compared to the intrinsic (control) fatty acid unsaturation (Table 3). At a concentration of 1 mM of each salt, *F. sambucinum* did not demonstrate any changes in unsaturation levels regardless of the salt employed. In contrast, the same concentration, 1 mM, significantly affected *R. solani* unsaturation levels. At 1 mM aluminum chloride, percent polyunsaturation and Δ /mol were significantly lower in *R. solani* fatty acids than in the control. At 1 mM sodium metabisulfite, *R. solani* unsaturation levels were lower than the control in all measured parameters. Moreover, percent unsaturation, percent polyunsaturation, U/S ratio, and Δ /mol were lower with 1 mM sodium metabisulfite than with 1 mM aluminum chloride in *R. solani*.

When a 0.1 mM concentration of each salt was tested, aluminum chloride did not significantly affect the unsaturation levels of *H. solani* compared to the control (Table 4). However, sodium metabisulfite significantly decreased the percent unsaturation, U/S ratio, and Δ /mol compared to the control. There was no significant difference in unsaturation between *H. solani* treated with 0.1 mM aluminum chloride and 0.1 mM sodium metabisulfite. In contrast to *H. solani*, a dose of 0.1 mM aluminum chloride or sodium metabisulfite significantly decreased all measured unsaturation parameters in *P. infestans* compared to the control. In *P. infestans*, the measured unsaturation parameters were not significantly different between 0.1 mM aluminum chloride and 0.1 mM sodium metabisulfite, with the exception of a lower percentage of polyunsaturated fatty acids in the sodium metabisulfite treatment.

Effects of salts on lipid peroxidation. MDA, a by-product of lipid peroxidation, was quantified to ascertain the implication of lipid peroxidation in the toxicity of aluminum chloride and sodium metabisulfite. Treatment with each salt has a variable effect on MDA presence in fungi. In *F. sambucinum*, there was

no difference in MDA concentration between salt treatments (1 mM) and the control (Table 3). In *R. solani*, MDA concentration in the salt treatments (1 mM) was two- and threefold higher than the control in the aluminum chloride and sodium metabisulfite treatments, respectively (Table 3). In *H. solani*, 0.1 mM of both aluminum chloride and sodium metabisulfite caused an increase in MDA concentration compared to the control (Table 4). Finally, both aluminum chloride and sodium metabisulfite (0.1 mM) caused a nearly twofold increase in MDA concentration in *P. infestans* relative to the control (Table 4).

DISCUSSION

Aluminum chloride and sodium metabisulfite have shown high efficacy at low doses in controlling postharvest pathogens on potato tubers (12, 20, 32). These salts have also been shown to cause loss of membrane integrity in sensitive pathogens (32; also T. J. Avis, M. Michaud, and R. J. Tweddell, unpublished data). In this study, four potato pathogens were used as living model membranes in order to elucidate the role of membrane lipids and lipid peroxidation in the relative sensitivity of microorganisms exposed to these salts and to shed light on the possible mode of action of these salts on pathogen membranes.

In general, *F. sambucinum* and *R. solani*, the fungi less sensitive to the antimicrobial salts, had a markedly lower intrinsic fatty acid unsaturation level than the more sensitive fungi *H. solani* and *P. infestans*. When fatty acid unsaturation was assayed in these fungi following exposure to a sublethal dose of the antimicrobial salts, shifts in fatty acid unsaturation were observed based on the sensitivity of the fungi. In the less sensitive fungi, *F. sambucinum* and *R. solani*, shifts in fatty acid unsaturation following salt treatment correlated well with the respective sensitivity level of each fungus. Indeed, the least

TABLE 3. Effects of 1 mM aluminum chloride (AlCl_3) and 1 mM sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) on lipid unsaturation and lipid peroxidation in *Fusarium sambucinum* and *Rhizoctonia solani*^a

Pathogen	Salt	% Fatty acids		U/S ratio	P/M ratio	Δ /mol	LPO (nmol/mg [dry wt]) ^b
		Unsaturated	Polyunsaturated				
<i>F. sambucinum</i>	Control	56.2*	30.9*	1.28*	1.22*	0.94*	19.7*
	AlCl_3	60.9*	36.8*	1.65*	1.52*	1.05*	20.0*
	$\text{Na}_2\text{S}_2\text{O}_5$	59.9*	34.1*	1.50*	1.34*	1.00*	19.3*
<i>R. solani</i>	Control	60.4*	41.7*	1.54*	2.31*	1.14*	15.9*
	AlCl_3	56.9*	36.8†	1.33*	1.83*†	1.02†	31.2†
	$\text{Na}_2\text{S}_2\text{O}_5$	40.3†	18.3‡	0.68†	0.86†	0.70‡	49.6‡

^a For each fungus, values within a column followed by the same symbol are not significantly different, according to Fisher's protected LSD test, at a *P* value of 0.05.

^b Lipid peroxidation (LPO) measured as MDA concentration.

TABLE 4. Effects of 0.1 mM aluminum chloride (AlCl_3) and 0.1 mM sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) on lipid unsaturation and lipid peroxidation in *Helminthosporium solani* and *Phytophthora infestans*^a

Pathogen	Salt	% Fatty acids		U/S ratio	P/M ratio	Δ /mol	LPO (nmol/mg [dry wt]) ^b
		Unsaturated	Polyunsaturated				
<i>H. solani</i>	Control	79.3*	51.8*	3.89*	1.88*	1.42*	16.0*
	AlCl_3	72.7*†	51.3*	2.67*†	2.39*	1.35*†	21.4†
	$\text{Na}_2\text{S}_2\text{O}_5$	67.5†	40.8*	2.08†	1.58*	1.19†	23.0†
<i>P. infestans</i>	Control	73.2*	57.1*	2.73*	3.55*	1.56*	21.2*
	AlCl_3	59.9†	43.4†	1.51†	2.65†	1.08†	39.5†
	$\text{Na}_2\text{S}_2\text{O}_5$	54.4†	38.7‡	1.22†	2.38†	0.94†	41.4†

^a For each fungus, values within a column followed by the same symbol are not significantly different, according to Fisher's protected LSD test, at a *P* value of 0.05.

^b Lipid peroxidation (LPO) measured as MDA concentration.

sensitive fungus, *F. sambucinum*, did not demonstrate changes in fatty acid unsaturation when exposed to a 1 mM concentration of either salt. Conversely, the same dose of these salts revealed a marked decrease in fatty acid unsaturation in *R. solani*, a fungus that was shown to be more sensitive to the salts. In the two more sensitive fungi, a sublethal dose of 0.1 mM sodium metabisulfite caused a decrease in unsaturation in *H. solani*. The same dose of both aluminum chloride and sodium metabisulfite caused a marked decrease in unsaturation in *P. infestans*. This also correlated well with sensitivity assays, in which *P. infestans* was more sensitive than *H. solani*.

Overall, higher sensitivity to the antimicrobial salts was consistent with higher intrinsic fatty acid unsaturation in the fungi. This indicated that fatty acids would have a role to play in the sensitivity of pathogens and would in part explain the loss of membrane integrity seen in pathogen cells exposed to these salts. This is not unexpected, as fatty acids are major components in cell membranes and are partly responsible for cell membrane fluidity, organization, and integrity (2).

Furthermore, more sensitive fungi with higher intrinsic unsaturation were shown to lose this unsaturation more readily than less sensitive fungi following exposure to the antimicrobial salts. This loss of membrane unsaturation in the more sensitive fungi points to a mechanisms of action that would affect double bonds in cellular fatty acids and, in particular, lipid peroxidation. In order to determine the role of lipid peroxidation in the tested fungi, MDA was measured as a stable biochemical marker. The MDA concentration remained constant in *F. sambucinum* treated with the salts, whereas *R. solani*, *H. solani*, and *P. infestans* showed an increase in MDA concentration in mycelium treated with these salts. Aluminum ion (Al^{3+}) and sulfite (SO_3^{2-}), from the dissociation of aluminum chloride and sodium metabisulfite, respectively, can form radical moieties that react with lipids, leading to lipid peroxidation (5, 6, 7). Peroxidation of membrane lipids is a complex process involving unsaturated fatty acids and, in particular, polyunsaturated fatty acids containing one or more methylene groups positioned between *cis* double bonds. These methylene groups are highly reactive to oxidizing agents and can form peroxy radicals that can set off a free radical chain reaction (propagation phase) to other methylene groups and generate new radical species and peroxidation by-products such as MDA (10, 19). This would explain the high sensitivity of fungi containing highly unsaturated fatty acids and, in particular, *P. infestans*, which contains an appreciable amount of

arachidonic acid. Indeed, arachidonic acid contains three methylene groups positioned between double bonds and is known to be exceptionally sensitive to peroxidation (10).

Taken as a whole, our data are best explained by a mechanism of action in which the lipid bilayer of fungal membranes is the initial structure affected by the antimicrobial salts. From a biochemical standpoint, the current data suggest that the antimicrobial salts would induce lipid peroxidation in the fungal membrane. Fungi with low intrinsic unsaturation would be less at risk, whereas fungi with highly unsaturated membrane lipids would be more vulnerable to peroxidation, which could promote the ensuing loss of integrity in the plasma membrane (14) and, eventually, lead to cell death. This direct effect on the fungal membrane would contribute, at least partially, to the sensitivity of fungi to these salts. Alternatively, by-products of lipid peroxidation have been shown to affect the mitochondrial respiratory chain (25) and are also implicated in DNA damage (19). This could account for the reported inhibitory effect of sodium metabisulfite on energetic metabolism (29) as well as DNA damage as a consequence of both aluminum and sulfites (9, 16, 31).

Although intrinsic fatty acid unsaturation would seem to be a biochemical determinant in the sensitivity of fungi against aluminum chloride and sodium metabisulfite, this does not exclude the possibility that other factors are involved. In particular, the presence of antioxidant activities in these fungi may have a bearing on the final outcome following lipid peroxidation. For example, fungi that are more tolerant to the antimicrobial effects of these salts may have greater antioxidant activities that would reduce or nullify the effect of peroxidation by breaking the propagation cycle (8). Furthermore, it is not improbable that other modes of action of aluminum chloride and sodium metabisulfite exist, as other antimicrobial activities of these salts (reactions with protein disulfide groups [4, 15, 26] and inhibition of enzyme activity through the inactivation of cofactors [30] and coenzymes [23, 26, 27, 28], in the case of sulfites, and replacement of divalent metal complexes [1] and complexation with ATP [11], DNA [31], and phosphates [24], for aluminum) have been reported and cannot be fully explained by lipid peroxidation. This study is the first, to our knowledge, to propose lipid peroxidation as a component of the inhibitory effects of aluminum chloride and sodium metabisulfite on fungal potato pathogens. These results also provided measurable biochemical determinants (intrinsic fatty

acid unsaturation) that could be useful in predicting the sensitivities of other pathogens.

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