Ultrastructural Alterations in *Fusarium sambucinum* and *Heterobasidion annosum* Treated with Aluminum Chloride and Sodium Metabisulfite

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ABSTRACT

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Aluminum chloride (AlCl_3) and sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) have received increasing attention as antifungal agents for the control of plant diseases. In an effort to understand their toxic action on fungi, ultrastructural changes and membrane damage in *Fusarium sambucinum* (Ascomycota) and *Heterobasidion annosum* (Basidiomycota) in response to salt exposure was investigated using transmission electron microscopy. Conidial membrane damage was quantified using SYTOX Green stain, which only enters altered membranes. The results showed that mortality

of the conidia was generally closely associated with SYTOX stain absorption in *F. sambucinum* treated with $\text{Na}_2\text{S}_2\text{O}_5$ and in *H. annosum* treated with AlCl_3 or $\text{Na}_2\text{S}_2\text{O}_5$, suggesting that these salts cause membrane alterations. For both fungi, ultrastructural alterations in conidia treated with AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ included membrane retraction, undulation, and invagination. At higher concentrations or exposure periods to the salts, loss of membrane integrity, cytoplasmic leakage, and cell rupture were observed. Ultrastructural alterations and increased SYTOX stain absorption in salt-treated conidia appear consistent with a mode of action where AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ alter membrane integrity and permeability.

Additional keywords: conidial mortality.

In the ongoing struggle with plant pathogens, synthetic pesticides remain the most widely used disease control measures. While relatively efficient in the short term, these compounds have demonstrated major drawbacks, the most prevalent being (i) their adverse effects on health and the environment and (ii) their lack of long-term efficacy as a result of the development of resistance by plant pathogens (2). Although great strides toward the use of alternative means of disease control have been achieved in the present context of safe and durable disease control, there remains an urgent need for measures with novel and efficient modes of action as well as reduced risks for the environment.

Among alternatives to synthetic chemicals, organic and inorganic salts have shown increasing evidence of efficacy in controlling plant pathogens (11,13,20,22,24). These compounds are widely used in industry as preservatives and antimicrobials (32) and have shown broad-spectrum antimicrobial activity with low mammalian toxicity (24), biocompatibility (13), and are generally recognized as safe. Moreover, they are less sensitive to environmental conditions than other alternative control measures such as biological control agents, which may render them particularly useful in the efficient and consistent control of plant pathogens (3). Among antimicrobial salts, aluminum chloride (AlCl_3) and sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) have received increasing attention for the control of plant diseases. In particular, these salts have demonstrated in vitro and/or in vivo inhibition of *Alternaria alternata*, *Botrytis cinerea*, *Erwinia carotovora*, *Fusarium* spp., *Hel-*

minthosporium solani, *Phytophthora* spp., *Rhizoctonia solani*, and *Verticillium* spp. (4,20,22,46).

Recent studies have postulated that, under certain conditions, AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ may act through cell disruption and compromised membrane permeability in affected cells (1,6,9,15,17,33,35,46,47). These results were mostly from studies on bacteria and only recently have studies been performed on the mode of action of these salts on fungal mycelia (4). In this work, lipid peroxidation afforded by these salts was shown to be involved in fungal mycelia growth inhibition and this was hypothesized to be a cause of compromised permeability of the fungal membrane that, when reaching levels over tolerable limits, would lead to cell death (4).

Although several studies have been carried out to elucidate the specific mode of action of aluminum and sulfite salts, there is, to our knowledge, no concrete or visual evidence of compromised cell permeability and cell disruption in fungi exposed to these salts. The objectives of this study were therefore (i) to quantify the loss of conidial membrane integrity following treatment with AlCl_3 or $\text{Na}_2\text{S}_2\text{O}_5$, and (ii) to visualize ultrastructural alterations in conidia exposed to these antimicrobial salts. This study was conducted with the fungi *Fusarium sambucinum* (phylum Ascomycota) and *Heterobasidion annosum* (phylum Basidiomycota). *F. sambucinum* is the predominant causal agent of dry rot of potato (*Solanum tuberosum* L.) in North America (42) and *H. annosum* is responsible for root and butt rot disease of many conifers throughout the northern hemisphere (45).

MATERIALS AND METHODS

Fungi. *F. sambucinum* was obtained from the Laboratoire de diagnostic en phytoprotection (MAPAQ, Québec, Canada) and

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maintained on potato dextrose agar (PDA; Becton Dickinson, Sparks, MD). *H. annosum* P group strain Ha014 was recovered from a fruiting body found on red pine (*Pinus resinosa* Soland.) stumps from Lac la Blanche (Québec, Canada) and maintained on malt agar (MA; Becton Dickinson).

Chemicals. Aluminum chloride (AlCl_3), sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$), safranin O, acrolein, low gelling temperature agarose, and sodium citrate (for preparing lead citrate) of high purity grade (>99%) were obtained from Sigma Chemical Co. (St. Louis, MO). SYTOX Green nucleic acid stain was purchased from Molecular Probes (Eugene, OR). Glutaraldehyde, sodium cacodylate, osmium tetroxide, and JEMBED 812 resin were from Canemco Inc. (Montréal, Québec, Canada), whereas toluidine blue O, uranyl acetate, and lead nitrate (for preparing lead citrate) were from Fisher Scientific International (Fairlawn, NJ).

Effect of salts on conidial viability. *F. sambucinum* and *H. annosum* were cultured at 24°C for 7 days in obscurity on petri dishes containing PDA and one-half MA (15 g liter⁻¹ malt extract and 7.5 g liter⁻¹ agar; Becton Dickinson), respectively. For each fungus, 2 ml of sterile distilled water was added to each petri dish and the mycelial mat was gently scrapped with a sterile glass rod in order to dislodge the conidia. Conidia were recovered and adjusted to 1×10^6 conidia ml⁻¹ with a hemacytometer. One milliliter of each conidial suspension was transferred to a sterile microcentrifuge tube and centrifuged at $16,000 \times g$ for 5 min. The supernatant was decanted off and replaced with 1 ml of the following saline solutions: 1 mM $\text{Na}_2\text{S}_2\text{O}_5$, 10 mM $\text{Na}_2\text{S}_2\text{O}_5$, 10 mM AlCl_3 , or 100 mM AlCl_3 . Sterile distilled water served as the control. Conidia were incubated in the saline solutions for either 0 (conidia were centrifuged immediately after addition of the solution), 10, or 60 min at 24°C. Following the incubation period, the conidial suspensions were centrifuged ($16,000 \times g$ for 5 min) and the saline solution was decanted off. The fungal pellet was washed with 1 ml of sterile distilled water, vortexed 10 s, and centrifuged ($16,000 \times g$ for 5 min), and the supernatant was decanted off. Conidia were resuspended in 250 μl of sterile water. Plate counts of the resulting suspensions were performed by immediately spreading the conidia on PDA to assess the viability of the treated conidia. Conidia were incubated 4 days at 24°C prior to counting. Conidia that did not germinate were considered dead. The proportion of dead conidia was determined as follows: number of nongerminated conidia/total number of conidia. The experiment was conducted according to a completely randomized factorial design with three repetitions for each fungus-salt-incubation period combination and was repeated twice. Analysis of variance was performed with the SAS System (SAS Institute, Cary, NC). When significant, mean comparison was performed with Fisher's protected least significant difference (LSD) test at $P = 0.05$.

Effect of pH on conidial viability. The effect of pH on conidial viability was tested for both *F. sambucinum* and *H. annosum*. The conidia (1×10^6 conidia/ml) prepared as described above were placed in microcentrifuge tubes containing 1 ml of sterile water adjusted to pH 3, 4, 5, 6, and 7 using HCl. Following a 60-min incubation at 24°C, the conidia were recovered and the rate of dead conidia was determined on PDA as previously described. The experiment consisted of three repetitions for each fungus and was repeated twice.

Effect of salts on membrane permeability. In parallel to the viability testing, the incorporation of SYTOX Green nucleic acid stain (a compound that penetrates damaged membranes) was assayed to evaluate the integrity of the plasma membrane in the two fungi exposed to the salts. The salt-treated conidial suspensions (prepared above) were immediately used as follows: 9 μl of each suspension (washed conidia resuspended in 250 μl of sterile water) was added to 1 μl of a 50 μM solution of SYTOX Green directly on a microscopy slide. The mixture was allowed to rest for 5 min. The slides were examined with a light microscope

(Olympus model BH2-RFCA; Olympus, Tokyo, Japan). For fluorescence microscopy, observations were carried out under blue light (488 nm) using the same microscope equipped with a BP490 filter (dichroic mirror cutoff 510 nm; Olympus) and a BH2-RFL-T3 mercury lamp (excitation range 450 to 490 nm; Olympus). For each treatment, at least 50 conidia were counted under visible and blue light. Percentage of fluorescent conidia (conidia that absorbed SYTOX Green) was calculated. Proportion of dead conidia (D) was divided by total fluorescent conidia (F) for each of the treatments to calculate D/F ratio. The experiment was conducted according to a completely randomized factorial design with three repetitions for each fungus-salt-incubation period treatment and was repeated twice. Analysis of variance was performed with the SAS System. When significant, mean comparison was performed with Fisher's protected LSD test at $P = 0.05$.

Effect of salts on conidial ultrastructure. Conidia of *F. sambucinum* and *H. annosum* treated with either 100 mM AlCl_3 , 1 mM $\text{Na}_2\text{S}_2\text{O}_5$, or distilled sterile water (control) for 0, 10, and 60 min were fixed with a mixture of 3% glutaraldehyde and 3% acrolein (pH 7.4) in 0.1 M sodium cacodylate buffer (SCB) under vacuum for 3 h and centrifuged ($16,000 \times g$ for 5 min at 4°C). Gels of 1 to 2 mm³ were prepared by adding 3% low gelling temperature agarose in SCB to the pellet. After thorough rinsing with 0.1 M SCB, the gels were post-fixed with 2% osmium tetroxide in 0.1 M SCB for 1 h at 20°C, dehydrated using a series of increasing ethanol concentrations, and embedded in JEMBED 812 resin. Thin (1 μm) and ultrathin (90 nm) sections were observed with a Leitz Orthoplan light microscope (Leitz, Wetzlar, Germany) and a transmission electron microscope (Model 300; Philips, Eindhoven, The Netherlands), respectively, after staining the sections with toluidine blue O and safranin O (31) or contrasting them with uranyl acetate and lead citrate (30). For each treatment, the test was repeated twice and at least two blocks by replicate were examined.

RESULTS

Effect of salts on conidial viability. The incubation of *F. sambucinum* conidia with salts markedly decreased their viability. Conidial mortality increased with higher AlCl_3 concentration (Fig. 1). Indeed, incubation with 10 mM AlCl_3 for 10 or 60 min significantly increased the proportion of dead conidia when compared with their respective controls. A concentration of 100 mM AlCl_3 revealed the significantly highest proportion of dead conidia in *F. sambucinum* when compared with the controls and with treatments with 10 mM AlCl_3 for both incubation periods. There were no significant differences in conidial mortality between a 10- and 60-min incubation in AlCl_3 at either concentration. All treatments with $\text{Na}_2\text{S}_2\text{O}_5$ resulted in complete conidial mortality in *F. sambucinum* (Fig. 1).

In *H. annosum*, the proportion of dead conidia also increased with higher salt concentrations and longer exposure periods (Fig. 2). *H. annosum* conidia incubated with 100 mM AlCl_3 for 10 min showed a significant increase in proportion of mortality when compared with the 10 min control. A 60-min incubation period with 10 mM AlCl_3 significantly increased conidial mortality when compared with the controls. A concentration of 100 mM AlCl_3 revealed the highest proportion of dead conidia following incubation for 60 min when compared with the controls and with the other AlCl_3 treatments. Results for AlCl_3 demonstrated a significant increase in conidia mortality with time. Indeed, the proportion of dead conidia was significantly higher following a 60-min incubation at both concentrations of AlCl_3 when compared with the 10-min incubation period. All treatments with $\text{Na}_2\text{S}_2\text{O}_5$ resulted in at least 95% mortality, which was significantly higher than the mortality observed following any of the AlCl_3 treatments in *H. annosum*.

Effect of pH on conidial viability. Results demonstrated that *H. annosum* conidial viability was not affected by pH 3 to 6. Moreover, *F. sambucinum* conidia incubated at pH 4 to 7 did not demonstrate any difference in viability. However, a 60-min incubation of *F. sambucinum* conidia at pH 3 caused an approximate 15% increase in conidial death relative to conidia incubated at pH 4 to 7 (data not shown).

Effect of salts on membrane permeability. In SYTOX Green incorporation studies, the percentage of fluorescent conidia was affected by the salt treatments. In *F. sambucinum* (Figs. 1 and 3A to C), the percentage of fluorescent conidia significantly increased with a 10-min incubation in 10 and 100 mM AlCl_3 when compared with the control. A 10-min incubation with 1 and 10 mM $\text{Na}_2\text{S}_2\text{O}_5$ further increased conidia fluorescence when

Fig. 1. Effect of aluminum chloride (AlCl_3) and sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) treatments on the proportion of dead (white bars) and fluorescent (black bars) conidia of *Fusarium sambucinum*. Percentage of dead conidia with the same uppercase letter and percentage of fluorescent conidia with the same lowercase letter are not significantly different, according to Fisher's protected least significant difference test ($P = 0.05$). Numbers in brackets indicate the ratio of dead to fluorescent conidia (D/F ratio).

Fig. 2. Effect of aluminum chloride (AlCl_3) and sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) treatments on the proportion of dead (white bars) and fluorescent (black bars) conidia of *Heterobasidion annosum*. Percentage of dead conidia with the same uppercase letter and percentage of fluorescent conidia with the same lowercase letter are not significantly different, according to Fisher's protected least significant difference test ($P = 0.05$). Numbers in brackets indicate the ratio of dead to fluorescent conidia (D/F ratio).

compared to the control and AlCl_3 treatments. A 60-min incubation with 100 mM AlCl_3 significantly increased proportion of fluorescent conidia relative to the 60-min incubation control. When compared with AlCl_3 treatments, a 60-min incubation in

1 mM $\text{Na}_2\text{S}_2\text{O}_5$ resulted in a significantly higher proportion of fluorescent conidia. The highest proportion of fluorescent *F. sambucinum* conidia was observed following a 60-min incubation in 10 mM $\text{Na}_2\text{S}_2\text{O}_5$. A significant increase in fluorescence with

Fig. 3. Effect of salt treatments on SYTOX Green incorporation and effect of AlCl_3 on ultrastructure of *Fusarium sambucinum* conidia. Using SYTOX Green, fluorescent conidia are barely visible with the control (A, after 10 min in water). They are more numerous after treatment with AlCl_3 for 10 min (B), and they are particularly abundant after exposure to $\text{Na}_2\text{S}_2\text{O}_5$ for 60 min (C). D, This control conidium appears normal after incubation for 60 min in water, although empty spaces or small vacuoles are noted, particularly in the periphery of the cytoplasm. N: nucleus. E to H, Treatments with 100 mM AlCl_3 . E, After 10 min, the cytoplasm of this conidium is altered and leakage through the wall is noticeable in this conidium (arrow) next to a lomasome (arrowhead). An abnormal mitochondrion (M) is also shown. F to H, After 60 min. F and G, Most conidia are greatly altered and organelles are barely recognizable. Cytoplasm leakage (arrows) is shown close to lomasomes (arrowheads). Asterisk: stain residue. H, The altered cytoplasm appears condensed in some areas (arrow) and abnormal mitochondria are also visible (arrowhead).

time was observed when conidia were treated with 1 or 10 mM of $\text{Na}_2\text{S}_2\text{O}_5$. No significant increase in fluorescence with time was observed in conidia treated with AlCl_3 .

In *H. annosum* trials (Figs. 2 and 4A to C), conidia incubated for 10 min with 100 mM AlCl_3 revealed increased proportion of fluorescent conidia relative to the control. A significantly higher proportion of fluorescent conidia was observed following a treatment with 10 mM AlCl_3 for 60 min and with 100 mM AlCl_3 for 10 or 60 min, when compared with the controls. Increasing incu-

bation period from 10 to 60 min significantly increased fluorescence in conidia treated with 10 and 100 mM AlCl_3 . The highest proportions of fluorescent conidia were observed following exposure to $\text{Na}_2\text{S}_2\text{O}_5$.

Dead to fluorescent conidia (D/F) ratios in *F. sambucinum* conidia incubated in distilled sterile water (control) were 2.4 and 1.9 for an incubation period of 10 and 60 min, respectively (Fig. 1). D/F ratios in *F. sambucinum* treated with AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ ranged from 2.2 (100 mM, 60 min) to 3.0 (100 mM, 10 min) and

Fig. 4. Effect of salt treatments on SYTOX Green incorporation and effect of AlCl_3 on ultrastructure of *Heterobasidion annosum* conidia. **A to C,** Fluorescence revealed under blue light using SYTOX Green stain. **A,** A few of these control conidia are fluorescent after 60 min in water. **B,** Fluorescent conidia are obvious after treatment with 100 mM AlCl_3 for 10 min. **C,** Numerous conidia are fluorescent after exposure to 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ for 60 min. **D to F,** Examination of conidia in transmission electron microscopy. **D,** This control conidium was collected after 10 min in water and its cytoplasm appears normal. Two nuclei (N) are visible with their well-defined envelopes. Inset (enlarged portion). The plasma membrane is obvious in this part of the conidium. **E and F,** Effect of 100 mM AlCl_3 after 10 min (**E**) and 60 min (**F**). **E,** Some vacuoles (V) are shown in the conidium as well as numerous small vesicles (arrowheads) adjacent to a lomasome (thin arrow). Although the cristae in mitochondria seem abnormal, the nuclear envelope is still visible. Some cytoplasm leaks out of the cell (large arrow). N: nucleus. **F,** An altered conidium in a plasmolysis state showing the ruptured plasma membrane (arrow) and several void spaces in the cell periphery (arrowheads). The wall is also thinner than normal.

from 1.0 (10 mM, 60 min) to 2.6 (1 mM, 10 min), respectively (Fig. 1). In *H. annosum*, D/F ratios were generally lower than those of *F. sambucinum*. In the AlCl_3 treatments, D/F ratios ranged from 1.1 (100 mM at 10 and 60 min) to 2.4 (10 mM, 10 min) (Fig. 2). All $\text{Na}_2\text{S}_2\text{O}_5$ treatments resulted in D/F ratios of unity (1.0) (Fig. 2).

Effect of salts on conidial ultrastructure. *F. sambucinum* conidia incubated in sterile water generally displayed integrity of the plasma membrane, organized cytoplasm, and visible nuclei and mitochondria with well-defined envelopes. With prolonged incubation in sterile water (60 min) as in Figure 3D, occasional vesicles and vacuoles appeared in the periphery of the cytoplasm, although the plasma membrane remained intact. The treatment of *F. sambucinum* with 100 mM AlCl_3 caused clear alterations in conidial ultrastructure. A 10-min incubation with 100 mM AlCl_3 revealed an altered cytoplasm, accompanied by occasional leakage of cytoplasmic content outside the plasma membrane into or through the cell wall (Fig. 3E). Following a 60-min incubation with 100 mM AlCl_3 , more apparent cytoplasmic leakage through the cell wall was observed (Fig. 3F and G). Presence of lomasomes was also frequently noted (Fig. 3E to G). Increased cytoplasmic condensation and aggregation were also characteristic of a 60-min treatment of *F. sambucinum* with 100 mM AlCl_3 (Fig. 3H).

In *F. sambucinum* conidia treated with 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ for 10 min, there was an apparent increase in the number of vesicles as well as alteration/deformation of the cytoplasmic membrane system (nuclear envelope and mitochondrial membranes) (Fig. 5A and B) accompanied by increased electron-opaque material covering the cell wall (Fig. 5C). In spite of these alterations, the internal membranous system was at times more contrasted than normal (Fig. 5B). When conidia were incubated for 60 min, disorganization and lysis of the cytoplasmic content (Fig. 5D to F), excessive vacuolization (Fig. 5E and F) as well as extensive fragmentation of the plasma membrane (Fig. 5E and F) were clearly visible.

Typical conidial structure of *H. annosum* is presented in Figure 4D. After 10 min in water, the conidia generally displayed integrity of the plasma membrane, organized cytoplasm and visible nuclei and mitochondria with well-defined envelopes. With prolonged incubation in sterile water (60 min), occasional vesicles and vacuoles containing granular material appeared, although the plasma membrane remained intact. A 10-min incubation of *H. annosum* conidia with 100 mM AlCl_3 showed the beginning of vacuolization and occasional cytoplasmic leakage (Fig. 4E). In this figure, small vesicles are shown next to a lomasome. A longer exposure period (60 min) to 100 mM AlCl_3 induced a state of plasmolysis as revealed by the retraction and/or rupture of the plasma membrane (Fig. 4F). The cytoplasm was then quite disorganized.

In *H. annosum* conidia exposed to 1 mM $\text{Na}_2\text{S}_2\text{O}_5$, a 10- or 60-min incubation period caused a general disorganization of the cytoplasm, cytoplasmic retraction, and plasma membrane breakage (Fig. 6A to D). As in *F. sambucinum*, some of the internal membranes appeared occasionally more contrasted than in controls (Fig. 6C). Overall, treatment with $\text{Na}_2\text{S}_2\text{O}_5$ caused highly degenerated conidia.

DISCUSSION

Aluminum and bisulfite salts are toxic to several microorganisms including fungi (11,20,21) and bacteria (1,14). Postulated mechanisms of sulfite and aluminum toxicity or inhibition of microbial growth include reactions with protein disulfide groups (7,25,32), ATP and ADP (10,18), phosphates (27), and DNA (23,38,44) as well as causing pH effect and inactivation of enzymes (14) or coenzymes (e.g., NAD) (26,32,39,41). Under certain conditions, AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ were shown to act through

cell disruption and compromised membrane permeability in affected cells (1,6,15,33,35,46). This effect was mostly observed on bacteria. The present study showed that AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ affected membrane permeability and caused concomitant ultrastructural alterations in fungi of the phylum Ascomycota and Basidiomycota.

Work performed with conidia of *F. sambucinum* and *H. annosum* showed that AlCl_3 and particularly $\text{Na}_2\text{S}_2\text{O}_5$ caused a high level of mortality following a 60-min exposure period. Indeed, a 60-min exposure of conidia to 100 mM AlCl_3 resulted in mortality proportions of 69% (*H. annosum*) and 81% (*F. sambucinum*) while exposure to 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ caused complete mortality of the conidia for both fungi. This is the first study, to our knowledge, reporting the antifungal activity of these salts on *H. annosum*, whereas previous work showed that AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ strongly affected *F. sambucinum* mycelial growth and conidial germination (4,20). It is also interesting to note that *H. annosum* and *F. sambucinum* showed comparable sensitivity to the salts and that a prolonged incubation of conidia in distilled water led to increased levels of conidial mortality for both fungi. Following a 60-min incubation period in distilled water, the proportions of conidial mortality were 40 and 36% for *F. sambucinum* and *H. annosum*, respectively.

With D/F ratios of 2.2 and 3.0, it seems that 100 mM AlCl_3 for the exposure periods tested did not cause *F. sambucinum* membrane damage severe enough for the SYTOX stain to migrate into the conidia although the mortality proportions were relatively high. This indicates that lethal effect of AlCl_3 on *F. sambucinum* conidia would be only partially correlated with increased membrane permeability and, therefore, mortality resulting from AlCl_3 exposure could be partially associated with membrane damage. There is also the possibility that the plasma membrane was actually damaged, but that the nucleic acids were too altered to react with SYTOX, as reported at times with bacteria (46). Although only partial correlation between mortality and increased membrane permeability could be shown, ultrastructural observations in *F. sambucinum* conidia appear rather consistent with a membrane-altering mode of action. Indeed, a 10-min incubation with 100 mM AlCl_3 revealed dilated and undulated plasma membranes, accompanied by diffuse cell walls in proximity to altered membrane regions. Following a 60-min incubation with 100 mM AlCl_3 , these alterations were more apparent and accompanied by occasional leakage of cytoplasmic content outside the plasma membrane. Exposure of *H. annosum* conidia for 60 min to AlCl_3 resulted in mortality proportions (54 to 69%) strongly associated with SYTOX incorporation with D/F ratios of ≤ 1.2 , indicating that the lethal effect of AlCl_3 on the fungus was strongly correlated with increased membrane permeability. Ultrastructural observations of the conidia treated with 100 mM AlCl_3 for 60 min confirmed membrane-associated damage including retraction and/or rupture of the plasma membrane accompanied by extensive cytoplasmic disorganization.

Incubation for a 60-min period with either 1 or 10 mM $\text{Na}_2\text{S}_2\text{O}_5$ revealed complete mortality of conidia for both fungi. Mortality was strongly associated with SYTOX incorporation (D/F ratios of ≤ 1.4) especially for *H. annosum* with D/F ratios of 1.0. This strongly suggests that conidial mortality results from membrane damage. This is corroborated by ultrastructural observations showing a general disorganization of the cytoplasm and cytoplasmic leakage associated with a discontinuous and highly undulating plasma membrane, suggesting severely altered membranes in both *F. sambucinum* and *H. annosum* exposed to $\text{Na}_2\text{S}_2\text{O}_5$.

AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ solutions are strongly acidic. In order to verify whether the observed toxic effects of salts were due to acidic pH, the influence of pH on conidial viability was evaluated. Conidial viability of both fungi was not affected by a 60-min exposure to pH values ranging from 4 to 6, while it was strongly affected by exposure to 100 mM AlCl_3 and 1 mM $\text{Na}_2\text{S}_2\text{O}_5$.

solutions (pH 4). This suggests that AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ per se are involved in the toxicity observed.

In general, the results provide evidence of a mechanism in which AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ would alter conidial membrane permeability, which could eventually lead to fungal cell death. Although AlCl_3 had been previously shown to cause membrane damage in the bacterium *E. carotovora*, this was not the case for $\text{Na}_2\text{S}_2\text{O}_5$ in

which membrane damage seemed less involved in its toxicity towards the bacterium (46). Conversely, recent results with fungi (namely *F. sambucinum*, *H. solani*, *Phytophthora infestans*, and *R. solani*) exposed to these two salts indicated that lipid peroxidation damage of fatty acids or fatty acyl chains would be involved in the antifungal effect of both AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ (4). It is not yet clear why $\text{Na}_2\text{S}_2\text{O}_5$ causes increased membrane per-

Fig. 5. Effect of $\text{Na}_2\text{S}_2\text{O}_5$ on ultrastructure of *Fusarium sambucinum* conidia. **A to C,** After exposure to 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ for 10 min, most of the conidia are affected. The cytoplasm is still obvious and dense but most organelles are affected. The thickness of the walls appears normal but electron-opaque material is at times observed covering them (arrowheads). **A,** A nucleus (N) is noticeable as well as many vesicles (arrows). **B,** The internal membranous system appears more contrasted around what appear to be the nucleus (N) and the mitochondria (arrows). **D to F,** After exposure to 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ for 60 min, the content of these conidia appears degraded. The dispersed cytoplasm is mixed with empty areas (arrowheads) or vacuoles (V). Electron-dense compounds are at times observed within the vacuoles (A). **D,** Numerous vesicles (arrows) are shown, particularly in areas where the plasma membrane is fragmented. M: mitochondrion.

meability in the tested fungi but not in *E. carotovora* whereas AlCl_3 affects membrane permeability in both the fungi and the bacterium.

Among the different ultrastructural observations, the presence of lomasomes in the conidia treated with AlCl_3 was noticeable. These structures have been mainly associated with cell wall formation or secretions in fungi (19,43). However, it has also been suggested that lomasomes could play a role in plant-pathogen interactions (5) and these structures have also been observed in cells treated with other antifungal compounds (29,36). In the present study, lomasomes were always composed of a system of well-organized membranes and in some cases, they were found adjacent to substances leaching through the cell wall (Fig. 3E to G). As they were present only in conidia that are not completely degenerated, they might be involved in mechanisms trying to counteract the toxic effect of AlCl_3 . Lomasomes have also been reported next to deposits in other fungi (34).

F. sambucinum and *H. annosum* are two important plant pathogens responsible for potato dry rot (42) and root and butt rot

disease of many conifers (45), respectively. Potato dry rot is controlled primarily by postharvest application of the fungicide thiabendazole (37). However, many strains of *F. sambucinum* have become resistant to thiabendazole (8,12,28). Consequently, alternative methods are necessary to control the disease. Fungitoxic salts, such as AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$, that were previously shown to reduce the development of the disease on potato tubers (20) are of particular interest and could eventually find application in the potato industry. Root and butt rot disease of conifers, which is considered to be one of the most destructive diseases in evergreen forests (40), also necessitates alternative control methods (16). The use of antifungal salts affecting the cell membrane may be appropriate through application of salts to freshly cut stumps to prevent subsequent infection by *H. annosum* and the development of root and butt rot in neighboring trees.

Overall, increasing concentrations and/or exposure periods to AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ caused an increased mortality in *F. sambucinum* and *H. annosum* conidia. This was accompanied by increased conidial membrane permeability as determined by the

Fig. 6. Effect of $\text{Na}_2\text{S}_2\text{O}_5$ on ultrastructure of *Heterobasidion annosum* conidia. **A and B**, After exposure to 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ for 10 min. **A**, This conidium is altered but many mitochondria are still identifiable (arrows). **B**, This conidium is clearly degraded and was probably not viable. The organelles are not recognizable and the plasma membrane is broken in many places (arrows). **C and D**, After exposure to 1 mM $\text{Na}_2\text{S}_2\text{O}_5$ for 60 min. **C**, The cytoplasm is still present but organelles are difficult to distinguish. Some membranous structures (arrows) might be remnants of mitochondria. **D**, The cytoplasm is retracted and only ribosomes and void spaces are visible within this degenerated conidium. Electron-opaque material is shown over the wall (arrowheads).

incorporation of SYTOX Green stain and related ultrastructural modifications (plasmalemma retraction, undulation, and invagination) in the affected fungi. These results indicate that both AlCl_3 and $\text{Na}_2\text{S}_2\text{O}_5$ exert their antimicrobial effect, at least in part, by affecting the conidial membrane. At high concentrations or long exposure periods, the ensuing loss of conidial membrane integrity would lead to cytoplasmic leakage, cell rupture and, eventually, cell death. The use of antifungal salts with activities that target the fungal membrane would seem promising as an alternative to synthetic fungicides for plant disease control.

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