Mycopathologia (2011) 171:291–298
DOI 10.1007/s11046-010-9356-8

Chemosensitization of Aflatoxigenic Fungi to Antimycin A and Strobilurin Using Salicylaldehyde, a Volatile Natural Compound Targeting Cellular Antioxidation System

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Received: 14 May 2010 / Accepted: 5 August 2010 / Published online: 29 August 2010

Abstract Various species of fungi in the genus *Aspergillus* are the most common causative agents of invasive aspergillosis and/or producers of hepatocarcinogenic mycotoxins. Salicylaldehyde (SA), a volatile natural compound, exhibited potent antifungal and anti-mycotoxigenic activities to *A. flavus* and *A. parasiticus*. By exposure to the volatilized SA, the growth of *A. parasiticus* was inhibited up to 10–75% at 9.5 mM $\leq$ SA $\leq$ 16.0 mM, while complete growth inhibition was achieved at 19.0 mM $\leq$ SA. Similar trends were also observed with *A. flavus*. The aflatoxin production, *i.e.*, aflatoxin B_1 and B_2 (AFB_1, AFB_2) for *A. flavus* and AFB_1, AFB_2, AFG_1, and AFG_2 for *A. parasiticus*, in the SA-treated (9.5 mM) fungi was reduced by $\sim$13–45% compared with the untreated control. Using gene deletion mutants of the model yeast *Saccharomyces cerevisiae*, we identified the fungal antioxidation system as the molecular target of SA, where *sod1*Δ [cytosolic superoxide dismutase (SOD)], *sod2*Δ (mitochondrial SOD), and *glr1*Δ (glutathione reductase) mutants showed increased sensitivity to this compound. Also sensitive was the gene deletion mutant, *vph2*Δ, for the vacuolar ATPase assembly protein, suggesting vacuolar detoxification plays an important role for fungal tolerance to SA. In chemosensitization experiments, co-application of SA with either antimycin A or strobilurin (inhibitors of mitochondrial respiration) resulted in complete growth inhibition of *Aspergillus* at much lower dose treatment of either agent, alone. Therefore, SA can enhance antifungal activity of commercial antifungal agents required to achieve effective control. SA is a potent antifungal and anti-aflatoxigenic volatile that may have some practical application as a fumigant.

Keywords Salicylaldehyde · *Aspergillus* · Antioxidation system · Mycotoxin · Antifungal · Chemosensitization

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Introduction

Resistance of pathogenic fungi to conventional antifungal drugs or fungicides is a widely recognized problem [1–3]. This problem necessitates the continuous development of new antifungal agents in addition to intensive efforts to discover mechanisms of resistance [4, 5]. Recent studies on a variety of fungal pathogens have demonstrated the effectiveness of natural compounds as antifungal or anti-mycotoxigenic agents. For example, naturally occurring phenols, such as vanillic and caffeic acid, inhibited the growth of fumonisin-producing *Fusarium verticillioides* [6]. Mycotoxin production by this organism was also inhibited by vanillic, caffeic, and ferulic

acids. Caffeic acid also effectively inhibited aflatoxin production by *Aspergillus flavus*, a fungal pathogen with medical and agricultural significance [7]. Modulation of the expression of fungal antioxidation gene(s) such as the alkyl hydroperoxide gene family has been postulated as the mechanism of anti-mycotoxigenicity of caffeic acid [7]. A number of benzo analogs were also effective as antimicrobials against several fungi and bacteria [8, 9]. Collectively, these studies demonstrated the potential for natural compounds to serve as potent antimicrobial and/or anti-mycotoxigenic agents.

Natural benzo analogs possess antifungal activity by targeting the oxidative stress-response system [10, 11], which is also a target of some commercial antimicrobial agents [12, 13]. Redox-active phenolics or sulfur-containing compounds can be potent redox-cyclers that inhibit microbial growth by interfering with cellular redox homeostasis and/or the function of redox-sensitive components [14–16]. In fungi, stress signals resulting from oxidative stress are integrated into mitogen-activated protein kinase (MAPK) pathways that eventually regulate genes countering/detoxifying the stress [17, 18]. In yeasts (*e.g.*, *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*), the Hog (high-osmolarity glycerol) MAPK system plays a key role in responding to oxidative stress [19]. Importantly, signal transduction and stress-response genes of fungal pathogens are also known to play roles for virulence and pathogenesis [20, 21]. Therefore, the antioxidative signal transduction and stress-response pathway(s) of fungi can potentially serve as a promising molecular target of redox-active agents for control of fungal pathogens.

Aflatoxins are hepato-carcinogenic difuranocoumarins produced most notably by *A. flavus* and *A. parasiticus*. Contamination by aflatoxins of food resources for human consumption has become a prominent international trade issue [22]. For example, in the United States, a maximum guidance level limit for tree nuts was set at 20 ppb [23]. The European Union has set lower threshold levels of 10 ppb for aflatoxin B_1 and 15 ppb for total aflatoxins [24]. These lower levels have a large impact on the exportability of certain U.S. crops. Therefore, methods are urgently needed to terminate fungal and mycotoxin contamination in food resources.

In this study, we described the use of salicylaldehyde (SA: 2-hydroxybenzaldehyde) as a volatile antifungal agent for the control of *A. flavus* and *A. parasiticus*. Noteworthy is that, in nature, SA is utilized as a natural antimicrobial agent by certain beetles [25]. Using gene deletion mutants of the model yeast *S. cerevisiae*, we identified the cellular antioxidation system as the molecular target of SA. The development and usage of SA as a potent chemosensitizing agent to the commercial inhibitors of mitochondrial respiration, *i.e.*, antimycin A or strobilurin, is also described.

Materials and Methods

Chemicals

Salicylaldehyde (SA) and antifungal drugs [antimycin A, strobilurin (Kresoxim-methyl)] were procured from Sigma–Aldrich (St. Louis, MO). Compounds were dissolved in dimethylsulfoxide (DMSO) before incorporation into media.

Microorganisms and Culture Condition

A. flavus NRRL3357 and *A. parasiticus* NRRL5862 were obtained from the National Center for Agricultural Utilization and Research, USDA-ARS, Peoria, IL. Fungi were grown at 28°C (5–7 days) on potato dextrose agar (PDA) medium. *S. cerevisiae* wild-type BY4741 (*Mat* a *his3*Δ1 *leu2Δ0 met15Δ0 ura3Δ0*) and selected gene deletion mutants were procured from Invitrogen (Carlsbad, CA) and Open Biosystems (Huntsville, AL), as follows: Gene regulation mutants: *yap1*Δ, *msn2*Δ, *msn4*Δ, *hot1*Δ, *sko1*Δ, *rim101*Δ; Transporter/assembly protein mutants: *flr1*Δ, *yor1*Δ, *pdr5*Δ, *vph2*Δ, *tfp1*Δ/*vma1*Δ; Signal transduction mutants: *sho1*Δ, *sln1*Δ, *ste50*Δ, *ste20*Δ, *ypd1*Δ, *ssk1*Δ, *ptp2*Δ, *ptp3*Δ, *hog1*Δ, *hog4*Δ, *ssk22*Δ, *ssk2*Δ, *ste11*Δ; Antioxidation mutants: *ctt1*Δ, *cta1*Δ, *osr1*Δ, *trr1*Δ, *trr2*Δ, *tsa1*Δ, *grx1*Δ, *grx2*Δ, *trx1*Δ, *trx2*Δ, *glr1*Δ, *gsh1*Δ, *gsh2*Δ, *sod1*Δ, *sod2*Δ, *ahp1*Δ; DNA damage control/energy metabolism mutants: *rad54*Δ, *sgs1*Δ, *acc1*Δ, *gpd1*Δ (Reference for the description of each deletion mutant: www.yeastgenome.org, accessed 27 April 2010). Yeast strains were grown on YPD (rich medium; 1% Bacto yeast extract, 2% Bacto peptone, 110 μM glucose) or SG (minimal medium; 0.67% Yeast nitrogen base w/o amino acids, 110 μM glucose with appropriate supplements: 180 μM uracil, 200 μM

amino acids) (Sigma–Aldrich, St. Louis, MO, USA) medium at 30°C (5–7 days).

Analysis of Aflatoxin

Spore suspensions (200 spores) were inoculated into the center of membrane filters (Poretics polycarbonate membrane filters, 0.2 μm pore size, 50 mm diameter; GE Osmonics, Minnetonka, MN) placed on top of the PDA. Cells were grown at 28°C for 5 days with or without SA (9.5 mM; Control: DMSO only).

Aflatoxin extracts were prepared by crushing fungal mats or agar media in 50 ml MeOH with a pestle, followed by filtration of a 1.0-ml aliquot through a 0.45-μm nylon syringe filter (Pall Co., Ann Arbor, MI). Extracts were analyzed for aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, AFG_2) using a HPLC system consisting of a degasser, autosampler, quaternary pump, and fluorescence detector (Agilent 1100, Santa Clara, CA) with injection volumes of 20 μl on a 4.6 × 250 mm Inertsil 5-μm ODS-3 column (GL Sciences, Torrance, CA) and a mobile phase consisting of $H_2O/CH_3CN/MeOH$ (45:25:30, v/v/v) at a flow rate of 1.0 ml/min. Fluorescence detection at 365 nm excitation and 455 nm emission was enhanced with "PHRED" post-column photochemical derivatization (Aura Industries, New York, NY). Aflatoxin standard solutions (AFB_1, AFB_2, AFG_1, AFG_2), used for the quantification of aflatoxins from salicylaldehyde-treated samples, were prepared as described in AOAC 971.22 [26]. HPLC quantification was linear in the range of 0.1–8.0 ng for AFB_1 and AFG_1 and 0.1–4.0 ng for AFB_2 and AFG_2, with retention times of 7.8 min for AFG_2, 8.7 min for AFG_1, 9.4 min for AFB_2, and 10.6 min for AFB_1.

Antifungal Bioassays

Sensitivities of filamentous fungi (*A. flavus* and *A. parasiticus*) to SA were measured based on percent radial growth of treated fungal colonies compared to control colonies, exposed only to DMSO [Vincent equation: % inhibition = 100 (C-T)/C, C: diameter of fungi on control plate; T: diameter of fungi on the test plate] [27]. Conidial suspensions (5×10^3 conidia/ml) were diluted in phosphate-buffered saline and spotted on the center of PDA plates (triplicates). SA was dissolved in DMSO (9.5–95 mM; final volume: 1 ml in DMSO) and placed next to the PDA plates inoculated with fungal spores. Fungal plates and SA were placed in the same sealed plastic container (739 ml volume; see Fig. 1a), rendering SA vapors directly contact fungal spores in a given space (vapor–agar contact method; [28]), and incubated at 28°C. The antifungal treatments, therefore, consist of: (1) control plates: *A. flavus* or *A. parasiticus* inoculum without SA vapor (DMSO only) and (2) treated plates: *A. flavus* or *A. parasiticus* inoculum with SA vapor. Results were means of three replicates. Growth was observed for 5–7 days.

To determine the chemosensitizing activity of SA, this volatile (12.0 or 13.5 mM) was placed next to the *Aspergillus*-inoculated PDA plates previously supplemented with antimycin A (10 μg/ml) or strobilurin (Kresoxim-methyl; 25 μM), which are inhibitors of mitochondrial respiration. Fungal radial growth was recorded as described above.

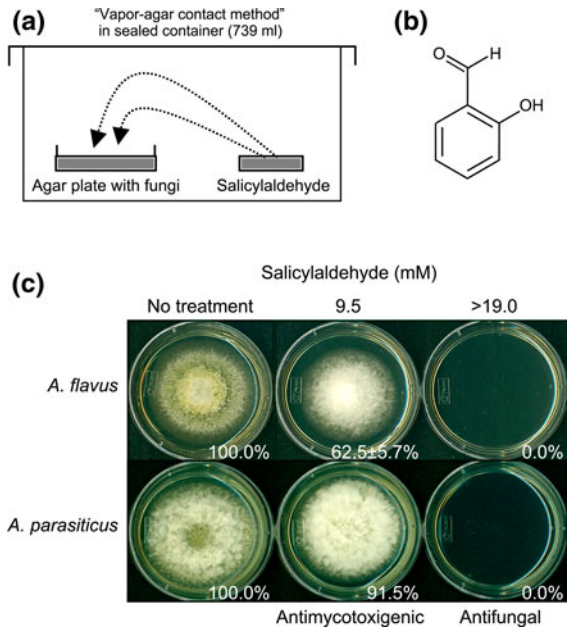

Fig. 1 Antifungal and anti-aflatoxigenic activity of salicylaldehyde (SA) against *Aspergillus flavus* and *A. parasiticus*. **a** Vapor–agar contact method showing how fungal cells were exposed to volatile SA. **b** Chemical structure of SA. **c** Effect of different concentrations of SA on fungal growth (see also Table 1). Note that 9.5 mM of SA, where spores lacked pigmentation, was used for anti-aflatoxigenic assays in this study

Sensitivities of gene deletion mutants of *S. cerevisiae* to SA (Concentrations: 0, 0.5, 0.6, 0.7, 0.8, 0.9 mM) were assessed by a yeast-cell dilution bioassay on SG agar as previously described [11]. Unlike the antifungal test in *Aspergillus*, SA was directly incorporated into the agar plate (absolute DMSO amount: <2% in media), and colony growth was monitored for 3–5 days.

Results and Discussion

Antifungal Activity of SA

Antifungal activity of SA was examined in *A. flavus* and *A. parasiticus* by vapor–agar contact method described by Sekiyama et al. [28] (Fig. 1a; see Fig. 1b for SA structure). The volatile molecules of SA were self-evaporated and distributed inside the sealed plastic container (739 ml), resulting in direct contact to fungal spores spotted on the PDA plates. At 9.5 mM $\leq$ SA $\leq$ 16.0 mM, the radial growth of *A. parasiticus* was gradually decreased (*i.e.*, Vincent equation: 10–75%; Table 1) as the concentrations of SA increased. Similar trends were also observed with *A. flavus* at 9.5 mM $\leq$ SA $\leq$ 13.5 mM (*i.e.,* Vincent equation: 35–87%; Table 1). Coinciding with this growth inhibition was the decrease in the formation of green pigments in fungal spores, leading to the development of pale colonies (Fig. 1c; at 9.5 mM SA). This result indicates that SA also has an inhibitory effect on the secondary metabolism for fungal pigment formation. At 16.0 mM $\leq$ SA or 19.0 mM $\leq$ SA, the growths of *A. flavus* or *A. parasiticus*, respectively, were completely inhibited, resulting in no colony formation on PDA plates (Table 1). Therefore, results proved that SA could act as an effective antifungal agent, where the level of antifungal activity was commensurate with SA concentrations applied.

SA Inhibits Aflatoxin Production in *A. flavus* and *A. parasiticus*

We then tested the anti-mycotoxigenic activity of SA in *A. flavus* and *A. parasiticus*. As mentioned earlier, volatilized SA could inhibit the development of green pigments in fungal spores. Considering that fungal pigments are synthesized by secondary metabolism in cells, we reasoned that SA could also inhibit the secondary metabolic pathway for aflatoxin production in *Aspergillus*.

As shown in Table 2, the aflatoxin production in SA-exposed fungi was reduced by $\sim$13–45% compared with the untreated control, depending on the types of fungi and/or types of aflatoxins synthesized. *A. flavus* produces AFB_1/B_2 only, while *A. parasiticus* produces AFG_1/G_2 as well as AFB_1/B_2. As expected, we did not detect AFG_1/G_2 in the culture of *A. flavus*, whereas the production of AFG_1/G_2 in *A. parasiticus* was reduced by 23–32% with the treatment of SA. These findings demonstrated that volatilized SA possesses both antifungal and anti-mycotoxigenic activities in *A. flavus* and *A. parasiticus*.

Target Identification: Sensitive Responses of *S. cerevisiae* Mutants Lacking Genes in Oxidative Stress-Response System and Vacuolar Function

Strains of yeast have been systematically developed where each open reading frame was functionally deleted, allowing for phenotypic characterization of individual genes [29]. Use of these deletion mutants of *S. cerevisiae* can serve to identify gene targets of chemicals or drugs [30]. A number of stress-response pathways, including oxidative stress, have also been well characterized in *S. cerevisiae* [19]. We present in this study the bioassays using *S. cerevisiae* as a model fungal system, which allows us to configure the mechanism of action of SA for its antifungal and/or

Table 1 Antifungal activity of salicylaldehyde (SA) volatilized in a sealed plastic container (739 ml)

SA conc. (mM)	0.0	9.5	10.5	12.0	13.5	16.0	19.0–95.0
Strains							
A. flavus	100.0	65.2 ± 5.7	60.9 ± 6.5	43.5 ± 5.7	13.0	0.0	0.0
A. parasiticus	100.0	91.5	76.6	72.3	51.1	25.5 ± 7.4	0.0

Numbers are in %, which is based on the Vincent equation (See Materials and Methods). SD < 5% except where noted

Table 2 Anti-aflatoxigenic activity of salicylaldehyde (SA; 9.5 mM)

Strains	SA (mM)	Partition	AFB_1	AFB_2	AFG_1	AFG_2
A. flavus 3357	0	Fungal mat (retained)	171.6 ± 16.6	3.7 ± 0.5	ND[a]	ND
		Media (secreted)	326.5 ± 42.5	11.8 ± 1.8		
		Total	498.1 ± 56.3 (100%)	15.5 ± 2.2 (100%)		
	9.5	Fungal mat (Retained)	148.3 ± 8.5	3.1 ± 0.2	ND	ND
		Media (secreted)	283.2 ± 25.2	10.0 ± 1.0		
		Total	431.5 ± 32.4 (86.6%)[b]	13.1 ± 1.1 (84.5%)		
A. parasiticus 5862	0	Fungal mat (retained)	93.3 ± 5.5	1.4 ± 0.2	42.5 ± 4.8	1.2 ± 0.2
		Media (secreted)	148.9 ± 2.8	4.0 ± 0.2	123.1 ± 4.8	2.8 ± 0.1
		Total	242.2 ± 3.2 (100%)	5.4 ± 0.3 (100%)	165.6 ± 9.3 (100%)	4.0 ± 0.2 (100%)
	9.5	Fungal mat (retained)	60.9 ± 16.1	0.8 ± 0.3	29.8 ± 5.9	0.8 ± 0.3
		Media (secreted)	112.8 ± 29.4	2.5 ± 0.9	98.0 ± 21.1	1.9 ± 0.4
		Total	173.7 ± 45.4 (71.7%)	3.3 ± 1.2 (54.8%)	127.8 ± 26.8 (77.2%)	2.7 ± 0.7 (67.5%)

Unit of aflatoxins produced: μg aflatoxin/100 mg dried fungal mat

[a] ND = Not detectable

[b] Treated percentages in parentheses are relative to control percentages

anti-mycotoxigenic activity in a relatively genetically uncharacterized fungi such as *A. flavus* and *A. parasiticus*. We recently confirmed structural homology of several signal transduction and antioxidative stress-response genes between *S. cerevisiae* and *A. flavus* [31].

Using gene deletion mutants of *S. cerevisiae* where the genes in gene regulation, signal transduction, antioxidation and DNA damage control/energy metabolism were systematically deleted, we performed yeast dilution bioassays on SG agar containing SA (see Materials and Methods). We observed the sensitive responses of the downstream antioxidation gene mutants, *i.e.*, *sod1*Δ [cytosolic superoxide dismutase (SOD) gene deletion], *sod2*Δ [mitochondrial SOD (Mn-SOD) gene deletion], and *glr1*Δ (glutathione reductase gene deletion) to SA, while none of the mutants for the upstream signal transduction pathway, *e.g.*, *yap1*Δ, controlling downstream antioxidation system, were sensitive to the same treatment (Fig. 2a). Sod2p and Sod1p detoxify mitochondrial and cytosolic superoxide radicals, respectively. *GLR1* encodes glutathione reductase, which converts GSSG (oxidized glutathione) to GSH (reduced glutathione: antioxidant). In *S. cerevisiae*, *GLR1* is a target gene of the Hog1p (MAPK) pathway [32], for replenishing GSH from GSSG. However, the *glr1*Δ mutant cannot reduce GSSG to GSH efficiently, resulting in higher sensitivity to SA. Our use of *S. cerevisiae* deletion mutants shows that SA targets *SOD1* and *SOD2*, and *GLR1* and *VPH2*. The particular hypersensitivity of these mutants, relative to all the others tested, suggests there is a high likelihood the mode of action of SA is through disruption of oxidative stress and detoxification responses; which can be verified with further complementation experiments in the future. Collectively, we hypothesized that the mechanism of antifungal action of SA is by targeting the downstream functional/structural genes in the antioxidation system (see also Fig. 2b).

In addition, the *vph2*Δ mutant was also sensitive to SA (Fig. 2a, b). The gene *VPH2* encodes a vacuolar ATPase (V-ATPase) assembly protein, and the

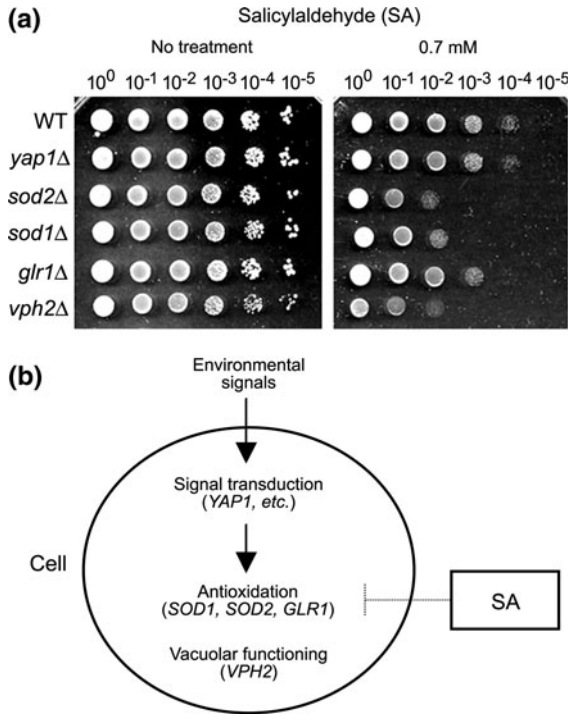

Fig. 2 **a** Yeast dilution bioassays showing sensitive responses of *Saccharomyces cerevisiae* gene deletion mutants (*sod2*Δ, *sod1*Δ, *glr1*Δ, *vph2*Δ) to SA (representative result shown from 0.7 mM treatment). Note that *yap1*Δ, lacking the *YAP1* gene controlling downstream antioxidation system, was not sensitive to the same treatment. **b** Diagrammatic representation of gene targets tested in this study showing the downstream antioxidation system was affected by SA

*vph2*Δ mutant has dysfunctional vacuolar and/or mitochondrial respiration resulting from intracellular fluctuations of pH [33]. In fungi, vacuolar compartmentalization of toxic substances (*e.g.*, xenobiotics through vacuolar transporters and ATPase) is a well-known mechanism for detoxification [34–36]. Acidification mediated by V-ATPases is also necessary for the accumulation of ions and metabolites in the vacuoles [37]. Since the *vph2*Δ mutant lacks the V-ATPase assembly protein, its cells are likely to be dysfunctional in transporting toxic compounds, such as SA, into vacuoles.

Chemosensitization of SA to Antimycin A or Strobilurin (Inhibitors of Mitochondrial Respiration)

Chemosensitization refers to the ability to synergistically enhance effectiveness of antimicrobial agents by co-applying a second compound. With regard to fungi, the second compound (chemosensitizing agent) debilitates the ability of the fungus to exert a protective/tolerant response to commercial, or other, antifungal agents. Such chemosensitizing agents can affect common cellular targets, such as the antioxidation system of fungi, which results in synergistic inhibition of fungal growth. In this study, the chemosensitizing effect of SA to antimycin A or strobilurin, both inhibitors of complex III of the mitochondrial respiratory chain which disrupts energy production [38], was tested. Coinciding with this disruption is an abnormal release of electrons that additionally damages cellular components by oxidative stress [39]. Therefore, antioxidation enzymes such as Mn-SOD play important roles in protecting cells from such oxidative damage.

As shown in Fig. 3, co-application of antimycin A (10 μg/ml) or strobilurin (Kresoxim-methyl, 25 μM) with volatilized SA (as low as 13.5 mM) completely inhibited the colony formation of *A. flavus* and *A. parasiticus*, while independent treatment of each reagent at the given concentrations still allowed the growth of *Aspergillus*. Thus, results demonstrated the efficacy of chemosensitization strategy by using volatile SA for enhancing activities of antimycin A or strobilurin. Considering the serious mammalian cytotoxicity caused by antimycin A (through the intra-cellular production of reactive oxygen species; [39]), and also the increased development of fungal resistance to strobilurin (http://www.frac.info, accessed 19 April 2010; target sites for mutations in various fungi are G143A, F129L, and additional mechanisms), our chemosensitization approach will, thus, advance effective control of fungal pathogens having either medical or agricultural importance.

We presented the potential for using SA as antifungal and/or anti-mycotoxigenic agent, and especially as a chemosensitizing agent. As shown in this study, SA can enhance activity, lower resistance, and alleviate health and environmental risks by reducing amounts of commercial antifungal agents required to achieve effective therapy and/or control. Further in vivo studies are necessary to determine whether the in vitro activities demonstrated herein can translate to in vivo treatment efficacy and safety. The use of SA has promising implications for medicine, especially in the chemotherapy of invasive fungal diseases of the lung, and for agriculture, as a

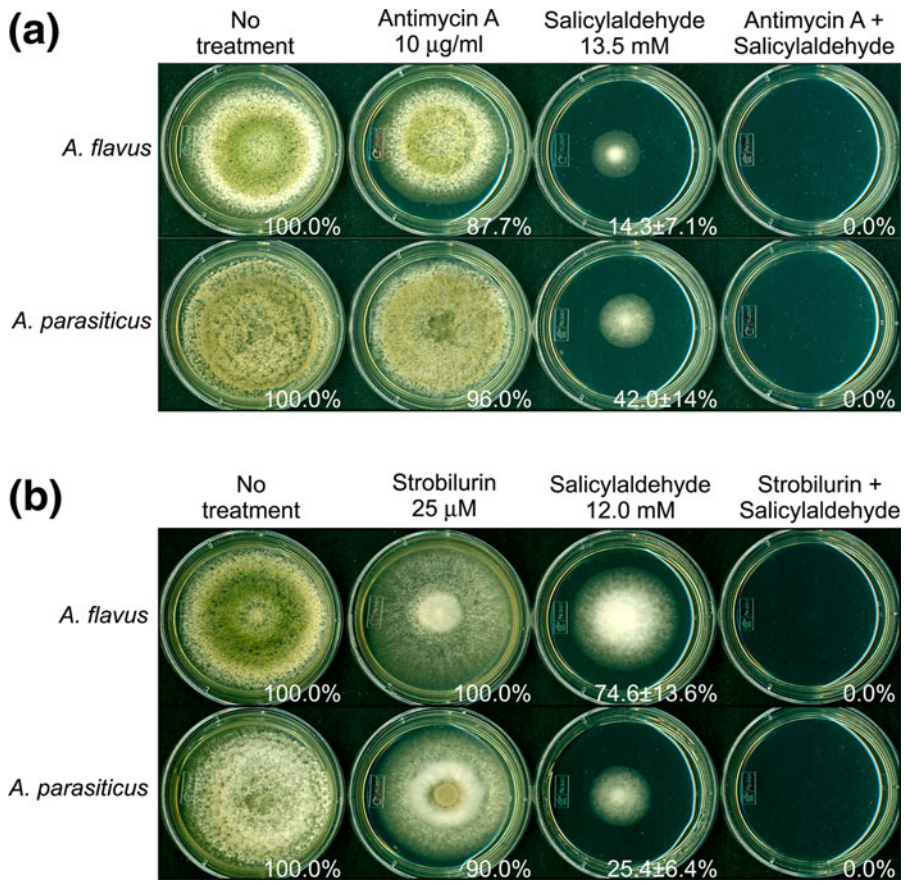

Fig. 3 Chemosensitizing activity of SA to inhibitors of mitochondrial respiration complex III [*i.e.*, **a** Antimycin A (*upper panel*) or **b** Strobilurin (*lower panel*)], resulting in complete inhibition of fungal growth by co-application

safe, natural fumigant to inhibit fungal growth and/or mycotoxin production in crops, or crop products, under storage.

Acknowledgments This research was conducted under USDA-ARS CRIS Project 5325-42000-035-00D. We thank the Almond Board of California for partially funding this research (Agreement No. 58-5325-9-156).

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