

ORIGINAL ARTICLE

Enhanced activity of strobilurin and fludioxonil by using berberine and phenolic compounds to target fungal antioxidative stress response

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Keywords

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Abstract

Aims: Identify natural products that effectively target antioxidative signal transduction/stress response systems [i.e., mitogen-activated protein kinase (MAPK) pathway, mitochondrial superoxide dismutase (Mn-SOD)] of fungi. Enhance activity of strobilurin or fludioxonil with discovered compounds.

Methods and Results: Enhancement of antifungal activity of strobilurins, inhibitors of complex III of the mitochondrial respiratory chain, was tested using berberine hemisulfate and different phenolic compounds. The *Saccharomyces cerevisiae sod2Δ*, a deletion mutant lacking Mn-SOD gene, was highly sensitive to berberine and veratraldehyde. Functional complementation analysis verified these compounds target Mn-SOD. Activity of strobilurin (25–50 μmol l⁻¹) was elevated on most aspergilli and *Penicillium expansum* by co-application with berberine or veratraldehyde (2–4 mmol l⁻¹). These compounds also prevented *Aspergillus fumigatus* MAPK mutants (*sakAΔ* and *mpkCΔ*) from escaping toxicity of fludioxonil (a phenylpyrrole fungicide potentiated by the MAPK pathway), a typical phenotype of fungal MAPK mutants.

Conclusions: Strobilurin activity or prevention of fungal escape from fludioxonil toxicity can be enhanced by co-application of certain alkaloids or phenolics. **Significance and Impact of the Study:** Natural products can be used to target cellular stress response systems in fungal pathogens and serve as alternatives/additives to commercial antifungal agents.

Introduction

Natural compounds can serve as alternatives or leads to conventional or new antimicrobials, respectively (Cos *et al.* 2006; Pauli 2006). For example, the alkaloid berberine, and its derivatives, possesses antibiotic activity against a variety of micro-organisms, including *Staphylococcus* (Stermitz *et al.* 2000; Yu *et al.* 2005), *Salmonella* (Wu *et al.* 2005) and *Candida* (Quan *et al.* 2006). Many natural phenolic compounds also have antifungal activities (Tawata *et al.* 1996; Florianowicz 1998; D'Auria *et al.* 2001; Beekrum *et al.* 2003). Phenolics can also act as antioxidants under oxidative stress conditions, but are potent

inhibitors of redox homeostasis (Guillen and Evans 1994; Shvedova *et al.* 2000), and disrupt cell growth when applied above antioxidant levels (Kim *et al.* 2006c).

The molecular target for strobilurin-related fungicides is the mitochondrial respiratory complex III (ubiquinol-cytochrome *c* oxidoreductase, EC 1.10.2.2). Strobilurins specifically bind to the Q_P (Q_O) centre of cytochrome *b* (Zheng *et al.* 2000; Hnatova *et al.* 2003), inhibiting mitochondrial respiration. This inhibition results in cellular oxidative stress triggered by electrons escaping from the respiratory chain, which can be detoxified by mitochondrial superoxide dismutase (Mn-SOD). Fludioxonil, a phenylpyrrole fungicide, hinders the protein kinase in

glycerol biosynthesis (Rosslenbroich and Steubler 2000), resulting in growth inhibition of pathogens (Ochiai *et al.* 2002). However, as mutants with defects in mitogen-activated protein kinase (MAPK) genes are tolerant to phenylpyrrole fungicides (Kojima *et al.* 2004, 2006), new approaches are needed to curtail the potential development of tolerance to this fungicide.

Many genes in *Saccharomyces cerevisiae* are orthologs of genes of fungal pathogens (Krantz *et al.* 2006). As such, *S. cerevisiae* has been used for identifying target genes of antifungal compounds (Parsons *et al.* 2004). Recently, stress response systems of fungi were recognized as potential targets for antifungal compounds (Smits and Brul 2005; Jaeger and Flohe 2006). We present how targeting the fungal antioxidative signal transduction/stress response systems with redox-active phenolics and the alkaloid berberine hemisulfate elevate fungicidal effectiveness of fludioxonil or strobilurin.

Materials and methods

Micro-organisms and culture conditions

Aspergillus fumigatus AF293 (wild type) and *A. fumigatus* *sakA*Δ and *mpkC*Δ (MAPK deletion mutants) (Xue *et al.* 2004; Reyes *et al.* 2006) were grown at 37°C on potato dextrose agar (PDA). *Aspergillus flavus* NRRL3357, *A. parasiticus* NRRL5862, *A. niger* NRRL326, *A. oryzae* FGSC A815, *A. ochraceus* NRRL 5175, *A. nidulans* A4 and *Penicillium expansum* NRRL 974 were cultured at 28°C on PDA. *Saccharomyces cerevisiae* wild type BY4741 (*Mat* a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and deletion mutants were obtained from Invitrogen (Carlsbad, CA, USA) and Open Biosystems (Huntsville, AL, USA) as follows (See also Kim *et al.* 2005): Gene regulation mutants: *yap1Δ msn2Δ, msn4Δ, hot1Δ, sko1Δ, rim101Δ, ure2Δ*; Transporter/assembly protein mutants: *flr1Δ, yor1Δ, pdr5Δ, vph2Δ, tfp1Δ/vma1Δ*; Signal transduction mutants: *sho1Δ, sln1Δ, ste50Δ, ste20Δ, ypd1Δ, ssk1Δ, ptp2Δ, ptp3Δ, hog1Δ, hog4Δ, ssk22Δ, ssk2Δ, ste11Δ*; Antioxidation mutants: *ctt1Δ, cta1Δ, osr1Δ, trr1Δ, trr2Δ, tsal1Δ, grx1Δ, grx2Δ, trx1Δ, trx2Δ, glr1Δ, gsh1Δ, gsh2Δ, sod1Δ, sod2Δ, ahp1Δ*; DNA damage control/energy metabolism mutants: *rad54Δ, sgs1Δ, acc1Δ, gpd1Δ, hor2Δ* (See <http://www.yeastgenome.org> for the description of each deletion mutant; accessed 12 December 2006). Yeast culture media were Yeast extract/Peptone/Dextrose (YPD; 1% Bacto yeast extract, 2% Bacto peptone, 110 μmol l⁻¹ glucose) or minimal medium (SG; 0.67% Yeast nitrogen base w/o amino acids, 110 μmol l⁻¹ glucose with appropriate supplements: 180 μmol l⁻¹ uracil, 200 μmol l⁻¹ amino acids), and cells were grown (5–7 days) at 30°C without light (See below, 'yeast complementation bioassay,' for other test media).

Chemicals and reagents

Fludioxonil, strobilurin (kresoxim-methyl), berberine hemisulfate, and phenolic agents vanillin (4-hydroxy-3-methoxybenzaldehyde), vanillylacetone (3-methoxy-4-hydroxybenzyl acetone), cinnamic acid (*trans*-3-phenylacrylic acid), *m*-coumaric acid (3-hydroxycinnamic acid), and veratraldehyde (3,4-dimethoxybenzaldehyde) were purchased from Sigma Co. (St Louis, MO, USA). Phenolic compounds were dissolved in dimethyl sulfoxide (DMSO; absolute amount <20 μl ml⁻¹ media) and berberine hemisulfate was dissolved in water before use.

Antifungal activity of compounds

Antifungal sensitivity of filamentous fungi was determined by harvesting spores from stock cultures and adjusting their concentration using phosphate-buffered saline (PBS). Spores (approx. 200) were then spotted onto the centre of PDA plates containing either berberine and/or test phenolic compounds w/ or w/o test fungicides (See Table and Figures for concentrations). Growth or germination of fungi on plates was monitored after 5–7 days. Berberine and/or phenolics were co-added with fludioxonil or strobilurin to media to test enhanced activity. Activity was measured by per cent difference in radial growth of fungal colonies grown on treated media compared with control colonies grown on PDA (receiving DMSO only).

Yeast responses to treatments were monitored using a cell dilution bioassay. This assay involved a series of five, sequential tenfold dilutions using SG liquid medium of a starting concentration of approx. 1 × 10⁶ cells, from cultures grown on YPD. Each dilution was spotted adjacently on SG agar plates containing treatment compounds. These spots provided six samples of approx. 10⁶–10 cells to determine sensitivity to treatments. Results were scored based on a designated value of the highest dilution, where a colony became visible after 5–7 days of incubation. Scores ranged from '0', highest antifungal activity, to '6', lowest activity, as follows: if no colonies were visible from any of the dilutions, the score was '0'; colonies visible in spots from all dilutions, the score was '6'; a colony visible from only the undiluted cell-spot (10⁶ cells), the score was '1', etc. Thus, each unit of numerical difference between yeast strains was equivalent to a tenfold difference in their respective sensitivity to a treatment.

A. flavus *sodA*/*S. cerevisiae* *sod2Δ* complementation bioassay

We examined if complementation of the *sod2Δ* yeast with the orthologous *sodA* gene from *A. flavus* (encodes

Mn-SOD; GenBank accession# AY585205) reversed hypersensitivity in the *sod2Δ* strain to berberine hemisulfate and phenolics. This complementation bioassay was carried out as described previously to examine effects of compounds on Mn-SOD (Kim *et al.* 2005). *Saccharomyces cerevisiae sod2Δ* with pYES2 empty vector (*sod2Δ* + pYES2; negative control; Invitrogen), wild type with pYES2 empty vector (WT + pYES2; positive control), wild type with pYES2 vector containing PCR-amplified *soda*, the *A. flavus* Mn-SOD gene (WT + *soda*; Mn-SOD overexpression), and *sod2Δ* with pYES2 vector containing PCR-amplified *soda* (*sod2Δ* + *soda*; functional complementation) were cultured in raffinose medium (*GAL1* noninducing medium; 0.67% Yeast nitrogen base without amino acids, 110 $\mu\text{mol l}^{-1}$ raffinose, 200 $\mu\text{mol l}^{-1}$ amino acids) at 30°C. Yeast cells were serially diluted as described above with raffinose liquid medium and spotted adjacently on SGAL (*GAL1* inducing medium; 0.67% Yeast nitrogen base w/o amino acids, 110 $\mu\text{mol l}^{-1}$ galactose, 200 $\mu\text{mol l}^{-1}$ amino acids). Functional expression of *soda* was achieved under induction of the yeast *GAL1* promoter (30°C, 10 days). Functionality of *soda* was assessed in the presence of berberine hemisulfate and phenolic compounds using the yeast cell dilution bioassay and the scoring system of visible colony growth, described above. If the dilution showing yeast growth was similar to the positive control or better than the negative control, *soda* was considered to have functionally complemented *sod2Δ*. Reduced sensitivity after complementation would signify test compounds targeted the yeast Mn-SOD, and thus, oxidative stress response system.

Results

Preliminary experiments examining the effect of berberine hemisulfate on growth of 46 signal transduction and anti-oxidation mutants of *S. cerevisiae* (See section 'Materials and methods') showed that the *sod2Δ* mutant, deleted Mn-SOD gene, was approx. ten times more sensitive than the wild type strain (Growth scores in yeast dilution bioassay: *sod2Δ*, 5 to 3 vs wild type, 6 to 4 at 1–3.5 mmol l⁻¹ berberine, respectively; Figure data not shown). This finding suggested Mn-SOD, one of the downstream targets of the MAPK pathway, is important for fungal tolerance to berberine hemisulfate.

The *sod2Δ* mutant was previously found to be sensitive to a number of phenolic compounds (See Kim *et al.* 2006a). In the study presented, here, of the phenolic compounds [vanillylacetone (10 mmol l⁻¹), vanillin (1 mmol l⁻¹), veratraldehyde (5 mmol l⁻¹), cinnamic acid (0.1 mmol l⁻¹) and *m*-coumaric acid (5 mmol l⁻¹)] co-applied with berberine, veratraldehyde and vanillylacetone were the most active, enhancing hypersensitivity >10⁴

times in wild type and *sod2Δ* strains (Dilution bioassay; Fig. 1a). This confirmed berberine and veratraldehyde (also vanillylacetone, which needed about a twofold higher concentration than veratraldehyde) affected a common cellular target, Mn-SOD. Each compound, at the given concentration, did not affect yeast cell growth when applied individually.

The *soda/sod2Δ* complementation assay (See also Kim *et al.* 2005) showed that Mn-SOD plays a role in tolerance to berberine hemisulfate and phenolics. The *sod2Δ* mutant overcame berberine- and phenolic-mediated growth inhibition by functional expression of *Aspergillus soda*. Co-application of berberine hemisulfate and veratraldehyde or vanillylacetone greatly reduced yeast survivability in all strains, including the wild type, compared with individual treatment of these compounds (Fig. 1b). The *sod2Δ*, complemented with the functional *soda* gene (*sod2Δ-soda*), had the same level of growth as the wild type when exposed to berberine and the phenolics. The *sod2Δ* with the empty vector, however, was 10–100 times more sensitive to these compounds. The *sod2Δ-soda* complementation also resulted in greater tolerance to individual treatments of veratraldehyde or vanillylacetone. However, over-expression of Mn-SOD (wild type + *soda*) had no effect on hypertolerance.

The antifungal interaction between berberine hemisulfate (0.5, 1 mmol l⁻¹) and veratraldehyde (2.5, 5 mmol l⁻¹), compounds having a common cellular target, Mn-SOD, on several different aspergilli and *P. expansum* showed that germination was completely inhibited in *A. fumigatus*, *A. parasiticus*, *A. oryzae* and *A. nidulans* at 0.5 mmol l⁻¹ berberine (1 mmol l⁻¹ for *A. niger*) with 5 mmol l⁻¹ veratraldehyde (Table 1; 2.5 mmol l⁻¹ for *A. fumigatus* and *A. nidulans*). However, growth of *A. flavus*, *A. ochraceus* and *P. expansum* was reduced, but not completely inhibited at the highest doses tested of both compounds (Table 1), reflecting different level of sensitivities of fungi to the treatments. Sensitivity was designated as either 's' (susceptible) if the growth level of the individual fungus was < the overall mean response of all eight fungi to the treatment. A designation of 'l' (less susceptible) meant that growth of the individual fungus was ≥ overall mean (Table 1). Trends also indicated the higher the concentrations of natural compounds in treatments, the higher the rate of growth inhibition (See Table 1), further demonstrating 'positive-interaction' of the compounds for antifungal activity.

Treating *A. fumigatus* MAPK mutants (*sakAΔ*, *mpkCΔ*) with berberine (as low as 0.5 mmol l⁻¹) prevented development of tolerance to fludioxonil (Fig. 2). Development of fludioxonil tolerance is a phenotype of these MAPK mutants. Supplementation with veratraldehyde, instead of berberine, also resulted in overcoming the tolerance to

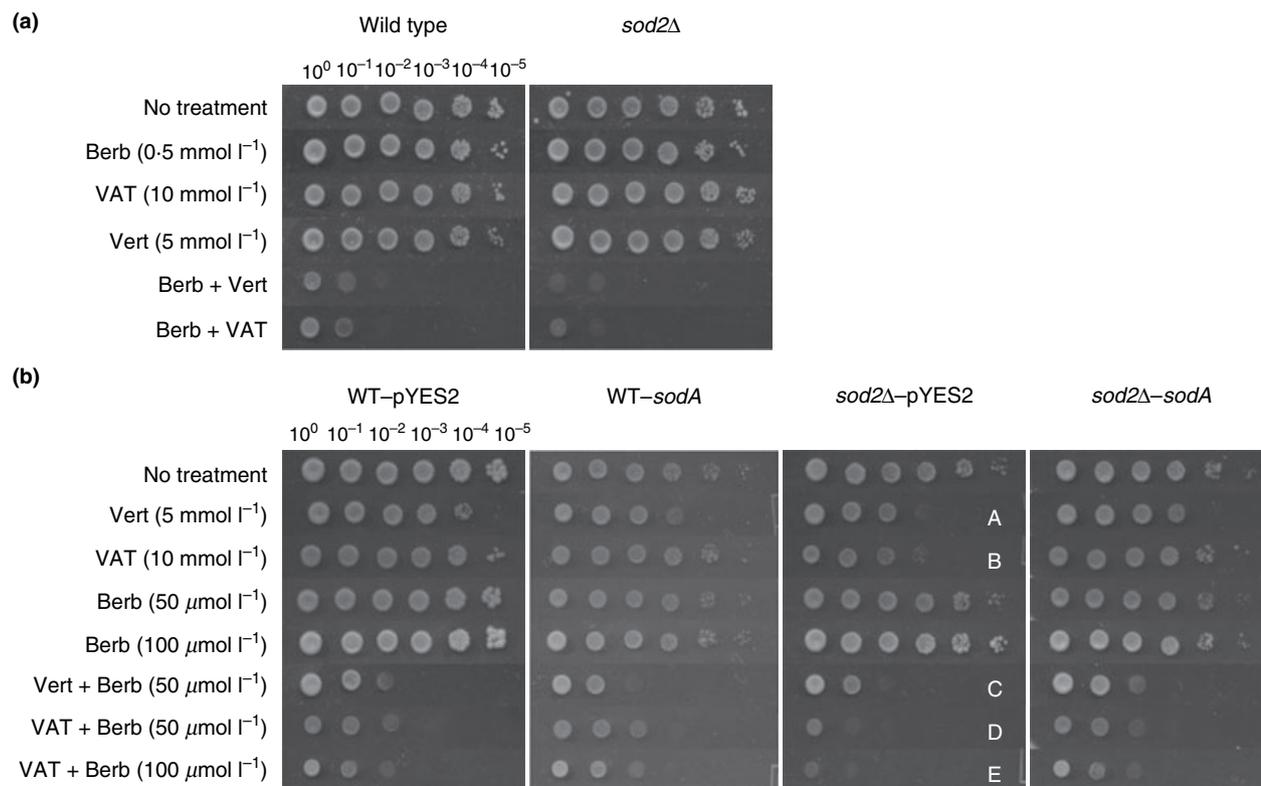


Figure 1 (a) Yeast bioassays showing berberine hemisulfate (0.5 mmol l⁻¹), veratraldehyde (5 mmol l⁻¹) and vanillylacetone (10 mmol l⁻¹) negatively affect the function of the common molecular target; i.e., Mn-SOD. Two replicates were monitored for the test. (b) Functional complementation assay of the Mn-SOD gene from *Aspergillus flavus* (*sodA*), using the vector pYES2, in yeast wild type (WT) or *sod2Δ*, lacking the orthologous gene. Assay shows *sod2Δ*-pYES2 (no Mn-SOD + empty vector) is 10–100 times more sensitive to 5 mmol l⁻¹ veratraldehyde (Vert) (A) and 10 mmol l⁻¹ vanillylacetone (VAT) (B) and to combined treatments with berberine (Berb) (C, D and E) than complemented *sod2Δ*-*sodA* and wild type (WT) strains. Two replicates were monitored for the test.

fludioxonil. However, a much higher concentration (≥ 5 mmol l⁻¹) was required for veratraldehyde than berberine, further indicating higher sensitivity of *A. fumigatus* to berberine than to veratraldehyde.

In co-application experiments of berberine with strobilurin, which inhibits mitochondrial respiration, *A. fumigatus* and *A. nidulans* were hypersensitive. Neither of these fungi germinated at 2 mmol l⁻¹ berberine and 25 μmol l⁻¹ strobilurin. While growth of *P. expansum* was not affected as greatly as that of the aspergilli (Fig. 3a). Combined application of veratraldehyde with strobilurin (as low as 25 μmol l⁻¹) completely inhibited the growth of *A. fumigatus* (4 mmol l⁻¹) and *A. nidulans* (2 mmol l⁻¹). The growth of other fungi was completely inhibited with 2–4 mmol l⁻¹ veratraldehyde and 25 μmol l⁻¹ strobilurin, including *A. ochraceous* (4 mmol l⁻¹), *A. parasiticus* (3 mmol l⁻¹), *A. oryzae* (3 mmol l⁻¹) and *A. niger* (2 mmol l⁻¹) (Figure data not shown). Whereas, growth of *P. expansum* was only partially inhibited at 4 mmol l⁻¹ veratraldehyde and 25 μmol l⁻¹ strobilurin (Fig. 3b). Of all fungi tested,

A. flavus showed the highest tolerance in co-application of veratraldehyde or berberine with strobilurin, with <30% growth inhibition at 4 mmol l⁻¹ berberine + 100 μmol l⁻¹ strobilurin; ≤40% growth inhibition with 4 mmol l⁻¹ veratraldehyde + 100 μmol l⁻¹ strobilurin. In summary, *A. fumigatus* showed higher sensitivity to berberine than to veratraldehyde (i.e., concentrations for no germination: 4 mmol l⁻¹ berberine, only, or 2 mmol l⁻¹ berberine vs 4 mmol l⁻¹ veratraldehyde, when combined with 25 μmol l⁻¹ strobilurin; See Fig. 3a,b). *Aspergillus nidulans* showed similar levels of sensitivity to co-application of either compounds (i.e., treatment with 2 mmol l⁻¹ berberine or veratraldehyde resulted in no germination when combined with 25 μmol l⁻¹ strobilurin; Fig. 3). Other aspergilli (*A. ochraceous*, *A. parasiticus*, *A. oryzae*, *A. niger*, *A. flavus*) and *P. expansum* were more sensitive to veratraldehyde than to berberine (See description above). The differential responses of fungi to these treatments further demonstrated a 'fungal strain compound' specificity for the antifungal activity of test compounds.

Table 1 Relative growth of aspergilli and *P. expansum* treated with varying amounts of veratraldehyde or berberine hemisulfate, individually or in combination*

	Veratraldehyde 0 (mmol l ⁻¹)								
	0			2.5			5		
Berberine (mmol l ⁻¹)	0	0.5	1.0	0	0.5	1.0	0	0.5	1.0
<i>A. fumigatus</i>	100	60† s	56 s	100 /	0 s	0 s	94 /	0 s	0 s
<i>A. flavus</i>	100	98 /	94 /	98 /	94 /	83 /	90 /	73 /	67 /
<i>A. parasiticus</i>	100	96 /	92 /	90 s	76 /	60 /	72 /	0 s	0 s
<i>A. ochraceous</i>	100	85 /	81 /	88 s	73 /	63 /	69 s	50 /	48 /
<i>A. oryzae</i>	100	89 /	83 /	87 s	62 /	51 s	70 s	0 s	0 s
<i>A. niger</i>	100	80 s	78 s	106 /	84 /	78 /	80‡ /	64§ /	0 s
<i>A. nidulans</i>	100	76 s	64 s	84 s	0 s	0 s	0 s	0 s	0 s
<i>P. expansum</i>	100	85 /	81 /	100 /	81 /	74 /	89 /	59 /	52 /
Mean value (eight fungi)	100	83.6	78.6	94.1	58.7	51.1	70.5	30.7	20.8

*Fungal growth is presented as a percentage of radial growth compared with control colonies grown on PDA plates receiving only DMSO. Values are means of three replicates. SDs of all measurements are <5%, except where noted. The mean values of all treatments were analysed using single-factor ANOVA, and a *P* value of <0.05 was considered significant. *s*, susceptible (<mean value from eight fungi for each treatment); *l*, less susceptible (≥mean value from eight fungi for each treatment).

†SD = 17.

‡SD = 27.

§SD = 13.

Discussion

Berberine hemisulfate and certain phenolics inhibit fungal growth, in part, by disrupting cellular redox homeostasis.

These compounds impede mitochondrial function by disrupting Mn-SOD activity, which, in turn, hinders cellular management of escaped electrons from the respiratory chain. As a result, there is an elevation in oxidative stress and inhibition of fungal growth. The phenolic vanillylacetone (Kim *et al.* 2006b) inhibits growth of *S. cerevisiae tsa1Δ* under oxidative stress (i.e., hydrogen peroxide). Thioredoxin peroxidase (cTPxI), encoded by *TSA1*, is essential for antioxidative defence of yeast having dysfunctional mitochondria (Demasi *et al.* 2001). The cTPx I gene deletion (*tsa1Δ*) renders cells hypersensitive to H₂O₂-induced oxidative stress compared with the wild type strain (Demasi *et al.* 2001). In our test, treatment of *tsa1Δ* with berberine and veratraldehyde also results in growth inhibition under oxidative stress conditions (Figure data not shown). The effect of these compounds is similar to antimycin A or strobilurin, fungicides inhibiting complex III in the mitochondrial respiratory chain (Slater 1973; Zheng *et al.* 2000). The inhibition of the cellular stress response system by berberine was previously shown in the fission yeast, *Schizosaccharomyces pombe* (Jang *et al.* 2002), where berberine treatment disrupted MAPK kinase (Wis1p) of the stress-activated protein kinase (SAPK) pathway.

This study shows that strobilurin antifungal activity is enhanced, and escape from fludioxonil toxicity is prevented, by targeting genes downstream of the MAPK pathway. The MAPK pathway triggers downstream antioxidative stress responses, such as *SOD2* in *S. cerevisiae* (Boy-Marcotte *et al.* 1998). By targeting Mn-SOD with berberine or certain phenolic compounds and co-applying

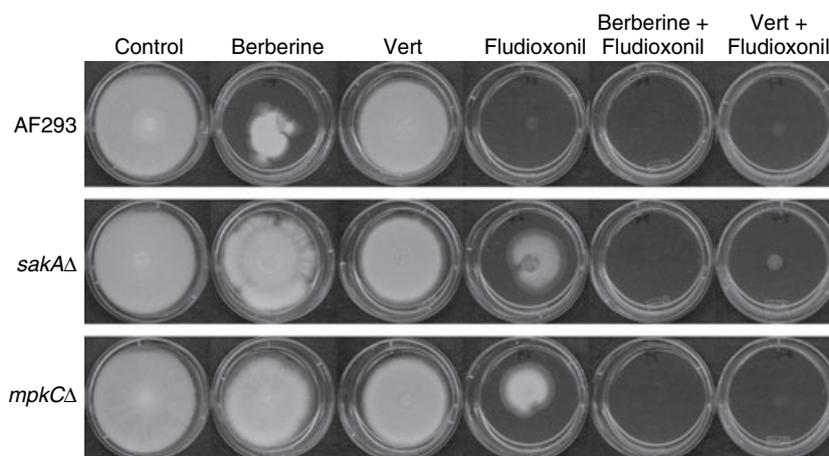


Figure 2 Bioassays of *Aspergillus fumigatus* AF293 (wild type) and MAPK mutants *sakAΔ* and *mpkCΔ* with no treatment (Control), or treated with berberine hemisulfate (0.5 mmol l⁻¹; % growth inhibition: wild type 55.6%, *sakAΔ* 15.0%, *mpkCΔ* 18.6%), veratraldehyde (Vert) (5 mmol l⁻¹; % inhibition: wild type 15.0%, *sakAΔ* 22.4%, *mpkCΔ* 18.6%), fludioxonil (50 μmol l⁻¹; % inhibition: wild type 100%, *sakAΔ* 66.8 ± 19.4%, *mpkCΔ* 73 ± 21.2%), fludioxonil (50 μmol l⁻¹) + berberine (0.5 mmol l⁻¹) (% inhibition: wild type 100%, *sakAΔ* 100%, *mpkCΔ* 100%), and fludioxonil (50 μmol l⁻¹) + veratraldehyde (5 mmol l⁻¹) (% inhibition: wild type 100%, *sakAΔ* 90%, *mpkCΔ* 100%). Results are means of three replicates. SDs were < 5% except noted.

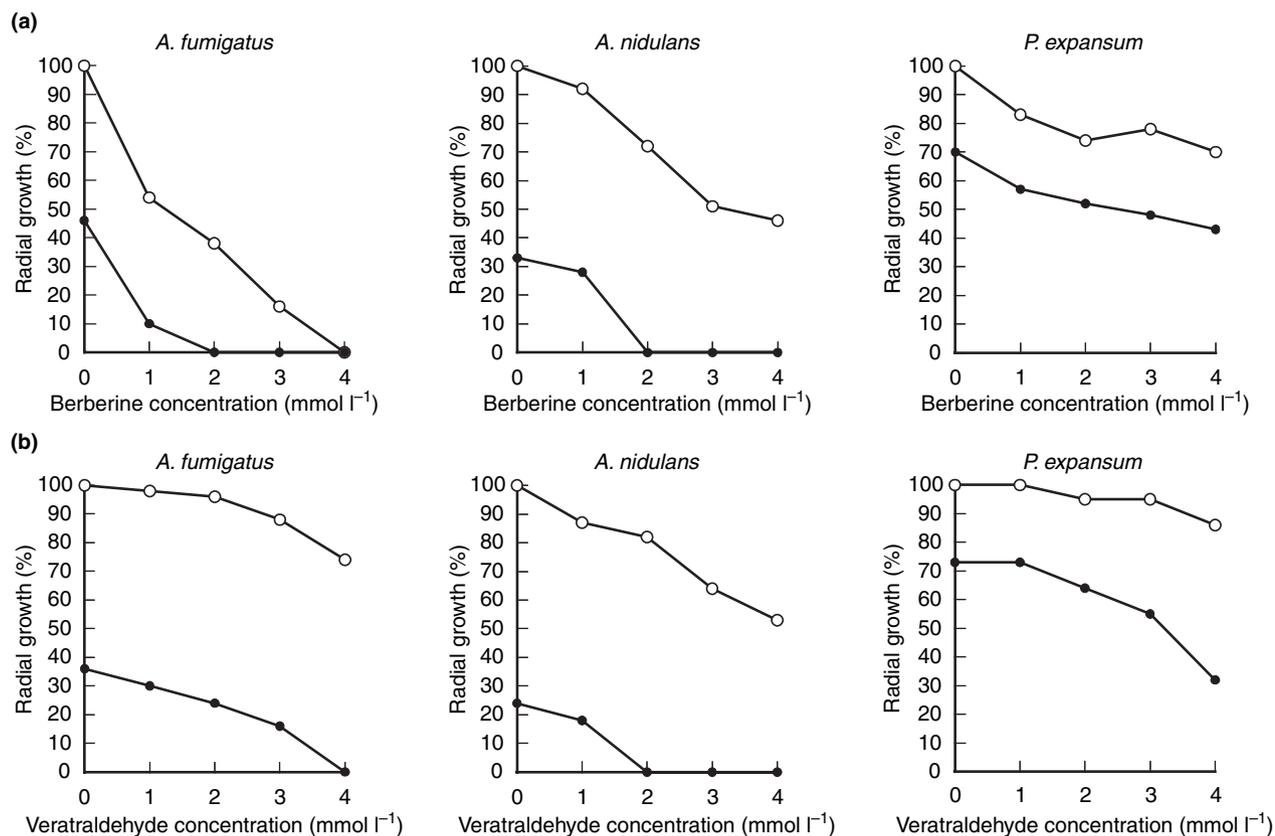


Figure 3 Graphic representations showing relative radial growth of *Aspergillus fumigatus*, *A. nidulans* and *Penicillium expansum* treated with strobilurin [0 (○) or 25 (●) μmol l⁻¹] and varying amounts (mmol l⁻¹) of (a) berberine hemisulfate or (b) veratraldehyde. Results are means of three replicates. SD < 5%.

fludioxonil, multiple genes in the same cellular pathway are targeted, greatly improving fungal control. Enhancing strobilurin activity with the identified synergists should help alleviate development of resistance in pathogens to this fungicide, the risk of which is considered to be high (<http://www.frac.info>; accessed 12 December 2006).

Our results show that the MAPK mutants *sakAΔ* and *mpkCΔ* of *A. fumigatus* escaped toxicity of fludioxonil. Kojima *et al.* (2004, 2006) similarly observed that certain MAPK mutants, *hog1Δ* and *osc1Δ*, purportedly involved in the signalling cascade for osmo-regulation in the pathogenic yeast, *Cryptococcus neoformans*, and the plant pathogen, *Colletotrichum lagenarium*, respectively, were also tolerant to phenylpyrrole fungicides, such as fludioxonil. The role of MpkC in *Aspergillus* has not been identified (Furukawa *et al.* 2005). We suggest that SakA and MpkC play a role in oxidative stress response in *A. fumigatus*. Both these MAPK mutants of *A. fumigatus* showed similar phenotypes with the treatments used in our study, escape from fludioxonil toxicity. Thus, MpkC probably has some overlapping role with SakA in responding to oxidative stress.

In summary, antioxidative stress response systems are molecular targets of phenolics or alkaloids. Combined application of alkaloids, or redox-active phenolics, with commercial antifungal agents shows promise for improving fungal control. These compounds can enhance activity, reduce costs, lower resistance, and alleviate health and environmental risks by reducing amounts of commercial antifungal agents required to achieve effective control.

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