ORIGINAL ARTICLE

Use of chemosensitization to overcome fludioxonil resistance in *Penicillium expansum*

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Abstract

Aim: To overcome fludioxonil resistance of *Penicillium expansum*, a mycotoxigenic fungal pathogen causing postharvest decay in apple, by using natural phenolic chemosensitizing agents.

Methods and Results: Fludioxonil-resistant mutants of *P. expansum* were co-treated with different oxidising and natural phenolic agents. Resistance was overcome by natural phenolic chemosensitizing agents targeting the oxidative stress–response pathway. These agents also augmented effectiveness of the fungicide, kresoxim-methyl. Results indicated that alkyl gallates target mitochondrial respiration and/or its antioxidation system. Fungal mitochondrial superoxide dismutase (Mn-SOD) plays a protective role against alkyl gallates.

Conclusions: Natural chemosensitizing agents targeting the oxidative stress–response system, such as Mn-SOD, can synergize commercial fungicides.

Significance and Impact of the Study: Redox-active compounds can serve as potent chemosensitizing agents to overcome resistance and lower effective dosages of fungicides. This can reduce costs with coincidental lowering of environmental and health risks.

Introduction

Resistance of fungal pathogens to drugs or fungicides is a continuing problem (Moore *et al.* 2000). This resistance frequently involves mutations triggered by environmental factors, such as oxidising agents, UV, *etc.* (Nakabeppu *et al.* 2006; Foster 2007). In fungi, stress signals resulting from osmotic, oxidative or cell wall stress, are integrated into mitogen-activated protein kinase (MAPK) pathways that regulate genes countering the stress (Schwartz and Madhani 2004; Levin 2005). In yeasts (*e.g. Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*), the Hog (high osmolarity glycerol) MAPK pathway plays a key role in responding to osmotic and/or oxidative stress (Toone and Jones 1998). Mutations in MAPK pathways, or upstream two-component (His-Asp phosphorelays) systems, relaying environmental signals to MAPK pathways, can result in tolerance to drugs or fungicides (Kojima *et al.* 2004; Motoyama *et al.* 2005; Hagiwara *et al.* 2007).

Natural benzo analogues possess antifungal activity by targeting oxidative stress response (Kim *et al.* 2008a, b); which is also a target of some antimicrobial agents (Smits and Brul 2005; Jaeger and Flohe 2006). Redox-active phenolics or sulfur-containing compounds can be potent redox-cyclers that inhibit microbial growth by interfering with cellular redox homoeostasis and/or the function of redox-sensitive components (Guillen and Evans 1994; Shvedova *et al.* 2000; Jacob 2006).

The fungicide fludioxonil disrupts the signalling systems of fungi resulting in excess stimulation of the histidine kinase (HK)-MAPK stress–response pathway or glycerol biosynthesis (Ochiai *et al.* 2001; Kojima *et al.* 2004; Kanetis *et al.* 2008). It is used to control a variety of phytopathogenic fungi, including *Penicillium expansum*. If there is a mutation in the HK-MAPK signalling system, a fungus can become resistant to fludioxonil (Ochiai *et al.* 2001; Kojima *et al.* 2004), but with reduced growth on normal media (Hagiwara *et al.* 2007).

Two strains of fludioxonil-resistant *P. expansum*, FR2 and FR3, were generated by UV treatment (Li and Xiao 2008). The current report shows that this resistance results from mutation(s) in cellular redox homoeostasis. Moreover, the investigation identifies a number of redox-active natural benzo analogues as chemosensitizing agents, targeting cellular stress response, to overcome this resistance. Overall, these results indicate that chemosensitization could improve effectiveness of, and overcome resistance to, commercial fungicides for apple production and processing. This may ultimately facilitate maintaining high apple quality and reduce potential for contamination by a mycotoxin synthesized by *P. expansum*, patulin.

Materials and methods

Micro-organisms

Saccharomyces cerevisiae wild type BY4741 (*Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and selected deletion mutants lacking antioxidation genes (*sod1Δ, sod2Δ, glr1Δ*) were obtained from Invitrogen (Carlsbad, CA, USA) and Open Biosystems (Huntsville, AL, USA; Reference: http://www.yeastgenome.org, Accessed 11 March 2010). Yeast strains were grown as described previously (Kim *et al.* 2008b). *Penicillium expansum* fludioxonil-resistant mutants, FR2 and FR3 (Li and Xiao 2008), and their parental strains, W1 and W2, respectively, were grown at 28°C on potato dextrose agar (PDA). *Aspergillus fumigatus* AF293, wild type, and *A. fumigatus* MAPK deletion mutants *sakAΔ* and *mpkCΔ* (Xue *et al.* 2004; Reyes *et al.* 2006) were grown at 37°C on PDA medium.

Chemicals

Thymol [5-methyl-2-(isopropyl)phenol]; 2,3-dihydroxy-benzaldehyde; gallic acid and ester analogues (methyl-, ethyl- and octyl-gallates); fludioxonil; kresoxim-methyl; antimycin A; menadione; hydrogen peroxide (H_2O_2); and diamide were purchased from Sigma Co. (St Louis, MO, USA). Compounds were dissolved in dimethylsulfoxide (DMSO; absolute DMSO amount: <2% in media), except H_2O_2 and diamide, which were dissolved in water, for incorporation into media.

Antifungal bioassays

Yeast strain sensitivities were assessed by a yeast-cell dilution bioassay on SG agar (Kim *et al.* 2008b). Sensitivities of filamentous fungi were based on per cent radial growth of treated (T) compared to control (C), receiving only DMSO, based on the Vincent equation [% inhibition of growth = 100 (C–T)/C, C: diameter of fungi on control plate; T: diameter of fungi on the test plate] (Vincent 1947). Spores (5×10^3) were diluted in phosphate-buffered saline and spotted in the centre of PDA plates (triplicates) with test compounds. Growth was observed for 3–7 days. Effectiveness of chemosensitization, by thymol (0·2–0·6 mmol l^{-1}), octyl gallate (0·05–0·2 mmol l^{-1}) or 2,3-dihydroxybenzaldehyde (2,3-D; 0·05–0·3 mmol l^{-1}), was assessed by co-application with fludioxonil or kresoxim-methyl (0·02, 0·04, 0·06 mmol l^{-1}). Oxidising agents, menadione (0·001–0·512 mmol l^{-1}), hydrogen peroxide (0·5–5 mmol l^{-1}) or diamide (0·5–5 mmol l^{-1}) were incorporated into media at respective levels. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of a compound where no fungal growth was visible.

Results

Characteristics of *Penicillium expansum* fludioxonil-resistant mutants

UV induction of *P. expansum* fludioxonil-resistant mutants FR2 and FR3 was previously reported (Li and Xiao 2008). In this study, we modified the growth condition for *P. expansum* by increasing concentration of fludioxonil (0·02–1 mmol l^{-1}) and the temperature (to 28°C) from that reported by Li and Xiao (2008) to provide conditions representative of an apple orchard environment. Other studies (Kanetis *et al.* 2008) also showed *Penicillium* mutants highly resistant to fludioxonil having an EC50 value >0·16 mmol l^{-1}. Both FR2 and FR3 mutants showed reduced radial growth on PDA (w/o fludioxonil) compared to respective parental strains, W1 and W2 (FR2: 11–17% reduction; FR3: 25–30% reduction).

Responses to oxidising agents

Some fungi having mutations in certain MAPK genes can escape toxicity of fludioxonil (see Introduction). Fludioxonil resistance in *P. expansum* FR2/FR3 may also have resulted from a mutation in the oxidative stress signalling system (e.g. HK-MAPK pathway). Characteristics of such a mutation, reduced hyphal growth on normal medium (Hagiwara *et al.* 2007), were observed in FR2/FR3. Because the HK-MAPK pathway is the key signalling system for fungal defence against oxidative stress, FR2/FR3 mutants should be hypersensitive to oxidising agents.

Menadione, a redox cycling quinone, is a source of toxic superoxide radicals (Castro *et al.* 2007). FR3 was approximately twice as sensitive as its W2 strain to menadione (MICs for menadione: 0·256 mmol l^{-1} < W2 < 0·512 mmol l^{-1} *vs* 0·128 mmol l^{-1} < FR3 < 0·256 mmol l^{-1})

and other oxidising agents, H_2O_2 (at 3 mmol l^{-1}) or diamide (at 3·5 mmol l^{-1}; thiol-oxidising agent) (Fig. 1a).

Alternatively, FR2 was almost twice as tolerant as its parental W1 strain to menadione (MICs for menadione: 0·128 mmol l^{-1} < W1 < 0·256 mmol l^{-1} vs 0·256 mmol l^{-1} < FR2 < 0·512 mmol l^{-1}) and was tolerant to H_2O_2 (at 4 mmol l^{-1}, where W1 showed no growth), and to diamide (at 2·5 mmol l^{-1}) (W1: 42% less growth than untreated vs FR2: 29% less growth than untreated) (Fig. 1a).

The hypersensitivity of FR3 to oxidising agents and characteristically reduced hyphal growth indicate its oxidative

stress–response system was defective. As fludioxonil activity is exerted through a normally functioning MAPK system, mutation of the HK-MAPK pathway may explain why the FR3 strain was able to escape fludioxonil toxicity (Kojima et al. 2004).

Alternatively, FR2 showed increased tolerance to the oxidising agents. This increased tolerance suggests a gain of function from increased antioxidative capacity that ameliorates cellular redox fluctuations under fludioxonil treatment. Heightened antioxidative activity under conditions without stress lowered hyphal growth of FR2. This reduced growth is similar to that seen in
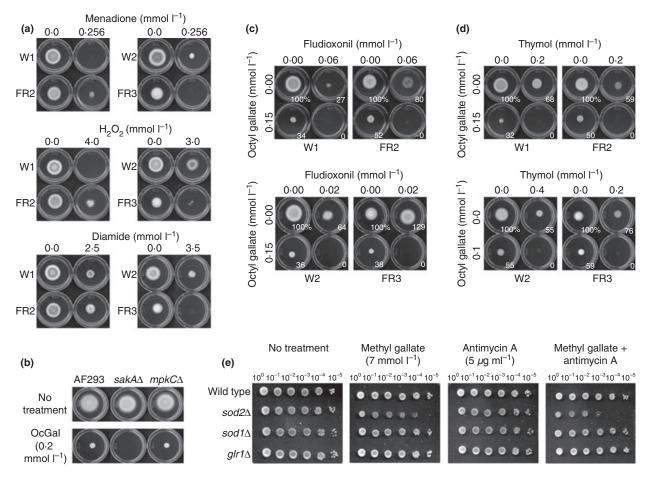

Figure 1 Responses of filamentous fungi and yeast to antifungal compounds. (a) Differential responses of *Penicillium expansum* parental strains, W1 and W2, and respective fludioxonil-resistant mutants, FR2 and FR3, to oxidising agents, menadione, hydrogen peroxide (H_2O_2) or diamide. (b) Effect of octyl gallate (OcGal) on growth of three strains of *Aspergillus fumigatus*, AF293 (wild type), *sakA*Δ mutant (osmotic/oxidative stress–sensitive mutant) and *mpkC*Δ mutant (mutant of the polyalcohol sugar utilization system). (c) Representative bioassay showing growth of FR2, FR3 and their respective parental strains in co-applications of fludioxonil (0·02 or 0·06 mmol l^{-1}) and octyl gallate (0·15 mmol l^{-1}). (See also Table 1). (d) Growth of parental and respective fludioxonil-resistant mutants of *P. expansum* to individual and co-treatments of thymol (as low as 0·2 mmol l^{-1}) and octyl gallate (as low as 0·1 mmol l^{-1}). Note: Radial growth of each strain w/o treatment was considered as 100% growth (control), and the relative growth rate among treatments was determined accordingly (SD < 5%; in triplicate assays for respective combinations of compounds). (e) Yeast dilution bioassays showing effects of methyl gallate and antimycin A, alone and in combination, against deletion mutants of *Saccharomyces cerevisiae*.

Table 1 Per cent growth of fludioxonil-resistant mutants (FR2 and FR3) and respective parental strains (W1 and W2) of *Penicillium expansum* to fungicides fludioxonil (Flud) and strobilurin (kresoxim-methyl; Kre-Me) and redox-active chemosensitizers (phenolics), alone and co-applied. The phenolics include octyl gallate (OcGal), 2,3-dihydroxybenzaldehyde (2,3-D) and thymol (Thy). Numbers in parentheses are concentrations (mmol l^{-1}) at which each compound was tested and are the lowest concentrations wherein combining the compounds resulted in no growth (0% under 'Co-applied'). Per cent numbers (%) are relative growth rate (radial growth) of fungi compared to 'no treatment' controls of each strain (=100%) (SD < 5%; triplicate for respective combination of compounds). *Penicillium expansum* strains were grown at 28°C on potato dextrose agar. Growth was observed for 3–7 days

Fungicide alone	Chemosensitizer alone	Co-applied (%)	Fungicide alone	Chemosensitizer alone	Co-applied (%)
W1			W2		
Flud (0·06)	OcGal (0·15)	0	Flud (0·02)	OcGal (0·15)	0
27%	34%		64%	36%	
Flud (0·06)	2,3-D (0·20)	c.0 (few colonies)	Flud (0·04)	2,3-D (0·30)	0
14%	71%		78%	75%	
Flud (0·04)	Thy (0·60)	0	Flud (0·04)	Thy (0·20)	0
44%	50%		26%	58%	
Kre-Me (0·02)	OcGal (0·05)	0	Kre-Me (0·02)	OcGal (0·05)	0
54%	69%		52%	73%	
Kre-Me (0·02)	2,3-D (0·15)	0	Kre-Me (0·02)	2,3-D (0·20)	0
50%	81%		54%	79%	
Kre-Me (0·02)	Thy (0·60)	0	Kre-Me (0·02)	Thy (0·40)	c.0 (few colonies)
44%	40%		47%	50%	
FR2			FR3		
Flud (0·06)	OcGal (0·15)	c.0 (few colonies)	Flud (0·02)	OcGal (0·15)	0
80%	52%		129%	38%	
Flud (0·06)	2,3-D (0·20)	48	Flud (0·04)	2,3-D (0·30)	96
78%	70%		119%	85%	
Flud (0·06)	Thy (0·60)	c.0 (few colonies)	Flud (0·04)	Thy (0·20)	c.0 (few colonies)
86%	36%		135%	83%	
Kre-Me (0·02)	OcGal (0·05)	0	Kre-Me (0·02)	OcGal (0·05)	0
59%	77%		67%	79%	
Kre-Me (0·02)	2,3-D (0·15)	0	Kre-Me (0·02)	2,3-D (0·20)	0
59%	77%		65%	82%	
Kre-Me (0·06)	Thy (0·40)	c.0 (few colonies)	Kre-Me (0·02)	Thy (0·20)	c.0 (few colonies)
41%	41%		61%	83%	

overexpression of the antioxidation gene *sodA*, encoding mitochondrial superoxide dismutase (Mn-SOD) of *Aspergillus flavus*, in an *S. cerevisiae* wild-type strain (Kim *et al.* 2006).

Chemosensitization using redox-active natural compounds

Chemosensitization by redox-active natural compounds, such as thymol or 2,3-dihydroxybenzaldehyde (2,3-D), was effective in overcoming resistance to antifungal drugs. Fungi having a mutation in their oxidative stress–response system (e.g. MAPK gene deletion) were more sensitive to thymol or 2,3-D than nonmutant strains (Kim *et al.* 2008a,b). Redox-active compounds probably disrupted cellular redox homoeostasis of FR2/FR3 by impairing cellular components and depleting activity of antioxidant enzymes, such as Mn-SOD. This disruption resulted in diminishing their ability to circumvent fludioxonil treatment.

Genomic tools are limited for *P. expansum*. We used available *A. fumigatus* mutants as models for *P. expansum*. We tested the responses of three strains of *A. fumigatus*, the AF293 (wild type), and two MAPK deletion mutants, *sakAΔ* and *mpkCΔ* (Xue *et al.* 2004; Reyes *et al.* 2006) against octyl gallate. *Aspergillus fumigatus sakAΔ* is an osmotic/oxidative stress–sensitive mutant, while *mpkCΔ* is a mutant of the poly-alcohol sugar utilization system. The *A. fumigatus sakAΔ* mutant was more sensitive to octyl gallate than AF293 or *mpkCΔ* mutant (Fig. 1b). This heightened sensitivity indicated, like thymol or 2,3-D, octyl gallate affected the SakA MAPK stress–response system.

Growth of FR2, FR3 and their parental strains was almost completely inhibited when fludioxonil (0·02 or 0·06 mmol l^{-1}) was co-applied with 0·15 mmol l^{-1} of octyl gallate (Fig. 1c). Thymol or 2,3-D also exhibited chemosensitizing activity to fludioxonil in these strains. However, this activity for 2,3-D was negligible in the mutant strains, for an undetermined reason (Table 1).

Co-applications of thymol (as low as 0·2 mmol l^{-1}) and octyl gallate (as low as 0·1 mmol l^{-1}) resulted in complete growth inhibition of all strains (Fig. 1d). This synergism suggests these compounds affect a common cellular target resulting from disruption of the oxidative stress–response system.

Chemosensitization to kresoxim-methyl

The chemosensitizing effect of phenolics to kresoxim-methyl, a fungicide that inhibits complex III of the mitochondrial respiratory chain resulting in a disruption of energy production (Wood and Hollomon 2003), was also tested. Coinciding with this disruption is an abnormal release of electrons that additionally damages cellular components by oxidative stress. Mn-SOD plays an important role in protecting cells from such oxidative damage. The responses of these mutants to kresoxim-methyl treatments were not substantially different from those of the respective parental strains (Table 1), indicating no resistance. Co-applying redox reactive chemosensitizing agents, targeting Mn-SOD, however, augmented fungicidal effects of kresoxim-methyl. Octyl gallate (as low as 0·05 mmol l^{-1}) combined with kresoxim-methyl (0·02 mmol l^{-1}) resulted in complete growth inhibition of all strains of P. expansum. This chemosensitization also occurred with co-application of 2,3-D or thymol (Table 1).

Protective role of fungal Mn-SOD against alkyl gallates

We used deletion mutants of S. cerevisiae [i.e. wild type and antioxidation mutants sod1Δ (cytosolic SOD gene deletion), sod2Δ (Mn-SOD gene deletion) and glr1Δ (glutathione reductase gene deletion), mutants not available in A. fumigatus/P. expansum] to investigate potential targets of alkyl gallates. Methyl gallate (7 mmol l^{-1}) was selected as a representative alkyl gallate, for experimental purposes. At this concentration, this compound had little effect on yeast growth. To confirm Mn-SOD played a protective role against alkyl gallates, methyl gallate was co-applied with antimycin A (5 μg ml^{-1}). Antimycin A is an antifungal drug and, like kresoxim-methyl, inhibits complex III of the mitochondrial respiratory chain (Lai et al. 2005). Of the yeast mutants examined, only the growth rate of the sod2Δ mutant was affected. This mutant showed c.10–100 times lower growth than treatments of either compound, alone (Fig. 1e). It was the only mutant affected by the co-treatment (Fig. 1e). If methyl gallate affected mitochondrial and Mn-SOD activities, then co-treatment of these compounds would be expected to be synergistic, with the sod2Δ mutant most sensitive.

These results strongly indicate alkyl gallates target mitochondrial respiration and/or its antioxidation system. Expression of the Mn-SOD gene is regulated by the HOG1 (MAPK) system in S. cerevisiae (Boy-Marcotte et al. 1998; Rep et al. 2001). Thus, the A. fumigatus sakAΔ mutant, a HOG1 ortholog deletion mutant in the oxidative stress MAPK pathway, was particularly sensitive to octyl gallate (Fig. 1b). The fact that both the Mn-SOD (sod2Δ) and MAPK (sakAΔ) mutants were hypersensitive further demonstrates the important role the oxidative stress–response system has in protecting exposure to alkyl gallates.

Discussion

These results are an in vitro based proof-of-concept that natural phenolic compounds can serve as potent chemosensitizing agents to enhance activity of antifungal drugs and fungicides. Many studies have shown antifungal or antimycotoxigenic activities of derivatives of benzoic or cinnamic acid (Tawata et al. 1996; Florianowicz 1998; Beekrum et al. 2003; Kim et al. 2004). Fungitoxic phenolics are produced or released during fungal infection in plants. The fact that fungi must detoxify these compounds to be infective demonstrates how such natural compounds could serve as potential sources of antimicrobial agents. We showed that a number of phenolic compounds greatly improved effectiveness of fludioxonil and kresoxim-methyl by disrupting the oxidative stress–response systems (e.g. Mn-SOD) common to many fungi (Roman et al. 2007). This disruption also activated a process for overcoming fludioxonil-resistance.

Other recent studies also showed the effectiveness of natural compounds as potent chemosensitizing agents. For example, in Candida albicans, the MIC of fluconazole was decreased when the drug was co-applied with an alkaloid berberine compared to the independent treatment of fluconazole (Quan et al. 2006; Iwazaki et al. 2010). Thymol also showed in vitro antifungal activity against fluconazole-resistant and susceptible clinical isolates and a standard strain, ATCC 10231, of C. albicans, as well as enhancing the activities of fluconazole and amphotericin B (Guo et al. 2009). Collectively, chemosensitization could be applied to a broad spectrum of fungal pathogens resulting in lowering effective dosages for drugs and fungicides. Accordingly, this would lower costs and reduce environmental and health risks.

Acknowledgement

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