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Redox-Active Antifungal Molecules Target Stress Defense System in Fungi

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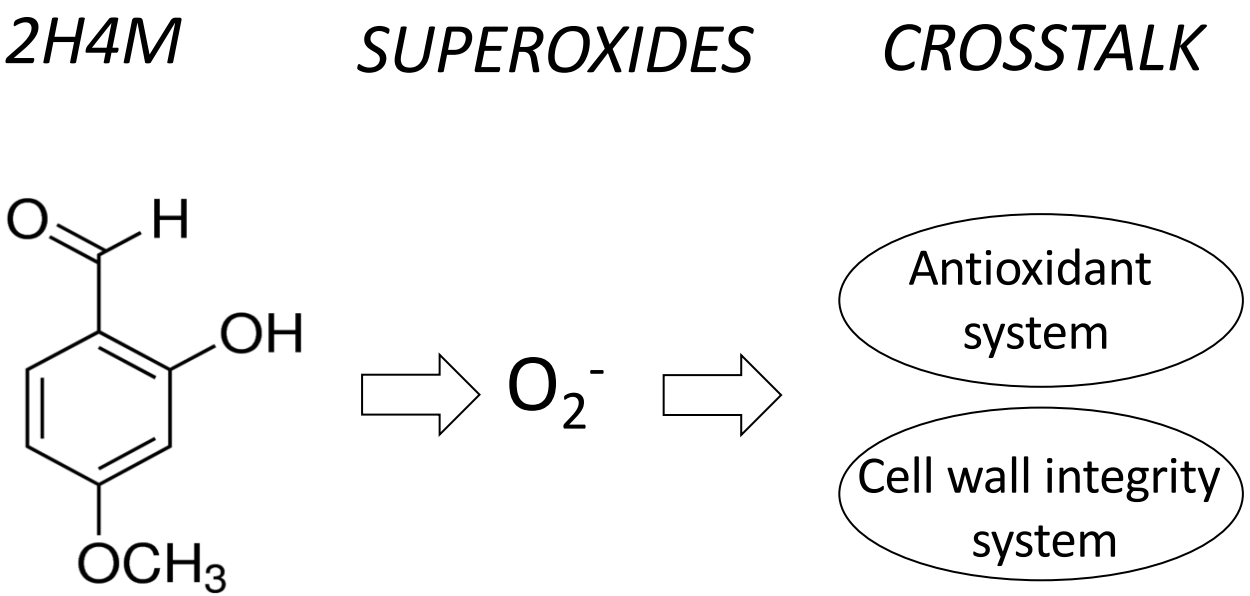
Graphical Abstract



Abstract

Redox-active molecules can function as potent redox-cyclers in fungi that contribute to the disruption of cellular redox homeostasis. Using *Saccharomyces cerevisiae* as a screening tool, we determined how the redox-active 2-hydroxy-4-methoxybenzaldehyde (2H4M) compound negatively affected both the antioxidant and cell wall integrity systems of fungi, indicating a crosstalk between the two systems under 2H4M-induced stress. The crosstalk contributes to the fungal defense against redox-active molecules, suggesting that it could be an effective target for antifungal treatment.

The redox-active polyene drug natamycin has also been used in food and crops for control of foodborne- and agricultural fungal pathogens. Studies indicated that invasive fungal infections in humans by *Candida*, *Aspergillus* and other species, could be acquired from contaminated food or nutritional supplements. Because such additive/fungicides might trigger the emergence of human pathogens that are cross-resistant to multiple polyenes (amphotericin B, nystatin, natamycin) via the food source, we examined the antifungal efficacy of natamycin against mycotoxin-producing, heat-resistant and invasive fungi. We observed differential susceptibility of the test strains to natamycin at both high and low acidic pH values. Our data provide information that could improve the safe and effective use of natamycin as an antifungal agent, thus enhancing food safety and public health.

Keywords: 2-Hydroxy-4-methoxybenzaldehyde; Fungal pathogens; Polyenes; Redox molecules; Stress signaling

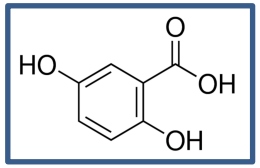
Introduction

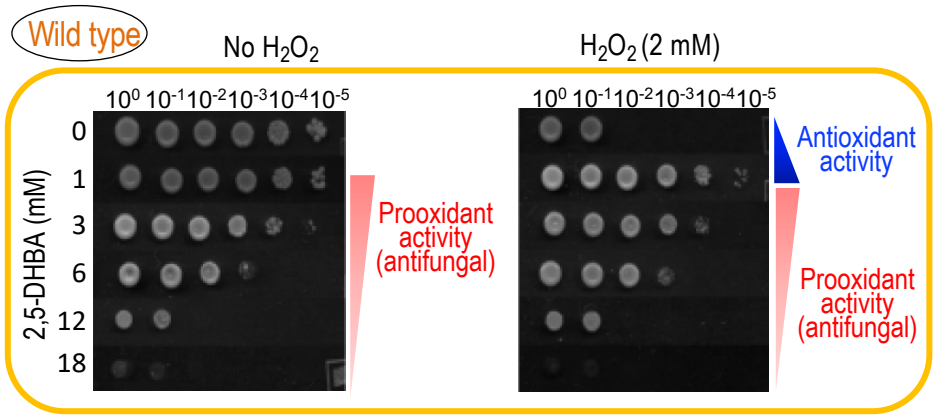
- One of the antifungal mechanisms of certain drugs involves **oxidative stress** response in **fungal pathogens**, which further contributes to the death of target fungi. Therefore, these types of drugs have been defined as **oxidative stress drugs**. Examples include **polyenes** such as amphotericin B (AMB) or nystatin. While AMB is known as a fungicidal drug which causes ion leakage, studies reported that ergosterol binding and pore formation in the fungal membrane was not the sole mechanism of AMB activity (Palacios et al., 2007). Instead, oxidative stress triggered by AMB could be one of the contributing mechanisms for AMB fungicidality.
- **Redox-active molecules** possessing both antioxidant and prooxidant potential, such as benzaldehyde analogs or sulfur-containing compounds, can be potent **redox-cyclers** in fungi and inhibit fungal growth by interfering with cellular **redox homeostasis** or the function of **redox-sensitive components** (Guillen and Evans, 1994; Jacob, 2006).
- For example, the natural phenolic compound **gentisic acid** has antioxidant or fungicidal activity, which is dependent on the levels of dose of the molecule applied and also whether chemically induced oxidative stress is present. Data showed that **lower doses** of gentisic acid **relieved oxidative stress**, while **higher doses** of gentisic acid has **antifungal activity** when cells are not under oxidative stress, through disruption of cellular redox homeostasis (Next page).

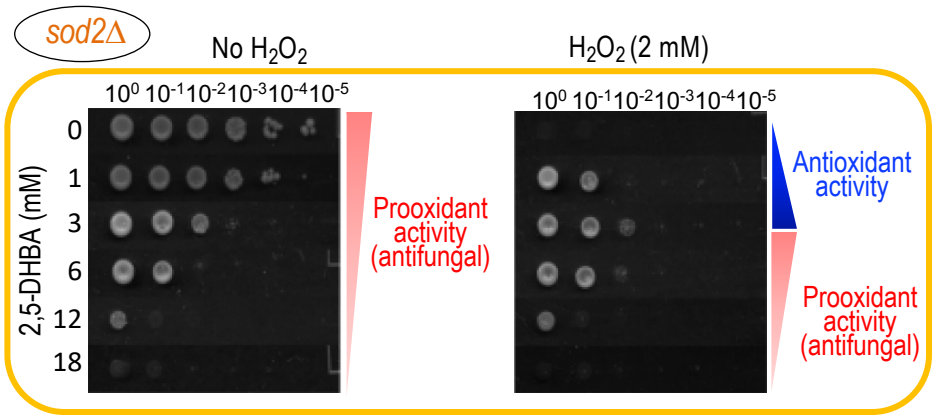
Natural Redox Molecules As Antifungal Alternatives: Prevention of Drug Resistance

❑ Background: Natural redox molecules possess both antioxidant and prooxidant activity

- Gentisic acid (2,5-Dihydroxybenzoic acid); aspirin metabolite
- Wild type, *sod2*Δ (mitochondrial superoxide dismutase mutant)







- **Lower doses** of gentisic acid relieved oxidative stress (H_2O_2)
- **Higher doses** of gentisic acid has antifungal activity when cells are not under oxidative stress, through disruption of cellular redox homeostasis

- Recently, the redox-modulatory anti-protozoal drug **chloroquine (CQ)** has shown to trigger the **crosstalk** between the **antioxidant** and **cell wall integrity** systems in the model fungus *Saccharomyces cerevisiae* (Baranwal et al., 2014). The CQ-induced stress was transmitted to both HOG1 (antioxidant) and SLT2 (cell wall integrity) signaling systems, namely, Mitogen-Activated Protein Kinase (MAPK) pathways (*Saccharomyces* Genome Database, 2020).
- CQ inhibited *S. cerevisiae* growth in a dosage-dependent manner, where the phosphorylated HOG1 MAPK enzyme translocated (from the cytosol) to the nucleus to activate glycerol synthesis. The SLT2 MAPK system was then activated to regulate the cell wall damage-induced defense responses (Baranwal et al., 2014).
- We previously identified the natural product **2-hydroxy-4-methoxybenzaldehyde (2H4M)** that targets **cell wall integrity** system of fungi (Kim et al., 2015). Fungi treated with 2H4M exhibited acute growth inhibition, while the sensitivity was alleviated by sorbitol. 2H4M should also serve as a potent redox-cycler in fungal pathogens by disrupting redox homeostasis and/or redox-sensitive structures.
- The **crosstalk between** the **HOG** and **cell wall integrity** systems has been documented further in: (a) ***Candida albicans*** where both HOG and Cek1 (cell wall construction) pathways were necessary to counteract the osmotic stress (Herrero-de-Dios et al., 2014), (Next page)

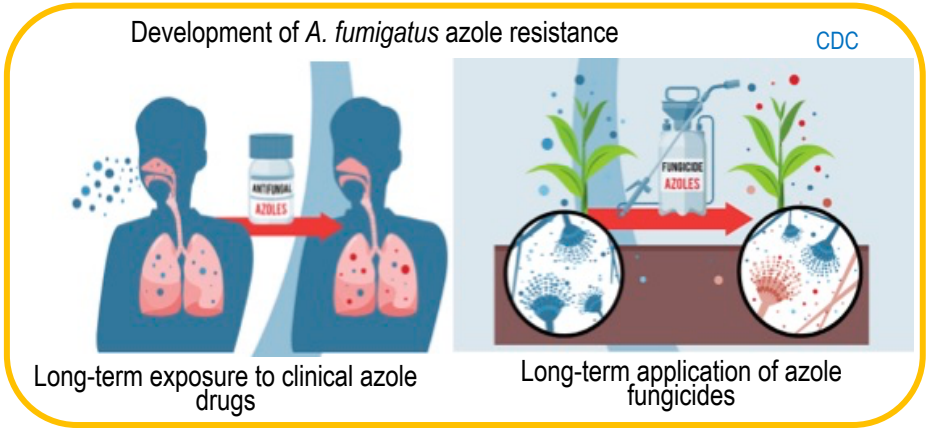
- (b) ***Pyrenophora graminea*** (phytopathogen) for which the pgpbs, a HOG pathway MAPK kinase (MAPKK) gene, was required for both osmotic stress and cell wall integrity response (Liang et al., 2019), and (c) ***Aspergillus fumigatus*** for which both SakA and MpkC (oxidative stress MAPKs) are necessary for the phosphorylation of MpkA (cell wall integrity MAPK) during cell wall damage; under the cell wall stress, SakA physically interacts with the MpkA (Mattos et al., 2020).
- **One Health** approach acknowledges that human, animal and environmental health are closely linked (Hernando-Amado et al., 2019). Studies indicated that invasive fungal infections in humans by *Candida*, *Aspergillus* and other species, could be acquired from contaminated food or nutritional supplements. **Natamycin**, the only US FDA-approved drug for curing **ophthalmic** fungal infections, is also used in the **food and agricultural industries** for the prevention of foodborne- or environmental fungal pathogens.
- The drug efficacy and/or pathogen susceptibility to drugs are often dependent on pH conditions, such as treatment of vaginal *C. albicans* (Chaillot et al., 2017). Therefore, determining the efficacy of the polyene drug natamycin in high and low acidic pH values mimicking food matrices is necessary for effective control of foodborne- or environmental fungi.

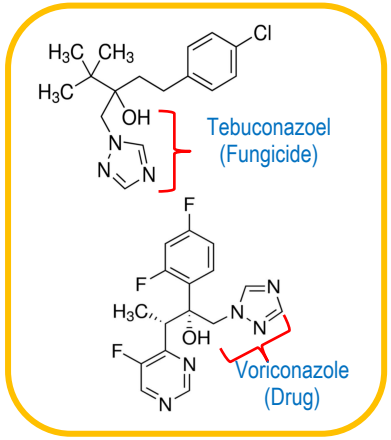
One health:
Human, animal & environmental health are closely linked

❑ Example: *A. fumigatus* azole resistance involves TR34/L98H mutations in CYP51A

- ***AZOLE DRUGS*** inhibit CYP51A, a cytochrome P450 enzyme involved in fungal membrane biosynthesis
- ***CYP51A TR34/L98H*** mutations: Identified first in > 90% of azole resistant *A. fumigatus* in European countries, subsequently in the United States (CDC 2018)

  TR34/L98H: two mutations, a 34-bp insertion in the CYP51A gene promoter region & Leu to His substitution at the codon 98 of CYP51A gene
- ***AZOLE FUNGICIDES***: MoA similar to azole drugs; environmental **selection pressure** for *A. fumigatus* pan-azole resistance
- More than **25%** of total fungicide sales are azoles





Results and Discussion

2-Hydroxy-4-methoxybenzaldehyde (2H4M)

- The compound-induced yeast gene deletion bioassays use heterozygous gene deletion mutants of *S. cerevisiae* that are susceptible to a particular compound, thus identifies both the candidate compound and its possible gene targets (Giaever et al., 1999).
- Agar plate *S. cerevisiae* dilution bioassay: wild type (BY4741 *MAT* a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), 25 mutants (Gene description, next page) to assess effects of the most potent benzaldehydes targeting the cell wall integrity system with a structure–activity relationship (Kim et al., 2015); 0.5 to 7.0 mM benzaldehydes.
- The level of yeast growth (5 to 7 days of incubation):
  Score '0'—no colonies were visible from any of the dilutions,
  Score '1'—only a colony from the spot with the undiluted cells (10^6 cells),
  Score '2' only colonies from the spots with the undiluted (10^6) and 10^5 cells were visible, etc., whereas
  Score '6'—colonies were visible from all dilution spots.
- Therefore, each unit (1 to 6) of numerical difference was equivalent to a 10-fold difference in the sensitivity of the yeast strain to the benzaldehydes applied. Results showed the differential susceptibility of yeast strains at the representative concentrations enough to detect the distinctive susceptibilities of the mutants. (Next page)

Characteristics of sugar metabolism genes examined in this study (*Saccharomyces Genome Database*)

Gene or Open Reading Frame	Protein
GLYCOGEN METABOLISM	
GPH1 [a]	Glycogen phosphorylase
GDB1 [a]	Glycogen debranching enzyme
UGP1 [a]	UDP-glucose pyrophosphorylase (UGPase)
YHL012W	Paralog of UGP1
PGM2 [a]	Phosphoglucomutase (Paralog of PGM1)
PGM1	Phosphoglucomutase
C_6 METABOLISM	
YDR516C [a]	Early Meiotic Induction 2 (EMI2)
HXK2 [a]	Hexokinase 2
HXK1	Hexokinase 1
GLK1	Glucokinase
PGI1	Phosphoglucose isomerase
CHITIN BIOSYNTHESIS	
CHS5	Chitin synthase-related
CHS1	Chitin synthase I
CHS3	Chitin synthase-related
CHS2	Chitin synthase

TREHALOSE METABOLISM	
TPS3 [a]	Trehalose-6-phosphate synthase/phosphatase regulatory subunit
TPS1 [a]	Trehalose-6-P synthase/phosphatase complex synthase subunit
TPS2 [a]	Trehalose-6-P synthase/phosphatase complex phosphatase subunit
NTH2 [a]	Neutral trehalase
ATH1 [a]	Acid trehalase
NTH1 [a]	Neutral trehalase
GLUCOSAMINE METABOLISM	
GFA1	Glutamine:Fructose-6-phosphate amidotransferase
GNA1	Glucosamine-6-phosphate acetyltransferase
PCM1	Phosphoacetylglucosamine mutase
QRI1 [a]	UDP-N-acetylglucosamine pyrophosphorylase

[a] Stress-regulated genes.

Sensitive Response of Cell Wall Integrity System Mutants to 2-Hydroxy-4-methoxybenzaldehyde (2H4M)

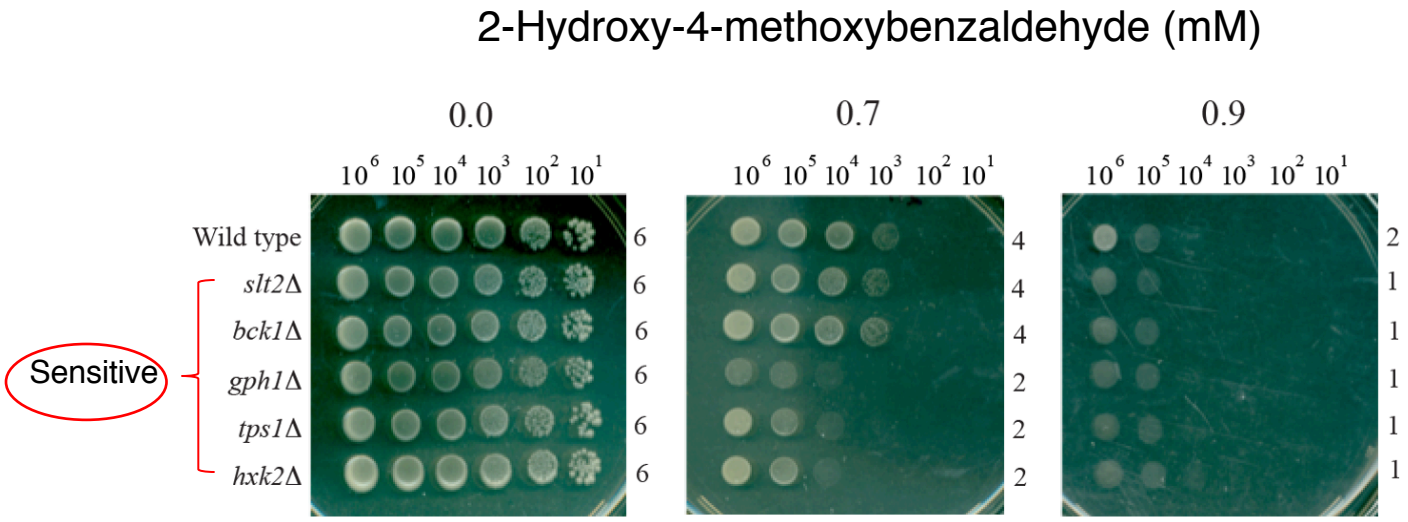


Figure 1. *S. cerevisiae* dilution bioassay showing the cell wall integrity system mutants are more susceptible to the treatment of 2-hydroxy-4-methoxybenzaldehyde compared to the wild type. Numbers on right side of each row (1, 2, 4 and 6) indicate growth scores (See Table 1) and 10^1 to 10^6 indicate cell numbers spotted.

*bck1*Δ, MAPK kinase kinase (MAPKKK); *slt2*Δ, MAPK; *gph1*Δ, *tps1*Δ, *hxk2*Δ; See slide 10

Table 1. Sensitive responses of the cell wall integrity/sugar metabolism mutants to six benzaldehydes determined at the representative concentrations. [a]

Benzaldehydes	Wild type	*slt2*Δ	*bck1*Δ	*gph1*Δ	*tps1*Δ	*hxk2*Δ
2-Hydroxy-4-methoxybenzaldehyde (0.9 mM)	2	1	1	1	1	1
4-Methoxy-2-methylbenzaldehyde (2.5 mM)	6	2	2	4	4	6
3,5-Dimethoxybenzaldehyde (1.5 mM)	6	5	5	4	4	5
2,3-Dimethoxybenzaldehyde (2.0 mM)	6	5	5	4	5	5
2,5-Dimethoxybenzaldehyde (1.0 mM)	6	5	5	5	5	6
2-Methoxybenzaldehyde (3.0 mM)	5	3	3	3	3	4

[a] Results showed that the five mutants, namely, *slt2*Δ & *bck1*Δ (signaling), *gph1*Δ (glycogen metabolism), *tps1*Δ (trehalose metabolism) and *hxk2*Δ (C_6 metabolism) exhibited increased susceptibility to 2H4M compared to the wild type (See also Figure 1). As determined previously, 2H4M possessed the highest antifungal activity. While all five mutants were susceptible to 2H4M (0.9 mM), the growth of the wild type was also greatly inhibited at the same condition. *S. cerevisiae* strains were obtained from Invitrogen (Carlsbad, CA, USA) and Open Biosystems (Huntsville, AL, USA).

Sensitive Response of Cytosolic Superoxide Dismutase Mutant to 2H4M

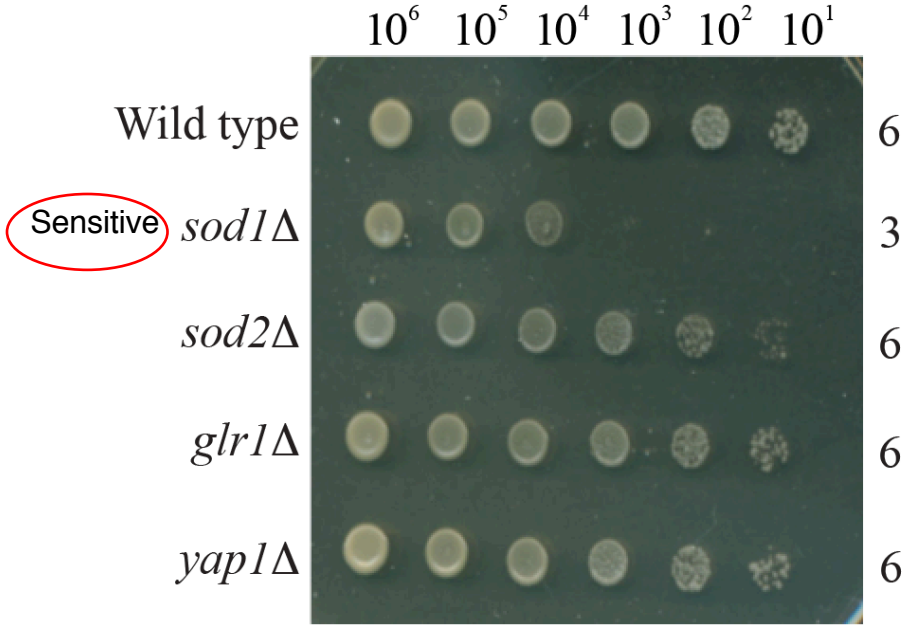


Figure 2. *S. cerevisiae* dilution bioassay showing the cytosolic superoxide dismutase gene deletion mutant (*sod1*Δ) is highly susceptible to the treatment of 2-hydroxy-4-methoxybenzaldehyde (0.6 mM) compared to the wild type or other antioxidant mutants. Numbers on the right side of each row (3 and 6) indicate growth scores (See Table 1) and 10^1 to 10^6 indicate cell numbers spotted.

*sod1*Δ, cytosolic superoxide dismutase; *sod2*Δ, mitochondrial superoxide dismutase; *glr1*Δ, glutathione reductase; *yap1*Δ, redox-responsive transcription factor

2H4M: Mechanisms of Action

- While the redox-active 2H4M could directly disrupt the fungal cell wall components, the prooxidant characteristic of 2H4M also induces cellular oxidative stress, for which cytosolic superoxide dismutase (SOD1) plays an important role for fungal defense by scavenging the cytosolic superoxide radicals.
- Fungal mutants lacking key genes in these systems, such as antioxidant and cell wall integrity MAPK mutants, are highly susceptible to 2H4M. Crosstalk between the antioxidant and cell wall integrity MAPK systems: The two MAPK systems are positively coordinated to counteract various stresses in S. cerevisiae (Rodriguez-Pena et al., 2010).
- For instance, the sequential activation of the HOG and cell wall integrity pathways has been determined during the yeast adaptation to zymolyase-treated cell wall stress. This cell wall-degrading enzyme activates both HOG1 and SLT2 MAPKs. The increased phosphorylation in SLT2 was through the 'transmembrane osmo-sensor' branch of the HOG1 MAPK pathway, which also required the upstream essential components of the cell wall integrity pathway such as MAPK kinase (MAPKK), MAPKK kinase (MAPKKK), protein kinase C1, etc. (Rodriguez-Pena et al., 2010).

Promotion of One Health Using Natural Redox Molecules as Fungicide Alternatives: Prevention of the Development of Azole Resistance of Human Fungal Pathogens in the Environment

❑ Redox molecule BA-1 as an antifungal & herbicide alternatives; fumigant activity

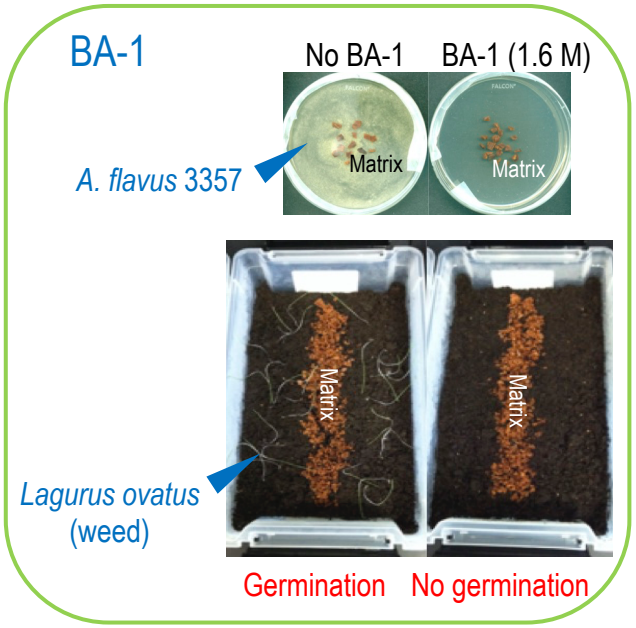
- BA-1 possesses both fungicidal & herbicidal characteristics; can lower pesticide burden in the crop fields
- Potential usage during early season soil solarization for both fungal pathogen & weed control

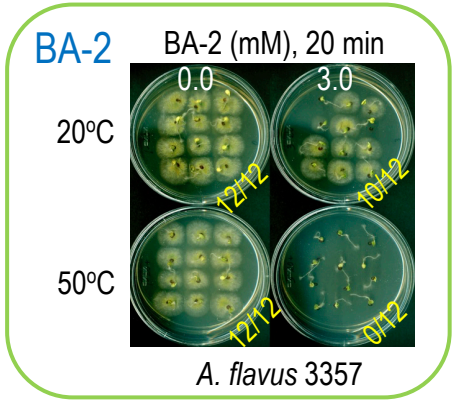
❑ Redox molecule BA-2 as a heat-sensitizer

- Co-application of BA-2 & mild heat achieved complete inhibition of fungal growth on the surface of cabbage seeds (*Brassica rapa* Pekinensis) inoculated with *A. flavus* 3357
- Potential alternatives to the seed sanitizing fungicides azoles, Thiram, etc.

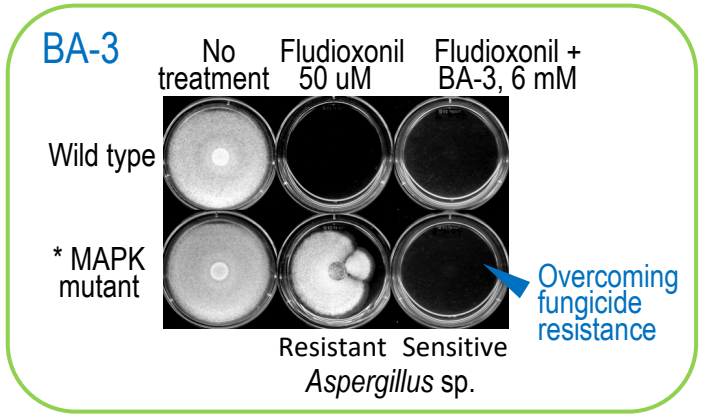
❑ Redox molecule BA-3 for overcoming fungicide resistance

- BA-3 prevents the development of fludioxonil resistance of stress signaling mutants (such as MAPK mutants) when co-applied
- BA-3 modulates the susceptibility of fungal antioxidant system to the co-treatment





contaminated vs. # germinated



* MAPK: Mitogen-Activated Protein Kinase

Results and Discussion

Natamycin (NAT)

- Natamycin (polyene drug) binds to ergosterol in fungal cell membrane, which inhibits cellular activity including membrane fusion, fission processes, endocytosis, plasma protein complexes (Lakhani et al., 2019).
- We evaluated the usage of natamycin, the only US FDA-approved drug for curing ophthalmic fungal infections, also in the food and agricultural industries for the prevention of foodborne- or environmental fungal contaminations.
- In the human pathogen *A. fumigatus*, the antifungal activity of natamycin was higher at pH 5.6 (low acidity) than at pH 3.5 (high acidity) (Figure 3).
- Whereas, natamycin showed higher antimycotic activity at pH 3.5 (high acidity) when compared to that observed at pH 5.6 (low acidity) in the foodborne fungal contaminants, thus showing opposite trends to that determined in *A. fumigatus* (Figure 3).

Utility of natamycin in food and agricultural industries for control of fungi

Target system	Fungi	Effects
Mandarin fruit	*Botrytis cinerea*	Postharvest disease control
Cherry tomato	*B. cinerea*	Iinhibit fruit decay in tomato fruit during storage.
Cheese	*Penicillium* spp.	Mold control on semi-hard cheese during ripening.
Cow milk	N/A	Protect cow milk exposed to light in refrigerated glass containers.
Apple juice	*Candida tropicalis, Rhodotorula mucilaginosa*	Reduced biofilm development from juice on stainless steel surfaces.
Blackberry fruits	*Aspergillus japonicus, Gilbertella persicaria*	Synergistic antifungal activity of ferulic acid and natamycin.
Phyllo pastry	Yyeasts, molds, enterococci	Delay the spoilage of phyllo pastry.
Strawberry crown Rot	QoI-Resistant *Colletotrichum acutatum*	Reducing disease severity and plant mortality in field studies.
Cheeses	Yeasts, molds	Inhibition of the growth of pathogenic or contaminant microorganisms (yeasts and molds).
Table olive	*S. cerevisiae, Candida boidinii*	Antagonistic effects between natamycin and citric acid in table olive packaging.
Phyllo, a dough-based wheat product	Yeast, mold	Reducing microbial species examined (mesophilic total viable counts), yeasts/molds.

Susceptibility of fungi to natamycin (NAT) is dependent on pH of matrices

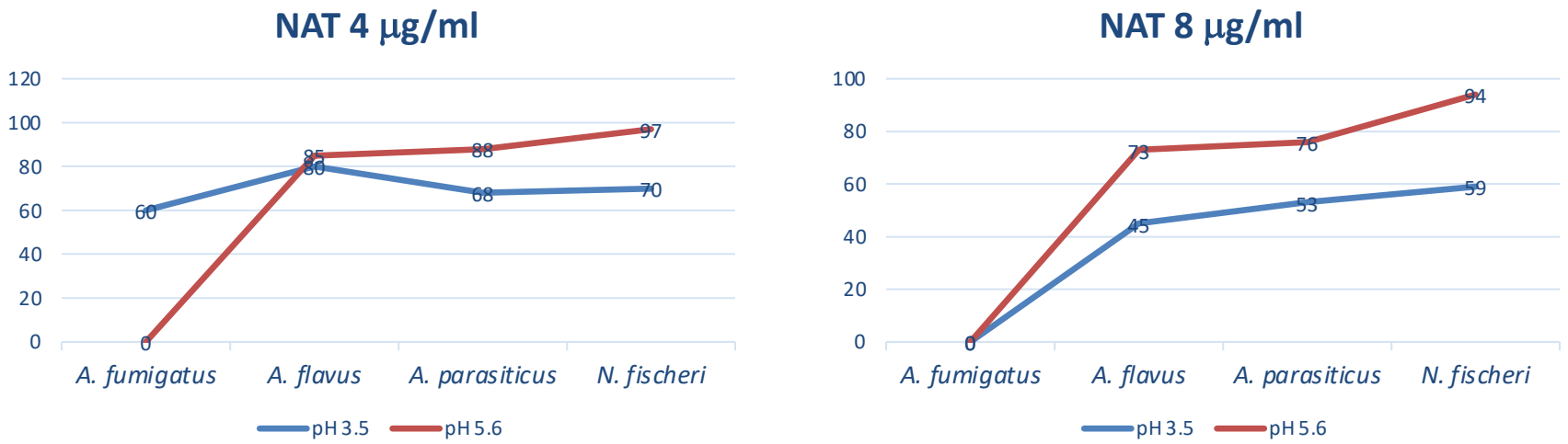


Figure 3. Antifungal activity of natamycin is higher at pH 5.6 (low acidity) than at pH 3.5 (high acidity) in the human pathogen *A. fumigatus*. However, natamycin showed higher antimycotic activity at pH 3.5 when compared to that observed at pH 5.6 in the foodborne fungi such as *A. flavus*, *A. parasiticus* and *N. fischeri*, thus showing opposite trends compared to that determined in *A. fumigatus*.

Screening natural, redox molecules for rapid elimination of invasive fungal pathogens, such as *A. fumigatus,* from food sources, thus promoting 'One Health'

Commercial food matrices:
e.g., organic fruit juices
(pH 3.0 – 3.5)

Compound screening

Redox-active molecules
+
Fungi (*Aspergillus, Penicillium*, *Neosartorya*)

IN PROGRESS:

2.5 hrs

> 24 hrs

Hours required to achieve ≥ 99.9% fungal death

REDOX-1

Effective candidate compound

Other compounds or commercial fungicides screened

Less effective

Conclusions

- Redox-active natural compounds, such as 2H4M, could serve as potent antifungal candidates where targeting cellular antioxidant and cell wall integrity systems is the proposed mechanism of action.
- The cytosolic oxidative stress signals, such as superoxide radicals (O_2^-), triggered by 2H4M are transmitted to activate the cell wall integrity pathway (via MAPK system), which then contributes to the maintenance of cell wall integrity.
- The crosstalk functions as a fungal defense against redox-active drugs/compounds or environmental stressors, thus could be an effective target for antifungal treatment.
- The oxidative stress drug natamycin showed promise for preserving foods by targeting the antioxidant system of fungal pathogens or contaminants.
- Differential susceptibility of fungi to natamycin was observed, where lower antifungal potency of natamycin was determined in the invasive pathogen *A. fumigatus* at the low acidic condition (which mimics commercial food matrices).
- The Redox-1, a redox-active molecule from compound screening could achieve rapid elimination of fungi; showing higher activity at lower pH. Identification of industry application which can promote One Health is currently underway.

References

Baranwal, S., Azad, G. K., Singh, V., Tomar, R. S. 2014. Signaling of chloroquine-induced stress in the yeast *Saccharomyces cerevisiae* requires the Hog1 and Slt2 mitogen-activated protein kinase pathways. Antimicrobial Agents and Chemotherapy, 58, 5552–5566.

Berkow EL, Nunnally NS, Bandea A, Kuykendall R, Beer K, Lockhart SR. 2018. Antimicrob Agents Chemother 62:e02240-17.

Chaillot J, Tebbji F, García C, Wurtele H, Pelletier R, Sellam A. 2017. pH-Dependant Antifungal Activity of Valproic Acid against the Human Fungal Pathogen *Candida albicans*. Front Microbiol. 8, 1956.

Giaever, G., Shoemaker, D. D., Jones, T. W., Liang, H., Winzeler, E. A., Astromoff, A., Davis, R. W. 1999. Genomic profiling of drug sensitivities via induced haploinsufficiency. Nature Genetics, 21, 278–283.

Guillen F., Evans C. S. 1994. Anisaldehyde and veratraldehyde acting as redox cycling agents for H_2O_2 production by *Pleurotus eryngii*. Appl. Environ. Microbiol. 60, 2811–2817.

Herrero-de-Dios, C., Alonso-Monge, R., & Pla, J. 2014. The lack of upstream elements of the Cek1 and Hog1 mediated pathways leads to a synthetic lethal phenotype upon osmotic stress in Candida albicans. Fungal Genetics and Biology, 69, 31–42.

Jacob C. 2006. A scent of therapy: pharmacological implications of natural products containing redox-active sulfur atoms. Nat. Prod. Rep. 23, 851–863.

Kim, J. H., Chan, K. L., Mahoney, N. 2015. Augmenting the activity of monoterpenoid phenols against fungal pathogens using 2-hydroxy-4-methoxybenzaldehyde that target cell wall integrity. International Journal of Molecular Sciences, 16, 26850–26870.

Kim, J.H., Chan, K.L., Tam, C.C., Cheng, L.W., Land, K.M. 2020. Crosstalk between the antioxidant and cell wall integrity systems in fungi by 2-hydroxy-4-methoxybenzaldehyde. Cogent Food & Agri. 6, 1823593.

Lakhani P, Patil A, Majumdar S. 2019. Challenges in the Polyene- and Azole-Based Pharmacotherapy of Ocular Fungal Infections. *J Ocul Pharmacol Ther*. 35, 6-22.

Liang, Q., Li, B., Wang, J., Ren, P., Yao, L., Meng, Y., Si, E., Shang, X., Wang, H. 2019. PGPBS, a mitogen-activated protein kinase kinase, is required for vegetative differentiation, cell wall integrity, and pathogenicity of the barley leaf stripe fungus Pyrenophora graminea. Gene, 696, 95–104.

Mattos, E. C., Silva, L. P., Valero, C., de Castro, P. A., Dos Reis, T. F., Ribeiro, L. F. C., Marten, M. R., Silva-Rocha, R., Westmann, C., da Silva, C. H. T. D., Taft, P., Al-Furaiji, C. A., Bromley, N., Mortensen, M., Benz, U. H., Brown, J. P., Goldman, G. H. 2020. The Aspergillus fumigatus phosphoproteome reveals roles of high-osmolarity glycerol mitogen-activated protein kinases in promoting cell wall damage and caspofungin tolerance. mBio, 11, e02962–02919.

Palacios D. S., Anderson T. M., Burker M. D. 2007. A post-PKS oxidation of the amphotericin B skeleton predicted to be critical for the channel formation is not required for potent antifungal activity. J. Am. Chem. Soc. 129, 13804–13805.

Rodriguez-Pena, J. M., Garcia, R., Nombela, C., Arroyo, J. 2010. The high-osmolarity glycerol (HOG) and cell wall integrity (CWI) signalling pathways interplay: A yeast dialogue between MAPK routes. Yeast 27, 495–502.

Saccharomyces Genome Database. Stanford University. Retrieved October 27, 2020, from https://www.yeastgenome.org

Wiederhold NP, Gil VG, Gutierrez F, Lindner JR, Albataineh MT, McCarthy DI, Sanders C, Fan H, Fothergill AW, Sutton DA. 2016. J Clin Microbiol 54, 168-171.

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