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Brief Note

Effect of the combinatorial iron-chelation and oxidative stress on the growth of *Aspergillus* species

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ABSTRACT

The growth of 14 *Aspergillus* strains belonging to nine species was studied under combinatorial deferriprone − H_2O_2 (iron-chelation − oxidative) stress. When deferriprone pretreated mycelia were subjected to even a weak oxidative stress, the growth inhibitory effect of iron-chelation stress was enhanced in 10 out of 14 strains. In contrast, oxidative stress pretreatment of conidia increased their deferriprone tolerance in 10 strains. Applying iron-chelators as antifungal agent or adjuvant can enhance the efficiency of the combinatorial iron withdrawal − oxidative stress strategy of our immune system and may reduce the survival of conidia escaped from the oxidative attack of pulmonary macrophages.

Most aspergilli are saprophytic molds occurring widely in our environment. Among them, *Aspergillus fumigatus* is the leading cause of invasive aspergillosis. The case fatality rate is high, typically 30–95%, and recently it was found that coinfection with COVID-19 increases it [1,2]. The human body, even in immunocompromised patients most susceptible to infections, is a stressful environment for microbes. Fungi have to cope with oxidative, nitrosative, or transition metal stresses; they have to adapt to oxygen, iron, zinc, and carbohydrate limitations; they must tolerate the temperature and pH of our tissues, as well as the possible presence of antifungal drugs if they want to survive in our body [3,4]. Previous studies demonstrated that stressors acting either simultaneously or sequentially can synergistically or antagonistically alter the stress tolerance of fungi [4], *e.g.,* iron-limitation stress increases oxidative stress susceptibility of *A. fumigatus* cultures [5]. Therefore, our immune system's strategy combining iron withdrawal with oxidative attack is an efficient way of control fungal growth [6,7]. It also concurs with the promising results reached by iron-chelation based antifungal therapies [8].

Here, we studied the growth inhibitory effect of combinatorial iron-chelation and oxidative stress treatments on 14 strains of 9 *Aspergillus* species (Table 1) [9,10]. *Aspergillus* strains were maintained on Barratt's minimal plates [11] at 24 °C, and conidia collected freshly from 6 d old cultures were used for the experiments. In some studies, agar plates were supplemented with deferiprone (DFP) at 1.35 or 2.25 mM final concentrations and conidia formed on these plates were used in the experiments. Diameters of conidia were determined by light microscopy. Iron-chelator and oxidative stresses were induced with deferiprone [8,12,13] and H_2O_2, respectively. Growth inhibitory effects of stress treatments were tested in 96-well microtiter plates at 37 °C. Each well contained 200 μl Barratt's minimal broth, DFP at 0, 0.45, 0.90, 1.35, 1.80, 2.25, or 3.60 mM final concentrations, H_2O_2 at 0, 0.625, 1.25, or 2.5 mM final concentrations as required, and 1000 conidia. Depending on the experiment, H_2O_2 was added to the cultures at inoculation or one day later. Growth was recorded at 600 nm (at 9 points in each well) on the third day. Growth inhibitory effects of DFP, or combined DFP − H_2O_2 treatments, were characterized with the lowest DFP concentration where the OD_{600} value did not exceed twice the starting value (MIC_{DFP}). Growth inhibitory effect of H_2O_2 was characterized with the relative growth value (OD_{600} of H_2O_2 treated cultures per OD_{600} of untreated cultures) measured in the absence of DFP. These data were used only to demonstrate that the selected H_2O_2 concentrations had no strong growth inhibitory effect after the three-day-long incubation time.

The iron-chelation tolerance (MIC_{DFP}) varied between 0.9 mM, and 3.6 mM depending on the strain (Table 1). The mean MIC_{DFP} of strains with "large" conidia (conidial diameter >3 μm; $MIC_{DFP} = 3.2 \pm 0.9$ mM) was significantly higher than that of the strains with "small" conidia (conidial diameter <3 μm;

Abbreviations: DFP, deferiprone.

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Table 1
Deferiprone (DFP) tolerance of different *Aspergillus* strains.

Strains	MIC^{a}_{DFP} (mM)	Relative growth in the presence of H_2O_2 (%)[b]		Conidium diameter[c] (μm)
		"conidium"	"mycelium"	
Aspergillus terreus NCCB IH2624	1.35[i]	38 ± 5[i]	45 ± 5[i]	1.9 ± 0.3[d]
Aspergillus fumigatus Af293[j]	1.80	57 ± 5[i]	94 ± 5	2.2 ± 0.3[d,e]
Aspergillus fumigatus SZMC3100[j]	0.90	82 ± 6[i]	97 ± 3	2.0 ± 0.2[d,e]
Aspergillus fumigatus SZMC3102[j]	0.90	88 ± 4[i]	85 ± 5[i]	2.0 ± 0.3[d,e]
Aspergillus fumigatus SZMC3104[j]	0.90	78 ± 3[i]	97 ± 4	2.0 ± 0.2[d,e]
Aspergillus fumigatus F.00056[j]	1.80	55 ± 6[i]	64 ± 3[i]	2.2 ± 0.2[d,e]
Aspergillus fumigatus F.00948[j]	1.35	32 ± 8[i]	48 ± 8[i]	2.0 ± 0.2[d,e]
Aspergillus fischeri CBS 544.65	1.35	99 ± 3	93 ± 5	2.6 ± 0.2[e]
Aspergillus nidulans FGSC A4	1.35	56 ± 8[i]	80 ± 6[i]	3.2 ± 0.2[f]
Aspergillus niger CBS 113.46	3.60	98 ± 2	92 ± 5	3.6 ± 0.3[f,g]
Aspergillus brasiliensis CBS 101.740	3.60	85 ± 6[i]	86 ± 4[i]	3.8 ± 0.5[f,g]
Aspergillus tubingensis CBS 134.48	3.60	85 ± 3[i]	97 ± 2	3.9 ± 0.4[g]
Aspergillus flavus CBS 128202	3.60	72 ± 3[i]	98 ± 3	4.0 ± 0.4[g]
Aspergillus oryzae Rib40	3.60	75 ± 4[i]	91 ± 3	4.8 ± 0.7[h]

[a] MIC_{DFP} values were determined in three independent experiments (with two technical replicates in each experiment) at 0 mM H_2O_2 concentration.
[b] Mean ± SD values of three independent experiments are presented. Growth recorded at 2.5 mM H_2O_2 is given in the percentage of growth of untreated cultures (relative growth). H_2O_2 was added at inoculation (before germination of conidia; "conidia") or at the first day of incubation (to the growing mycelia; "mycelium"). The relative growth values at 0.65 or 1.25 mM H_2O_2 concentrations were higher than 90% in each strain.
[c] A total of 100 conidia isolated from 10 independent plates were measured. The mean value of conidium diameters from the same plate was calculated and the mean ± SD value of the ten means is presented.
[d-h] –Means marked with the same letter do not differ significantly (one way ANOVA followed by Tukey post-hoc test; adj. p value < 0.05, n = 10) from each other.
[i] Growths of the H_2O_2 treated cultures were significantly lower (Student's t-test; $p < 0.05$, n = 3) than those of the untreated ones.
[j] *A. fumigatus* SZMC3100, SZMC3102, and SZMC3104 were isolated from human keratitis and are available in the Szeged Microbial Collection (University of Szeged, Hungary; http://www2.sci.u-szeged.hu/microbiology/collection.htm). *A. fumigatus* F.00056, and F.00948 were isolated from soil and are available in NCAIM (National Collection of Agricultural and Industrial Microorganisms; Budapest, Hungary; http://ncaim.uni-corvinus.hu). *A. fumigatus* Af293 (FGSC1100) was isolated from a patient with systemic aspergillosis.

MIC_{DFP} = 1.3 ± 0.4 mM) (Student's t-test; p = 0.0015). When *A. fumigatus* Af293 and *Aspergillus flavus* CBS 128202 were maintained under "iron-limited conditions" (*i.e.,* they formed conidia on DFP containing media), their conidia showed reduced DFP tolerance (Table 2). This is in line with the results of Kang et al. [14] who found that the environment of sporulating *A. fumigatus* cultures determines the properties of the germinating spores including their virulence. Our results can be easily explained by that conidiogenic hyphae help the accumulation of iron in the conidia formed [15] to aid their germination. On DFP containing plates, it was not efficient enough and the smaller iron stores of conidia reduced their success to survive under iron limited conditions. Our data suggest that species with "large", rather than "small", conidia may have a better chance of colonizing iron-limited habitats like the human body. However, small conidia have a much better chance of avoiding mucociliary clearance and reaching pulmonary alveoli. Therefore, there must be an optimal range for iron-limitation stress tolerance depending on conidial size that is the most optimal to colonize the human lung.

Table 2
Changes in the deferiprone (DFP) tolerance of different *Aspergillus* strains in the presence of H_2O_2.

Species	MIC_{DFP} (mM)[a]						
	H_2O_2 concentration (mM):						
	0	0.63	1.25	2.5	0.63	1.25	2.5
		"Pretreated with H_2O_2"			"Pretreated with DFP"		
Aspergillus terreus NCCB IH2624	1.35	1.35	0.90	0.45	0.90	0.45	0.45
Aspergillus fumigatus Af293	1.80	2.25	2.25	0.90	1.35	0.90	0.45
Aspergillus fumigatus Af293 (DFP)[b]	0.90	1.35	1.35	0.45	0.90	0.45	0.00
Aspergillus fumigatus SZMC3100	0.90	1.35	1.80	1.80	0.90	0.90	0.90
Aspergillus fumigatus SZMC3102	0.90	1.35	1.35	1.35	0.90	0.90	0.45
Aspergillus fumigatus SZMC3104	0.90	1.80	1.80	0.45	0.90	0.90	0.90
Aspergillus fumigatus F.00056	1.35	1.80	1.80	0.45	0.90	0.45	0.45
Aspergillus fumigatus F.00948	1.35	1.35	1.35	0.45	1.35	0.90	0.45
Aspergillus fischeri CBS 544.65	1.35	1.35	1.35	1.35	1.35	1.35	1.35
Aspergillus nidulans FGSC A4	1.35	1.80	1.80	1.35	0.90	0.90	0.90
Aspergillus niger CBS 113.46	3.60	3.60	3.60	3.60	3.60	3.60	3.60
Aspergillus brasiliensis CBS 101.740	3.60	>3.60	>3.60	3.60	1.35	1.80	1.80
Aspergillus tubingensis CBS 134.48	3.60	>3.60	>3.60	>3.60	1.80	1.80	1.80
Aspergillus flavus CBS 128202	3.60	>3.60	>3.60	>3.60	3.60	2.25	1.35
Aspergillus flavus CBS 128202 (DFP)[b]	1.80	2.25	2.25	1.35	1.80	1.35	0.90
Aspergillus oryzae Rib40	3.60	>3.60	>3.60	>3.60	3.60	2.25	1.35

[a] MIC_{DFP} values were determined in three independent experiments (with two technical replicates in each experiment) at 0 mM H_2O_2 concentration. H_2O_2 was added at inoculation (before germination of conidia; "pretreated with H_2O_2") or at the first day of incubation to the growing mycelia ("pretreated with DFP").
[b] *A. flavus* and *A. fumigatus* conidia used in these experiments were produced on Barratt's minimal medium supplemented ("DFP") or not ("control") with DFP at 2.25 mM and 1.35 mM final concentration, respectively.

Importantly, H_2O_2 had no strong growth inhibitory effect even at 2.5 mM final concentration (Table 1). Despite of this observation, the applied H_2O_2 treatment markedly modified the DFP tolerance of the strains (Table 2). The outcome of the experiments highly depended on how the two stressors (DFP and H_2O_2) were combined. If conidia that had already germinated in the presence of DFP were exposed to H_2O_2, the MIC_{DFP} values generally decreased (Table 2). In this case, mycelia suffering from iron (and probably other metal ion [16]) limitation were treated with oxidative stress ("pretreated with DFP"). The decreasing MIC_{DFP} values suggest that even low iron-chelation stress could enhance the growth inhibitory effect of H_2O_2. Oxidative stress sensitivity of iron-limited *A. fumigatus* cultures is a well-documented phenomenon [5,17]. Our data demonstrate that this phenomenon can be general within the *Aspergillus* genus which concurs well with the strategy of mammalian immune systems combining iron withdrawal with oxidative attack to suppress microbial growth. Moreover, the antifungal effect of iron chelators can be higher *in vivo* – where iron-chelation stress may combine with oxidative stress – than that based on *in vitro* studies. When H_2O_2 (final concentration 0.65 mM or 1.25 mM) was added together with DFP to the conidia, it surprisingly increased the MIC_{DFP} in most strains (Table 2). In this case, germinating conidia had to cope with oxidative stress relying on their own iron storages, and the negative consequences of iron-chelation stress was developed only following that ("pretreated with H_2O_2"). This observation suggests that adaptation to oxidative stress can counterbalance the growth inhibitory effect of iron-chelation stress. There are several data in the literature indicating that oxidative stress, depending on its type and strength, can either enhance or reduce iron acquisition [5,18–20]. The upregulation of iron uptake by oxidative stress could explain our results. This behavior suggests that inhaled conidia surviving the oxidative attack of alveolar macrophages may have better chance to germinate and penetrate into the lung tissue, despite of the iron-limited conditions occurring there. Actually, this may represent an Achilles' heel of the combined iron withdrawal – oxidative attack strategy of our immune system. However, making the iron-limitation stress stronger by applying iron-chelators as antifungal adjuvants may reduce the risk of the successful invasion of the host. Interestingly, those strains, where the growth inhibitory effect of H_2O_2 on conidia was strong (*Aspergillus terreus* NCCB IH2624, and *A. fumigatus* F.00948) or weak (*Aspergillus fischeri* CBS 544.65, and *Aspergillus niger* CBS 113.46) (Table 1), did not show this phenomenon (Table 2). It raises the possibility that if the oxidative stress tolerance is too weak or too strong, oxidative stress is unable to initiate the appropriate changes in the physiology of germinating conidia to enhance their iron-limitation stress tolerance.

In conclusion, our results suggest that opportunistic human pathogens not necessarily have superior stress tolerance attributes. Any stress tolerance that is within an appropriate range (i.e. neither too strong nor too weak) may support pathogenicity. Our results also confirm the validity of antifungal approaches based on iron-chelating agents.

Declaration of competing interest

No conflict of interest.

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