Current Medical Mycology

2024, 10: e2024.345251.1549

In vitro enrichment of trace elements promotes rapid germination of *Aspergillus* conidia: a clinical concern for immunosuppressed and hyperglycemic patients

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Article Info

Article Type:
Original Article

Article History:
Received: 20 Jun 2024
Revised: 12 Oct 2024
Accepted: 02 Nov 2024

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A B S T R A C T

Background and Purpose: Trace elements play crucial roles in metabolic processes and serve as cofactors for various enzymes, although their specific involvement in fungal pathogenesis remains unclear. This study aimed to explore the impact of essential trace elements, iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu), in conjunction with D-dextrose, on conidial germination and growth of *Aspergillus fumigatus* and *A. flavus*.

Materials and Methods: The research involved determining the minimum inhibitory concentrations (MIC) of Fe, Mn, Zn, and Cu for *Aspergillus* ATCC strains. The study commenced by determining the MIC of the four trace elements, followed by evaluating the impact of selected trace elements on the kinetic growth and germination rates of Aspergillus species by the micro-broth method. Following MIC assessment, optimized concentrations of the trace elements (~140 and 550 pM) and various concentrations of D-dextrose (1-3% w/v) were introduced to assess their effects on fungal growth in RPMI 1640 broth. Growth was measured in optical density, while conidial germination rates were also observed.

Results: The MICs for Fe, Mn, and Zn exceeded 35 µM, while Cu exhibited lower MICs of 2 and 7.6 µM against *A. fumigatus* and *A. flavus*, respectively. Fe, Mn, Zn, and Cu significantly enhanced fungal growth in both *Aspergillus* species at optimized concentrations. Additionally, growth rates increased proportionally with higher D-dextrose concentrations. Notably, combining enriched trace elements and D-dextrose resulted in up to 98% conidial germination.

Conclusion: The findings demonstrate that optimized concentrations of essential trace elements and D-dextrose significantly promote conidial germination and growth of *Aspergillus* species *in vitro*. These results suggest that trace element supplementation might have important implications for immunocompromised and hyperglycemic patients. Further studies are warranted to explore the interactions between these micronutrients in fungal physiology and pathogenesis.

Keywords: Aspergillosis, *Conidia* germination, COVID-19, Diabetes, Immunocompromised patients, Trace elements

➢ **How to cite this paper**
Nikhil A, Tiwari A K, Tilak R, Bhatia M, Gupta M K. *In vitro* enrichment of trace elements promotes rapid germination of *Aspergillus* conidia: a clinical concern for immunosuppressed and hyperglycemic patients. Curr Med Mycol. 2024; 10: e2024.345251.1549. DOI: 10.22034/cmm.2024.345251.1549

Introduction

Aspergillus is a ubiquitous filamentous saprotroph that can cause life-threatening invasive fungal infections in immunosuppressed individuals [1]. Further, among the fungus that can lead to airborne infections, *Aspergillus* stands out as a primary contributor to indoor contamination in hospitals such as *A. niger* (11.28%) and *A. flavus* (8.95%) respectively [2]. *Aspergillus* conidia enter humans through inhalation and primarily affect the paranasal sinuses and lungs, where they manifest as severe asthma with fungal sensitization, allergic bronchopulmonary aspergillosis, sinus aspergillosis, chronic pulmonary aspergillosis, and invasive pulmonary aspergillosis (Figure 1) [3-6]. During the second wave of the SARS-CoV-2 pandemic, a significant surge in respiratory aspergillosis cases was noted particularly in India, manifested as fever, cough with or without hemoptysis, breathing difficulty, and pleuritic chest pain [7, 8]. Additionally, Healthcare centers have reported instances of candidiasis, aspergillosis, and fungal infections that introduce additional complications to the already lethal viral outbreak. Furthermore, other less common fungal infections have been documented, including

histoplasmosis, blastomycosis, pneumocystis pneumonia, and cryptococcosis [9]. A multifaceted interplay of elements results in systemic immune changes in individuals with COVID-19, leading to secondary infections that affect the overall survival rates of those affected [10]. This process significantly impairs innate immunity by decreasing the number of T lymphocytes, CD_4+, and CD_8+ cells in the body [11]. One of the primary clinical manifestations of COVID-19 is the presence of host iron in patients with mucormycosis. Owing to its angioinvasive nature, mucormycosis obtains its main iron source through heme, either intracellularly via heme oxygenase or through iron strips using a reductase-permease system [11, 12]. Furthermore, hyperferritinemic syndrome, a common manifestation in COVID-19 inflammatory responses, triggers a degenerative process that can eventually lead to the death of hepatic cells and the release of the stored Fe^{2+} extracellularly [13, 14]. Also, the virus itself may target hemoglobin or induce hepcidin dysregulation which can elevate the amount of free iron available for fungal acquisition, thereby facilitating mucormycosis in COVID-19 patients. An impaired immune system resulting from COVID-19 is believed to be a key factor in the development of fungal diseases. Although a clear and direct link between COVID-19 and mucormycosis has not been established. Further, research conducted by Muthu et al. 2022, revealed that zinc enhanced the *in vitro* growth of certain *R. arrhizus* isolates [15]. The researchers hypothesized that an observed increase in fungal biomass correlated with zinc levels could potentially facilitate the development of COVID-19-associated mucormycosis (CAM) in individuals with competent immune systems. In addition to diabetes mellitus and other conditions that suppress the immune system, zinc could be an unexpected factor behind the recent global increase in COVID-19-related mucormycosis. This potential link is due to patients self-administering zinc to boost their innate immunity, and healthcare providers overprescribing multivitamin supplements. However, further research is necessary to establish a definitive connection between zinc intake and the surge in mucormycosis cases [16]. Therefore, it is prudent to investigate the role of different trace elements supplementation on *Aspergillus* conidial growth and germination behavior. Hence, an *in vitro* study was performed to determine the enrichment effect of different trace elements, such as Fe, Mn, Cu, and Zn at variable concentrations, along with D-dextrose, on the conidial growth and germination behavior of *A. fumigatus* and *A. flavus* strains.

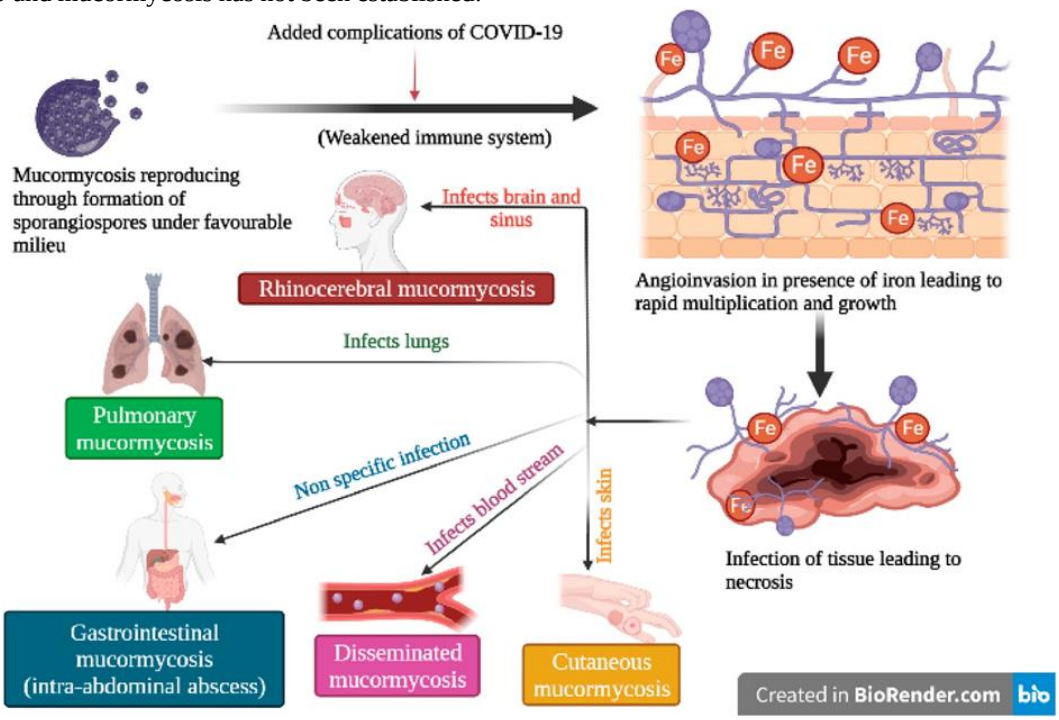

Figure 1. SARS-CoV-2 (COVID-19) associated mucormycotic infection. Adopted with permission [6].

Materials and Methods

Materials

Salt of trace elements (i.e., $FeSO_4$, $CuSO_4$, $MnSO_4$, $ZnSO_4$), and D-dextrose were procured from Sigma-Aldrich (USA). Fungal culture media potato dextrose agar (PDA), RPMI 1640 (Roswell Park Memorial Institute, Buffalo, NY, USA) without Sodium bicarbonate with Morpholinepropane sulfonic acid (MOPS) buffer, and L-Glutamine were procured from Hi-Media Laboratories Limited (India). Other solvents and plastic wares were purchased from Merck and Tarson Product Private Limited (India). It should be mentioned that all reagents used were of analytical grade.

Fungal strains, growth conditions, and harvesting of conidia

Lyophilized strains of *A. fumigatus* (ATCC 204305) and *A. flavus* (ATCC 204304) strains were acquired from the American Type Culture Collection (Rockville, MD, USA) and stored at -80°C. The fungal strains were revived on PDA medium and incubated at 37°C for 3-6 days. Freshly grown cultures were used for further experiments. *A. fumigatus* and *A. flavus* were sub-cultured over a PDA slant and incubated at 37°C for a week. Conidial harvesting was performed by adding 100 µL of Tween-20 (0.01% concentration), followed by adding 5 ml of normal saline with gentle shaking of the tubes. The resulting suspension was initially filtered using a vacuum membrane filter with a pore size of 0.45µm. The filtered suspension was pelleted at 4000 rpm for 6 min and washed twice with PBS to remove media and surfactant traces. Subsequently, the conidial suspension was adjusted to an optical density of 0.08-0.1 OD at 565 nm to a solution containing 10^6 conidia/ml.

Antifungal susceptibility test of selected trace elements

The Antifungal activity of the selected trace elements was evaluated by agar well diffusion against *A. fumigatus* and *A. flavus* strains, as described in Clinical and Laboratory Standards Institute (CLSI) M38-A3 guidelines. Briefly, freshly harvested conidia of both strains were adjusted to 10^6 conidia/mL. The prepared conidial suspension was swabbed over a freshly prepared Mueller-Hinton agar plate and left for 10 min to dry under a laminar hood followed by wells cutting with a sterile cork-borer. The well get poured with 50 µL of 100 nM voriconazole (positive control), 50 µL of distilled water (negative control), and 50 µL of 230 nM each of trace element salts ($FeSO_4$, $CuSO_4$, $MnSO_4$, and $ZnSO_4$) into each well. The plates were then incubated for 24 h at 37 °C, and the zone of inhibition was measured. The entire process was performed in triplicates for validation and reproduction.

Minimal Inhibitory Concentration determinations of trace elements

The MIC of the trace elements (Fe, Cu, Mn, and Zn) against *A. fumigatus* and *A. flavus* was determined by following CLSI M38 A3 guidelines. Briefly, a 40 µM stock solution of each trace element salt was prepared and serially diluted by following a two-fold serial dilution method in a sterile 96-well flat-bottom microtiter plate containing 100 µL of RPMI-1640 along with MOPS buffer into each well, except the 1st and 11th well. a 100µl suspension of 10^4 conidia/ml in RPMI-1640 was poured into all wells except the 11th well, followed by incubation at 37^0C for 48 h, and 100% growth inhibition was noted as the MIC value. The experiment was conducted in triplicate to ensure the reliability of the results.

Effect of trace elements and D-dextrose on Aspergillus growth by kinetic study

The effects of trace elements and D-dextrose (1-3% w/v) on the growth rate (depending on optical density) of *A. fumigatus* and *A. flavus* were monitored accordingly [16]. Initially, 230 µL of RPMI-1640 was dispensed into each well of a 96-well sterile flat-bottom microtiter plate, excluding the first column well. Subsequently, 10 µL (concentrated) of the trace element solution was added (making up to predetermined optimum conc.) to each well, except for the first column well, which served as a culture control. In the culture control well, 240 µL of RPMI-1640 was added without the trace elements. Further, 10 µL of freshly harvested conidia of *Aspergillus* spp. were dispensed into each well, including the first column well. A well-containing RPMI-1640 medium alone was used as the medium control. The same procedure was followed for D-dextrose with varying concentrations (1-3% w/v). Optical density was monitored for 8 hours at 37°C on a Multiskan Sky-High Plate Reader by recording the absorbance at 565 nm in triplicate. Subsequently, the mean OD values were calculated and plotted using Origin 8.5 software.

Effect of trace elements and D-dextrose on the germination rate of Aspergillus conidia

The effects of trace elements and D-dextrose on the conidial germination of *A. fumigatus* and *A. flavus* were evaluated. Briefly, 10^6/ml conidial inoculum was prepared in RPMI-1640, as described above followed by adding trace elements at concentrations of ~140 and 560 pM and D-dextrose (1-3% w/v) in a 15 ml sterile tube separately. Subsequently, the tubes were incubated at 150 RPM at 37 °C on an incubator shaker. Then, at 0, 2, 4, 6, and 8 h of incubation, 50 µL of culture was withdrawn from each tube, and germination was observed under a bright-field compound light microscope (Olympus, Tokyo, Japan) at 400X magnification. The germination percentage of conidia was calculated using the following formula followed by statistical graph plotting:

Spores' germination rate (%) = number of germinated conidia /total number of observed conidia

Statistical analysis

Data obtained from the in vitro supplementation of micronutrients from different growth rates of *Aspergillus* were analysed using the SPSS ver21. Therefore, we analysed the correlation coefficient relationships between multiple independent variables using Spearman's correlation coefficient. Supplementation with trace elements had a significant positive relationship. This shows that trace element supplementation affects the germination of *Aspergillus* conidia. Statistical analysis was also performed using the Kruskal-Wallis test, Friedman test, and Two-way ANOVA of non-parametric tests using Graph Pad Prism software 8.4. We used a non-parametric test because our data were distributed in numerical and categorical values, and it was more reliable with small sample sizes. Our data are also less sensitive to outliers and extreme values with significance (p-value<0.05), indicating that micronutrient supplementation had a potential effect on the growth of *Aspergillus* species.

Results

Antifungal activity of trace elements

The results demonstrated that all tested elements i.e., Fe, Mn, Zn, and Cu had not shown any significant zone of inhibition whereas positive control (voriconazole) showed 25 mm of the zone of inhibition in *A. fumigatus* and *A. flavus* zone of inhibition was 22 mm as shown in Figure 2. Further, the calculated MIC values of trace elements against *A. fumigatus* and *A. flavus* (as shown in Table 1) were more than 35 µM for $FeSO_4$, $ZnSO_4$, and $MnSO_4$ while, $CuSO_4$ demonstrated an inhibitory effect at a concentration of 2 and 7.6 µM respectively.

Effect of trace elements over Aspergillus growth rate by kinetic study

We found that the addition of trace elements ($FeSO_4$, $MnSO_4$, $CuSO_4$, and $ZnSO_4$) significantly enhanced the growth of *A. fumigatus* and *A. flavus* after 8 h of incubation compared to without enrichments, as shown in Figures 3 and 4. We determined the increased growth by comparing the optical density (OD) of RPMI-1640 alone with that of containing *Aspergillus* conidia with and without trace elements. Among these elements, Zn had a strong influence on the growth of *A. fumigatus* at a higher concentration of 532pM, as shown in Fig. 3b. However, at lower concentrations (≈140 pM), Zn and Mn had a stronger influence on growth than Fe and Cu, as shown in Fig 3a. At lower concentrations (≈140 pM), the elements promoted conidial growth of *A. fumigatus* to Mn>Zn>Fe>Cu (Figure 3a); however, at higher concentrations (≈550 pM), elements influenced growth as Zn>Fe>Mn>Cu, as shown in Fig. 3b. Moreover, the noted increase in growth rate was due to the enlarged size followed by germination of conidia. For this, we performed a microscopic examination to confirm *Aspergillus* conidial germination in the presence of different trace elements and D-dextrose. Furthermore, it has been noted that fungal conidia achieved the log phase shortly and were attributed to the rapid origin of germ tubes compared to the control culture. Similarly, the conidial growth behaviour of *A. flavus* was recorded after 8 h of incubation following enrichment with varying concentrations (≈140 and 550 pM) of elements, as shown in Figure 4. Unlike *A. fumigatus*, *A. flavus* demonstrated significantly enhanced growth in the presence of Zn ions at both concentrations (130.1 and 532.7 pM). The remaining elements had the least effect on conidial growth compared to the control culture.

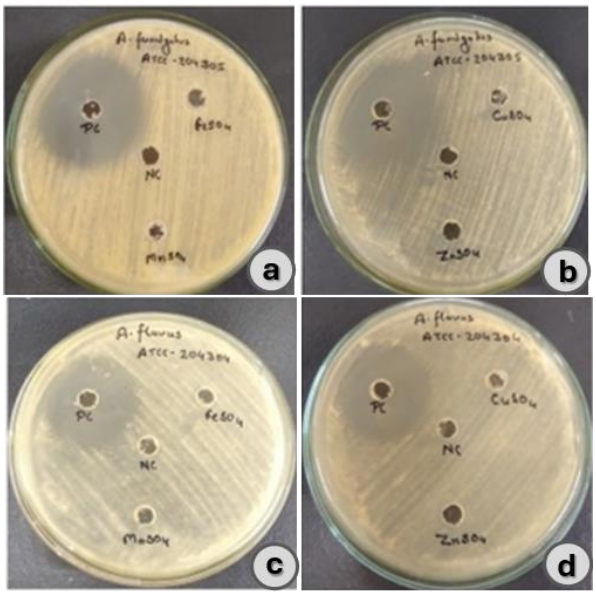

Figure 2. Antifungal evaluation of trace elements along with negative control (NC; Distilled Water) and positive control (PC; voriconazole) against *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus flavus* (ATCC 204304). (a & b), $FeSO_4$, $MnSO_4$, $CuSO_4$ and $ZnSO_4$ against *A. fumigatus*; (c & d), $FeSO_4$, $MnSO_4$, $CuSO_4$ and $ZnSO_4$ against *A. flavus*.

Table 1. Minimum inhibitory concentration of trace elements against *Aspergillus fumigatus* and *Aspergillus flavus*

Trace elements	MIC against *A. fumigates* (ATCC 204305)	MIC against *A. flavus*(ATCC 204304)
$FeSO_4$	>35µM	>35µM
$MnSO_4$	>35µM	>35µM
$CuSO_4$	2µM	7.6µM
$ZnSO_4$	>35 µM	>35 µM

ATCC: American Type Culture Collection

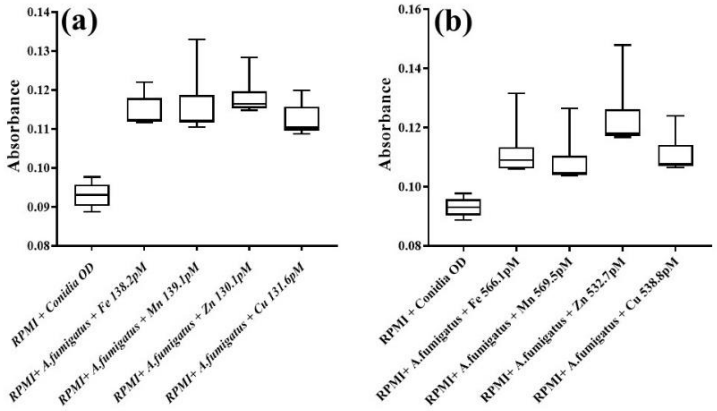

Figure 3. Whisker plot showing the effect of trace elements supplementation for 8 h over the conidial overall growth (optical density) of *Aspergillus fumigatus* at low (a) and (b) high (Fe, 566; Mn, 569.5; Cu, 538.8 & Zn, 532.7 pM) concentrations (Kruskal-Wallis Statistics, Spearsman Correlation of X (Time) value with each Y (Trace element) value, Friedman test Statistics and two-way ANOVA; $p < 0.0001$).

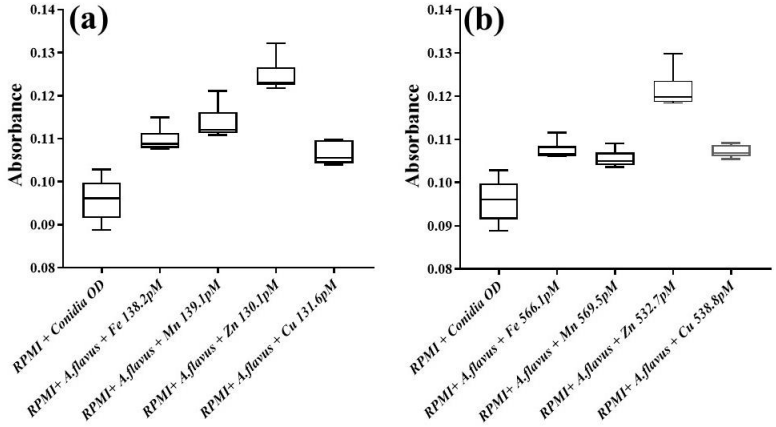

Figure 4. Whisker plot showing the effect of trace supplementation over the conidial growth rate of *Aspergillus flavus* both at low (a) and (b) high concentrations. (Kruskal-Wallis Statistics, Spearsman Correlation of X (Time) value with each Y (Trace element) value, Friedman test Statistics, and ordinary two-way ANOVA; $p < 0.0001$)

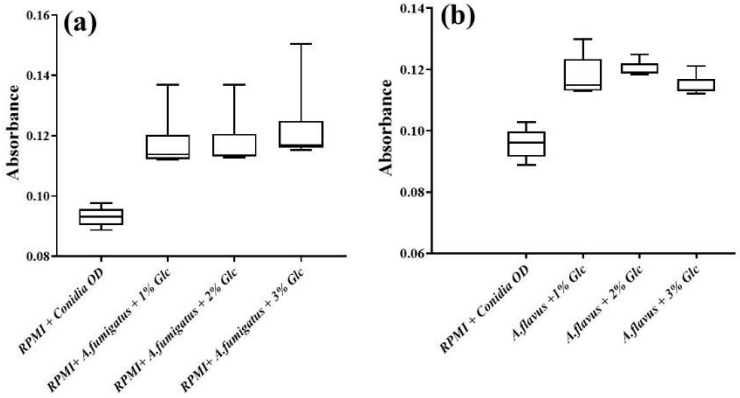

Figure 5. Whisker plot showing the effect D-dextrose supplementation over the conidial growth rate of A. fumigatus and flavus at variable (1-3% w/v) concentrations. (Kruskal-Wallis Statistics, Spearsman Correlation of X (Time) value with each Y (Trace element) value, Friedman test Statistics, and ordinary two-way ANOVA; $p < 0.0001$)

Effect of D-dextrose over kinetic growth of Aspergillus species

Dextrose plays an indispensable role in the growth and pathogenesis of filamentous fungi, such as *Aspergillus* spp. Therefore, the effect of varying concentrations of D-dextrose on *Aspergillus* conidial growth and germination was determined using kinetic analysis to understand the nutrient availability and growth behaviour. Similar to the results obtained with trace element enrichment, the growth rate was enhanced after

the addition of D-dextrose in a concentration-dependent manner. Interestingly, *A. fumigatus* demonstrated higher conidial growth (25-30 % increased optical density) at higher D-dextrose enrichment (3% *w/v*) as compared to lower concentrations (1% and 2%), as shown in Fig. 5a. While, *A. flavus* showed (10-15 %) increment at lower concentrations of 1% and 2% as compared to 3% D-dextrose depicted in Figure 5b.

Evaluation of the influence of trace elements and D-dextrose on conidial germination rate

The results demonstrated that the germination of *A. fumigatus* conidia was variable in the presence of a predetermined concentration of trace elements and D-dextrose, as shown in Fig 6a. However, *A. flavus* had a high germination rate in the presence of elements and D-dextrose, as shown in Fig 6b. Under *in vitro* conditions, conidial fungal germination did not follow a synchronous germination pattern. Therefore, we also observed that only 40-50% of the conidia of *A. fumigatus* and *A. flavus* germinate after 8h of incubation. Under enrichment conditions, in this case of *A. fumigatus*, 98% Mn and Zn promoted conidial germination, followed by Fe (~94%) and Cu (50%), as shown in Figure 6a. After enrichment with 1, 2, or 3% d-dextrose, conidial germination increased by 10, 20, and 80%, respectively. On the other hand, *A. flavus* demonstrated maximum conidial germination in the presence of Mn (98 %) followed by Zn (90 %) and Fe (88 %). However, *A. flavus* had slow germination in the presence of Cu (78 % at a lower concentration), as depicted in Fig 6b. Overall, in vitro enrichment of selected trace elements and D-dextrose promoted the synchronous germination of *A. fumigatus* and *A. flavus* conidia within 8 h of incubation.

Statistical Analysis

Data obtained from the *in vitro* supplementation of micronutrients from different growth rates of *Aspergillus* were analyzed using the SPSS software. Therefore, the correlation coefficient relationships between multiple independent variables were analyzed using Spearman's correlation coefficient. Supplementation had a significant positive relationship with trace elements. This shows that trace element supplementation affects the germination of *Aspergillus* conidia. Statistical analysis was also performed using the Kruskal-Wallis test, Friedman test, and Two-way ANOVA of non-parametric tests using GraphPad Prism software (version 8.4). A non-parametric test was used since the data were distributed in numerical and categorical values, and it was more reliable with small sample sizes. The collected data were also less sensitive to outliers and extreme values with significance (P<0.05), indicating that micronutrient supplementation had a potential effect on the growth of *Aspergillus* spp. Statistical data are provided in the supplementary form.

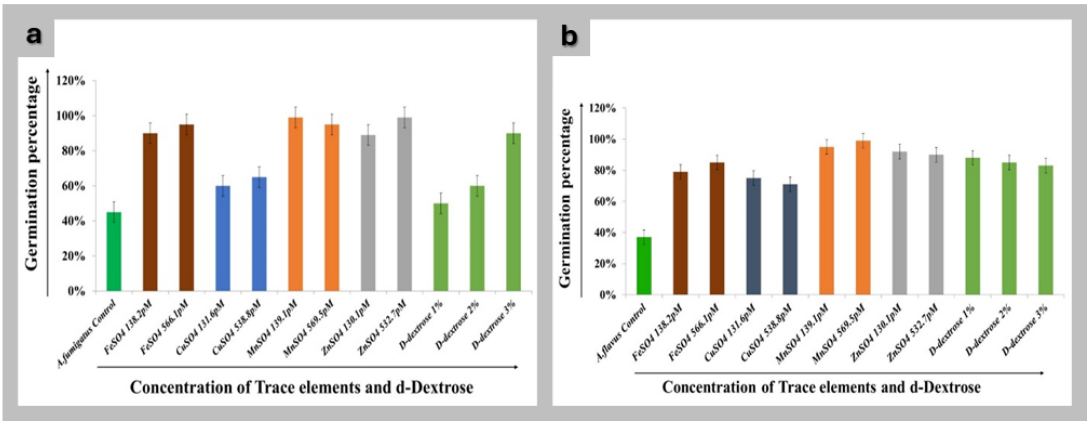

Figure 6. Graphical representation of conidial germination %age after the enrichment of variable concentration of trace elements and D-dextrose. (a) *Aspergillus fumigatus* and (b) *Aspergillus flavus*.

Discussion

Trace elements are indispensable nutrient factors required for the normal metabolism of fungal cells [17]. These elements are active centres of various enzymes and participate in the synthesis and metabolism of substances [18]. Moreover, trace elements have a significant influence on the stability of biomacromolecules and cell structures. They control the redox potential of cells and serve as an energy source for the proliferation of microorganisms [19]. Trace elements are also essential for conidial germination and play a key role in host-fungal interactions [20, 21]. Hence, the rationale behind this study was associated with observations during the second wave (April 2021-July 2021) of SARS-CoV-2 infection in India that, supplementation of zinc and iron resulted in an increased incidence of mucormycosis and aspergillosis in hyperglycaemic and immunosuppressed patients [22, 23]. Therefore, it is prudent to investigate the relationship between the growth behaviour and supplementation of trace elements in *Aspergillus* spp. However, iron is a cofactor for catalase and peroxidase enzymes that neutralize cell-damaging reactive oxygen species (ROS) generated during fungal metabolism [20]. The capacity of iron to readily exchange electrons between its two oxidation states (Fe2+ and Fe3+) has made it a valuable cofactor in redox biochemistry. Many enzymes depend on iron- or iron-containing cofactors to perform specific functions. These cofactors include

mononuclear or dinuclear nonheme-iron centers, iron-sulfur (Fe S) clusters, heme, and siroheme [24–26]. Moreover, fungal pathogens take up the supplemented iron through siderophores and utilize it in processes such as DNA replication, chromatin remodeling, and mitochondrial respiration, thereby enhancing fungal growth [27]. Under aerobic conditions, Fe forms complexes with low solubility. To address iron's limited bioavailability, a widely conserved strategy involves extracellular reduction of ferric iron to ferrous iron, enhancing its solubility and availability. In this process, plasma membrane metalloreductases, primarily FreB in *A. fumigatus* [28], convert ferric to ferrous iron for mobilization. Subsequently, a multicopper oxidoreductase, FetC in *A. fumigatus*, re-oxidizes ferrous iron to ferric iron for uptake through the ferric-iron transporter FtrA. Although these reduction and oxidation steps seem contradictory, their rationale remains unclear. Increased free iron has been reported as a predisposing risk factor for fungal infections [29]. Therefore, to prevent fungal infections, the human host has a stringent regulatory mechanism that makes iron as inaccessible as possible to fungal conidia, especially during infection and accompanying inflammation, such as overproduction of hepcidin and the presence of natural iron chelators, both of which reduce free iron availability and fungal virulence and pathogenicity (Figure 7) [20]. In mammals, iron exists mainly as heme in haemoglobin, ferric iron in the storage protein ferritin, ferric iron in the transport protein transferrin, and as Fe-S clusters and heme in enzymes. The innate immune system restricts iron to combat infections, a process called "nutritional immunity," which leads to inflammation-induced anemia [30 ,31]. Normally, plasma iron available to cells is bound to transferrin, typically only 30% saturated with iron, allowing efficient capture of free iron. However, pathological iron overload can exceed transferrin binding capacity, resulting in non-transferrin bound iron (NTBI), also known as enhanced labile plasma iron (eLPI). A recent study showed that eLPI promoted in vitro growth of *A. fumigatus* in serum from patients who had undergone hematopoietic stem cell transplantation [32]. During SARS-CoV-2 infection, reduced hepcidin levels and hyperferritinemia have been observed in patients with mucormycosis and aspergillosis [33, 34].

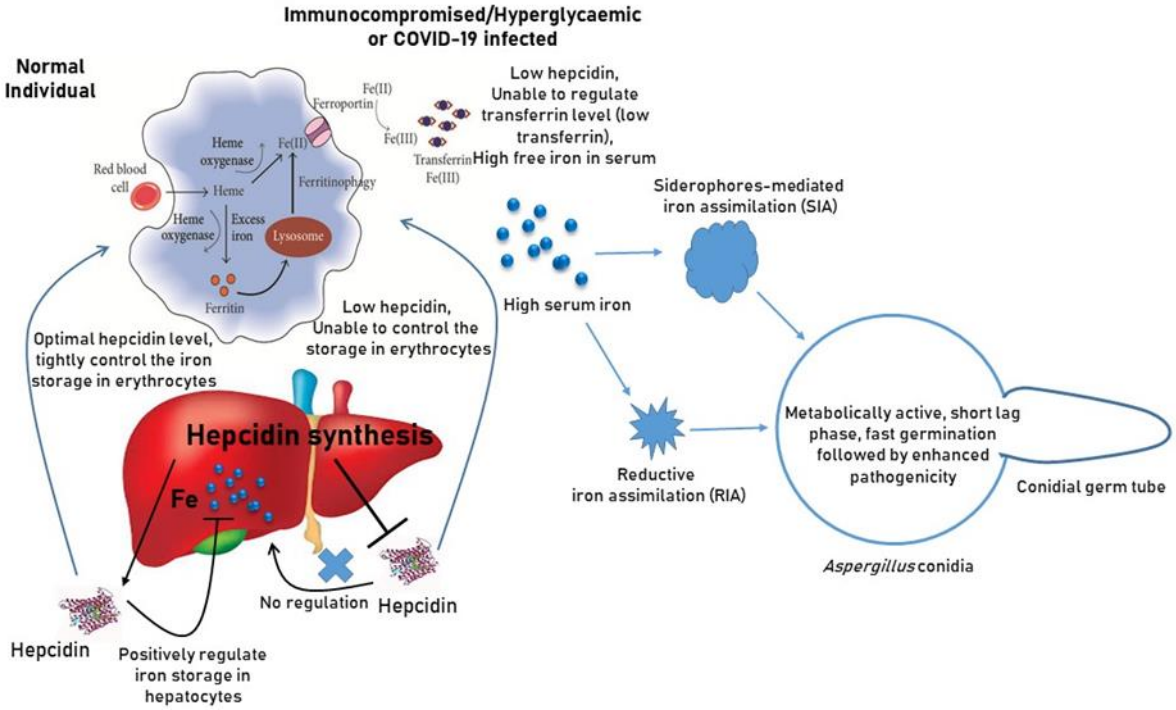

Figure 7. Simplified diagram of the relationship between host serum iron level and *Aspergillus* growth.

Additionally, LV et al. examined patients with severe COVID-19 and discovered that their serum transferrin levels were reduced [35]. These levels show an inverse relationship with the likelihood of negative outcomes. Specifically, elevated transferrin was linked to a decreased risk of severe disease, while diminished transferrin was associated with an increased risk of COVID-19 severity [35]. Hence, supplementation of iron plays a significant role in conidial growth and promotes rapid germination in *Aspergillus* spp. demonstrated in this *in vitro* study as depicted in (Figures 4 & 6). Therefore, iron depletion or chelation therapy in SARC-CoV-2 patients might positively influence the control of these fungal infections.

Similarly, Mn plays an indispensable role in the catalysis and functionality of various enzymes that regulate housekeeping biological processes, including oxidative phosphorylation, glycosylation, and signal transduction [36]. Specifically, Mn is required for fungal survival, growth, and virulence. In contrast, the

human host utilizes nutritional immunity to sequester Mn to prevent the establishment of these fungal infections [36]. Naturally, neutrophils produce metal ion-binding calprotectin, which is secreted into the microenvironment to sequester essential metals from pathogens [37]. These strategic pathways significantly reduce the levels of Mn in the serum and tissues, protecting the host from invasion by fungal pathogens [38]. Moreover, Mn potentially influences the innate immune system of the human host by increasing myeloid cells and NK cell activity, thereby facilitating a strong defense against fungal infections [39]. While there is no direct evidence supporting manganese's active role in mucormycosis, Clark et al. examined the impact of high manganese levels on promoting fungal keratitis. Their findings revealed an increase in manganese-binding calprotectin and demonstrated that neutrophils exhibited antihyphal activity [40]. However, under hyperglycaemic and immunocompromised conditions, the sequestration of Mn from serum and extracellular fluids is impaired, resulting in easy access to Mn by the *Aspergillus* [40]. Additionally, external supplementation with Mn negatively affects the immune system. Compared to iron, Mn had a slight influence on the conidial growth (biomass). While, Mn highly promoted the conidial germination in *A. fumigatus* as well as *A. flavus*, a higher conidial germination rate (99%) was observed at lower concentrations compared to that of the culture control (Figure 4 & 6). Overall, the presented data could be a decisive factor in establishing the relationship between Mn and fungal pathogenesis in immunocompromised as well as COVID-19 conditions however, a more detailed investigation is needed to establish the argument.

Similar to Fe and Mn, zinc is an important trace element, providing a favorable niche for fungal growth, as it regulates the DNA-binding proteins present in Class 3 zinc finger proteins (zinc cluster proteins). Class III zinc finger proteins, or zinc cluster proteins, feature a distinctive DNA-binding domain (DBD) with six cysteine residues linked to two zinc atoms, hence also known as zinc binuclear cluster or Zn(II)2Cys6 (Zn2C6) proteins [41, 42]. These transcription factors are unique due to their single zinc finger unit binding two zinc atoms and can interact with DNA as monomers, homodimers, or heterodimers [43]. Exclusively found in fungi, these proteins, like other transcription factors, include multiple functional domains beyond the cysteine-rich DBD, such as regulatory and activation regions [44]. Research shows zinc cluster proteins recognize elements with trinucleotide sequences, which may be single or repeated, and symmetrical or asymmetrical. CGG triplets are common, but variations in binding sites also exist [44]. Zinc cluster proteins can form homodimers and bind to CGG triplets in everted, inverted, or direct repeats. For proteins like Gal4p, Put3p, and Ppr1p, binding occurs at inverted repeats with zinc clusters of each monomer facing each other. Transcriptional activity regulation involves mechanisms such as nuclear-cytoplasmic shuttling, DNA binding, phosphorylation, and activation domain exposure [45]. In addition, zinc also mediates fungal virulence by enhancing the production of gliotoxins in the phagocytic pathway, which hampers fungicidal activity [46]. Zinc-binding proteins, such as superoxide dismutase (SOD), generate ROS during host-pathogen interactions [46]. Other zinc-binding proteins, such as aspartic proteases, serine proteases, and metalloproteases, cause tissue degradation, with subsequent fungal invasion and dissemination. In humans, zinc homeostasis is induced by the natural defense system, where specific membrane transporters or zinc-binding proteins regulate zinc uptake [47]. Zn intake was associated with fungal infections during the COVID-19 pandemic. Interestingly, Kumar et al., noted a significant correlation between zinc consumption and COVID-19-associated mucormycosis, with 89.1% of affected individuals reporting zinc intake compared to 52% of the control group. Additionally, diabetes mellitus showed a strong association with COVID-19-related mucormycosis, affecting 83.6% of cases versus 16% of controls. These findings suggest that diabetes mellitus is a considerable risk factor for developing COVID-19-associated mucormycosis [16]. Moreover, reduced serum zinc levels have been reported to suppress fungal growth [48]. Our findings support a previous report stating that zinc levels modulate the pathogenicity of *Aspergillus*. Thus, the administration of trace amounts of zinc under in vitro conditions potently promoted the growth and conidial germination rate of both species of *Aspergillus*.

Aspergillus spp., an opportunistic human pathogen, requires copper as an essential micronutrient. Its survival and pathogenicity rely on maintaining proper copper balance [49]. A sophisticated network, managed by copper-responsive transcription factors AceA and MacA, regulates the response to environmental copper to sustain equilibrium. *A. fumigatus* uses copper exporters to mitigate toxicity, while also encoding importers and small molecules to ensure sufficient copper for vital processes like nitrogen acquisition and respiration. Recently identified isocyanides produced by *A. fumigatus* may contribute to copper homeostasis, potentially binding copper similarly to bacterial isocyanides copper chelators [49]. Further copper is a cofactor in the cytochrome C oxidase enzyme, which is involved in respiratory chains, and plays a role in reductive iron assimilation, superoxide anion detoxifying superoxide dismutase, and melanin formation [50]. In *A. fumigatus*, the protein CycA, is a widespread eukaryotic enzyme crucial for energy production. It functions by catalysing the final reaction in the electron transport chain [51]. During respiration, a harmful reactive oxygen species (ROS) called superoxide is generated as a by-product. To neutralize this toxin, organisms employ copper-containing superoxide dismutase (SODs) [52]. In fungi, these copper SODs transform superoxide into hydrogen peroxide. Subsequently, an iron-dependent catalase converts this hydrogen peroxide into water and oxygen

[53]. We observed significantly enhanced growth rate and germination with ~ 140 and 550 pM CuSO4. However, the MIC values (2 and 7.6µM) for copper ions were noted against *A. fumigatus* and *A. flavus* respectively. Our findings suggest that a lower concentration of Cu enhances fungal growth and promotes rapid germination. The effects of different concentrations of D-dextrose on the conidial growth and germination behaviours of *A. fumigatus* and *A. flavus*. Interestingly, an enhanced growth rate was recorded in proportion to sugar concentration compared to culture control. A higher growth rate was observed with 3% D-dextrose in strains of *Aspergillus*. D-dextrose provides energy for conidial germination, as a combination of D-glucose and water has been reported to initiate conidial germination in *A. nidulans* [54]. In addition, D-mannitol and D-trehalose are mobilized during the initial phases of germination, where D-trehalose metabolizes and produces D-glucose, which promotes germination [55]. It should be noted that diabetic patients are 1.38-fold more prone to fungal infections as hyperglycaemia induces fungal growth [56] and, on the other hand, raised sugar levels hamper the degranulation and neutrophil extracellular traps (NET) required for microbial killing [57]. In addition, higher sugar levels weaken natural immune barriers, thereby promoting fungal adhesion and invasion. It also increased ROS levels and pro-inflammatory cytokines, subsequently inhibiting phagocytic activity by affecting complement receptors [58]. Therefore, in this *in vitro* study, all tested essential trace elements have promoted conidia growth and germination, even at low concentrations however, there is a need for *in vivo* correlation. Additionally, it is also needed to study the interplay between trace elements and host immunity in fungal infections for future research work.

Conclusion

In conclusion, we concluded that essential trace elements (Fe, Zn, Cu, and Mn) enrichments, *A. fumigatus*, and *A. flavus* conidia had demonstrated a shortened lag phase and grew faster with enhanced germination rate under *in vitro* conditions. Similarly, D-dextrose pushes a proportionate conidial growth and germination rate. Consequently, healthcare providers should exercise caution when prescribing corticosteroids or immunomodulators to diabetic patients and routinely monitor them for mucormycosis symptoms and signs. Given the significant link between essential elements intake and COVID-19-associated mucormycosis, medical professionals should limit the use of trace elements-containing multivitamins and other metals that may promote fungal growth. However, additional research is necessary to firmly establish the relationship between these elements and COVID-19-associated mucormycosis, as well as to investigate the potential use of metal chelators in treating mucormycosis.

Acknowledgments

The authors are thankful to the Head of the Department of Microbiology of Instrument Facility, Institute of Medical Science, BHU. For availing the facility of the Multi-scan-sky High Plate Reader and Bright Field light Microscope, the authors are also thankful to Mr.Zinnu Rain and Mr. Alakh Narayan Singh for their kind support.

Authors' contributions

A. N. and A. K. T. contributed equally; the Study was planned by M. K. G., R. T., and M. B.; A.N., A. K. T. performed experiments and drafted the manuscripts. AN and A. K. T. analyzed the data. M. K. G., and R. T., supervised the experiment; M. K. G., R. T., M. B., and A. K. T. revised the manuscript. A. K. T. edited the final version of the manuscript; All authors read and approved the submitted version.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper and consent for its publication.

Financial Disclosure

Dr. M.K. Gupta has been awarded an Incentive Grant under the Institution of Eminence program, as per reference R/Dev/D/IoE/seed and Incentive grant_III/2022-23/49235, acknowledging outstanding contributions and providing support for continued excellence in research and development.

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