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A morphological, enzymatic and metabolic approach to elucidate apoptotic-like cell death in fungi exposed to *h*- and α- molybdenum trioxide nanoparticles

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Supporting information

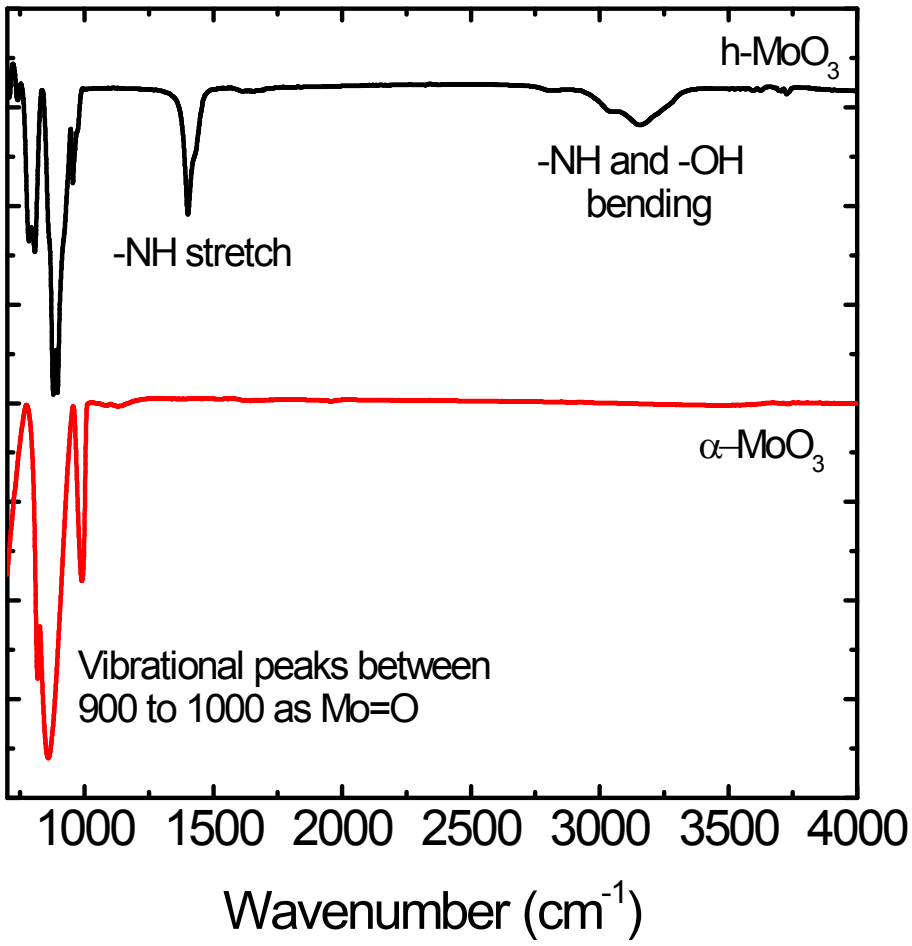

Figure S1. FTIR spectra of MoO_3 (h and and α-MoO_3). The spectrum of h-MoO_3 shows a peak of the -NH and -OH bending distinguishing h-MoO_3 from α-MoO_3, which does not possess this peak.

Table S1. Enzymes tested in this study and detected through API ZIM test

Enzyme	Substrate
Phosphate alkaline	2-nephtyl phosphate
Esterase (C4)	2-naphthyl butyrate
Esterase lipase	2-naphthyl caprylate
Lipase (C14)	2-naphthyl myristate
Lucine arylamidase	L-leucyl-2-napthylamide
Valine arylamidase	L-valyl-2-napthylamide
Cystine arylamidase	L-cystyl-2-naphtylamide
Trypsine	N-benzoyl-L-arginine-2-napthylamide
Chymotrypsine	N-glutaryl-phenylalanine-2-naphtylamide
Acid phosphatase	2-naphthyl phosphate
Napthol-AS-BI-phosphohydrolase	Napthol-AS-BI-phosphate
α-galactosidase	2-naphthyl-α-D-galactopyranoside
β-galactosidase	2-naphthyl-β-D-galactopyranoside
β-glucoronidase	Napthol-AS-BI-β-D-glucopyranoside
α-glucosidase	2-naphtyl-α-D-glucopyranoside
β-glucosidase	2-naphtyl-β-D-glucopyranoside
N-acetyl-β-glucosaminidase	1-naphtyl-N-acetyl-β–D-glucosaminide
α-mannosidase	6-Br-2-naphthyl-α-D-manopyranoside

Stability of the nanoparticles in the Czapek media (dissolution experiment)

The sterile czapek medium (pH 4.63) was filtered through 0.22 μm pore size filter (VWR). The sterile *h*-MoO_3 and α-MoO_3 were used for the dissolution experiment at 200 mg L^{-1} concentration. The dissolution experiments were done as described in table S2. For the dissolution in the supernatant obtained after growing the fungi (the spent media), both fungi were grown under the same conditions as described in the growth inhibition section. The growth cultures were centrifuged and then the supernatant was filtered using 0.22 μm syringe filter to remove all fungal cells. The supernatants were used as the media for one of the dissolution experiments (Table S2

sample 16 to 21). The pH of czapek before and after growth as well as all the solutions used in the experiment were recorded for the dissolution experiment set-ups. The reason we did different experimental setups is because the fungi secrete metabolites and byproducts that change the composition of the media, as well as the pH. These changes could have impacted the dissolution of the nanoparticles. A total of 23 samples were prepared for this experiment, including controls (Table S2). All the samples were incubated at 28 ^{o}C for 10 days, which was the length of the inhibition experiment for the fungi. After the incubation period, all the samples were filtered with a EMD Millipore Amicon™ Ultra-15 Centrifugal Filter Units before analysis to remove all the nanoparticles and leave only the ions. The concentration of MoO_3 ions from the samples were analyzed by using atomic absorption spectroscopy (AAS, Perkin Elmer, U.S.A.). In addition, the dissolution of the MoO_3 was also analyzed with DI water. This experiment is to show the different dissolution behavior of these nanoparticles under different pH and media conditions used in this investigation. The experiment was conducted with the highest concentration used in this study, meaning 200 mg L^{-1} of nanoparticles.

Table S2 Dissolution samples with corresponding pH in the experiment

	Medium used for dissolution experiments	**pH**	**Nanomaterial Concentration**	**Note**
1	Czapek media as prepared	4.63	None	Control (blank)
2	Czapek media as prepared	4.63	200 mg L^{-1} *h*-MoO_3	Dissolution experiment
3	Czapek media as prepared	4.63	200 mg L^{-1} α-MoO_3	Dissolution experiment
4	Supernatant of *A. niger* spent media (media with biomolecules secreted by *A. niger*)	2.57	None	Control (blank)
5	Supernatant of *A. niger* spent media	2.57	200 mg L^{-1} *h*-MoO_3	Dissolution experiment
6	Supernatant of *A. niger* spent media	2.57	200 mg L^{-1} α-MoO_3	Dissolution experiment
7	Supernatant of *A. flavus*	4.63	None	Control (blank)

	spent media (media with biomolecules secreted by *A. flavus*)			
8	Supernatant of *A. flavus* spent media	4.63	200 mg L^{-1} *h*-MoO_3	Dissolution experiment
9	Supernatant of *A. flavus*	4.63	200 mg L^{-1} α-MoO_3	Dissolution experiment
10	Czapek as prepared with pH adjusted to the final pH of *A. niger* spent media (no biomolecules secreted from any fungi)	2.57	None	Control (blank)
11	Czapek as prepared with pH adjusted to the final pH of *A. niger* spent media (no biomolecules secreted from any fungi)	2.57	200 mg L^{-1} *h*-MoO_3	Dissolution experiment
12	Czapek as prepared with pH adjusted to the final pH of *A. niger* spent media (no biomolecules secreted from any fungi)	2.57	200 mg L^{-1} α-MoO_3	Dissolution experiment
13	Czapek as prepared with pH adjusted to the final pH of *A. flavus* spent media (no biomolecules secreted from any fungi)	4.63	None	Control (blank)
14	Czapek as prepared with pH adjusted to the final pH of *A. flavus* spent media (no biomolecules secreted from any fungi)	4.63	200 mg L^{-1} *h*-MoO_3	Dissolution experiment
15	Czapek as prepared with pH adjusted to the final pH of *A. flavus* spent media (no biomolecules secreted from any fungi)	4.63	200 mg L^{-1} α-MoO_3	Dissolution experiment
16	Czapek+ *A. niger* mycelium	4.63	None	Control (blank)
17	Czapek+ *A. niger* mycelium	4.63	200 mg L^{-1} *h*-MoO_3	Inhibition experiment condition
18	Czapek+ *A. niger* mycelium	4.63	200 mg L^{-1} α-MoO_3	Inhibition experiment condition
19	Czapek+ *A. flavus* mycelium	4.63	None	Control (blank)
20	Czapek+ *A. flavus*	4.63	200 mg L^{-1} *h*-	Inhibition experiment

	mycelium		MoO_3	condition
21	Czapek+ *A. flavus* mycelium	4.63	200 mg L^{-1} α-MoO_3	Inhibition experiment condition
22	DI	6.3	200 mg L^{-1} *h*-MoO_3	Dissolution experiment
23	DI	6.3	200 mg L^{-1} α-MoO_3	Dissolution experiment

Results and discussions

All the prepared samples showed no presence of ions in the media after 10 days, except for the DI water (pH 6.3) that showed high dissolution after 24 hrs. The dissolution results for the nanoparticles in the media were all under the detection limit of the AAS instrument. The low dissolution observed can be expected and explained by the range of pH in the growth media. The pH of the media is acidic, since the optimal pH for *Aspergillus* spp. growth is in the range of 2 to 6.[1-3] The analysis of the spent media (the pH of the media at the end of the fungal growth) made the media even more acidic in the case of *A. niger* (pH 2.57). For *A. flavus,* the pH remained the same as the initial pH of the media (i.e. pH 4.63) (see table S2). In previous studies, the high dissolution rate of MoO_3 was reported to happen at neutral and high pH values. When the pH is shifted to more acidic, MoO_3 is more stable in terms of the hydrolysis process.[4] In addition, the stability of the nanoparticles can be explained by the nanoparticles being coated by biomolecules in the complex media. Media and fungal supernatants contain large amounts of macromolecules such as carbohydrates, proteins, lipids, and enzymes (as seen by the enzymatic and also VOC analysis of the spent media, i.e. the media supernatant after growing the fungi for 10 days). Therefore, complex surface interactions between macromolecules and nanoparticles can be developed in the fungi growth media. These interactions could inhibit the dissolution of MoO_3 in the media by coating the nanoparticles. This observation has been previously discussed under different environmental contexts.[5] Therefore, the dissolution results confirmed that there is no

significant dissolution of MoO_3 nanoparticles in the czapek medium under 10 days, ruling out the potential toxic effects of ions released by the MoO_3 nanoparticles. In the case of MoO_3 in DI water (pH 6 to 6.5), there was 38% and 10% of dissolution of MoO_3 found after 24 h. This result confirms that MoO_3 nanoparticles are not stable at pH close to neutral and tend to dissolve into molybdenum ions. However, in acidic medium and complex environments, such as the growth conditions used for fungi, these nanoparticles have different behavior and have low (below the detection limit of the instrument) to no dissolution.

Table S3 Dissolution of MoO_3 results

Medium	**Nanomaterials**	**Molybdenum ionic concentrations (mg L^{-1}) after 10 days**
Czapek	None	Blank
Czapek	200 mg L^{-1} *h*-MoO_3	Not detected
Czapek	200 mg L^{-1} α-MoO_3	Not detected
Supernatant *A. niger*	None	Blank
Supernatant *A. niger*	200 mg L^{-1} *h*-MoO_3	Not detected
Supernatant *A. niger*	200 mg L^{-1} α-MoO_3	Not detected
Supernatant *A. flavus*	None	Blank
Supernatant *A. flavus*	200 mg L^{-1} *h*-MoO_3	Not detected
Supernatant *A. flavus*	200 mg L^{-1} α-MoO_3	Not detected
Czapek pH adjusted	None	Blank
Czapek pH adjusted	200 mg L^{-1} *h*-MoO_3	Not detected
Czapek pH adjusted	200 mg L^{-1} α-MoO_3	Not detected
Czapek pH adjusted	None	Blank
Czapek pH adjusted	200 mg L^{-1} *h*-MoO_3	Not detected
Czapek pH adjusted	200 mg L^{-1} α-MoO_3	Not detected
Czapek+ *A. niger* mycelium	None	Blank
Czapek+ *A. niger* mycelium	200 mg L^{-1} *h*-MoO_3	Not detected
Czapek+ *A. niger* mycelium	200 mg L^{-1} α-MoO_3	Not detected
Czapek+ *A. flavus* mycelium	None	Blank
Czapek+ *A. flavus* mycelium	200 mg L^{-1} *h*-MoO_3	Not detected
Czapek+ *A. flavus* mycelium	200 mg L^{-1} α-MoO_3	Not detected
DI water (24 h)	200 mg L^{-1} *h*-MoO_3	75.3 mg L^{-1} detected after 24 h
DI water (24 h)	200 mg L^{-1} α-MoO_3	20 mg L^{-1} detected after 24h

References

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