Evaluation of fungi exposure to CuO-NPs in *in vitro* tests

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Objectives:

Copper Oxide Nanoparticles (CuO-NPs) are a promising alternative to conventional Cu^{2+}-based fungicides due to enhanced adhesion and controlled Cu^{2+} release[1,2]. However, the fate, transformations and performance of NPs-based fungicides are highly modulated by the biochemical conditions of the surrounding environment, notably by the presence of fungi [3]. It is expected that the fungi will be the main driver to CuO-NPs dissolution due to the secretion of organic compounds, decreasing the surroundings' pH[4].

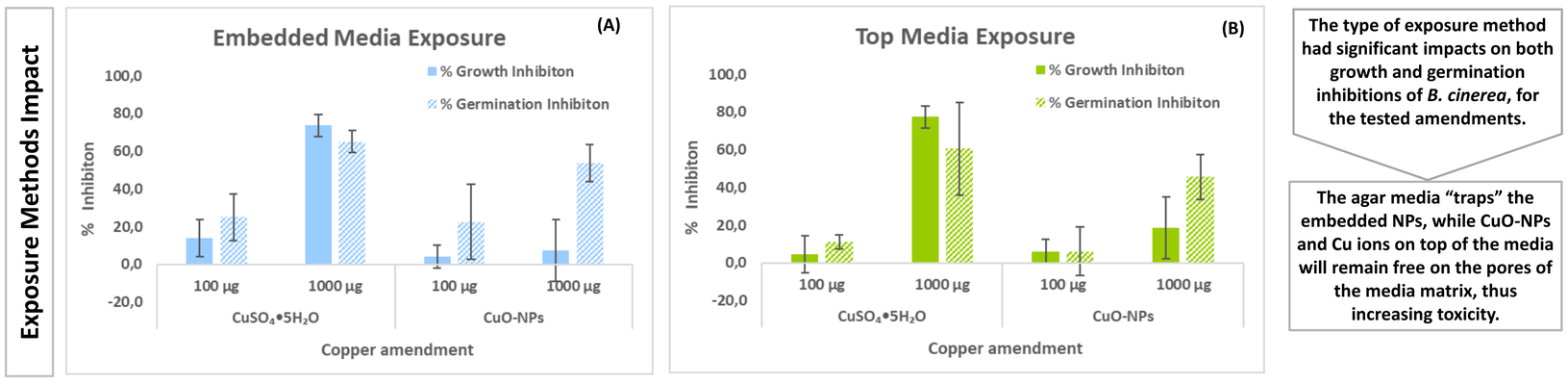
The main objective of this study was to assess the impact of applying variable exposure conditions for similar Cu doses in the toxicity response of *Botrytis cinerea* in CuO-NPs *in vitro* tests. Two Cu exposure methods (Top Deposition VS Media Embedded) and two inoculation strategies (Mycellium VS Spores) were tested to this end.

Methods & Results:

Cu exposure dosages: **100 and 1000 µg/plate;**
Copper formulations: **$CuSO_4$ and CuO-NPs;**

Exposure Methods:

- **Top Media Deposition: deposition of 1 mL of Cu amendment on top of PDA plates media, or**
- **Embedded Media: 1 mL of each Cu formulation was added to the media before solidifying.**



The type of exposure method had significant impacts on both growth and germination inhibitions of *B. cinerea*, for the tested amendments.

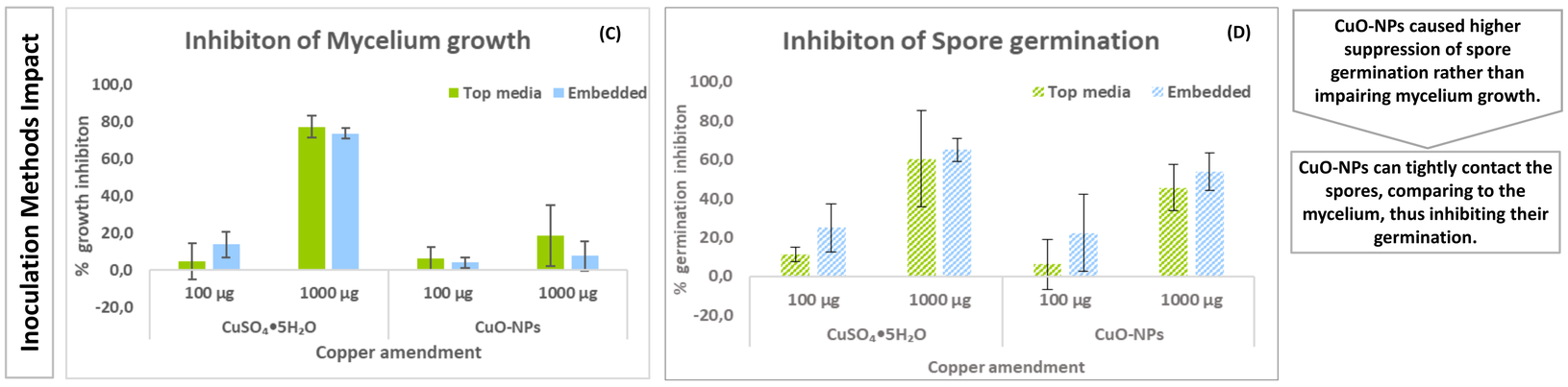
The agar media "traps" the embedded NPs, while CuO-NPs and Cu ions on top of the media will remain free on the pores of the media matrix, thus increasing toxicity.

Figure 1 – Percentage of growth and germination inhibition according to the exposure method: Embedded Media (A) and Top Media (B) Exposure.

Botrytis cinerea Inoculation Methods*:*

- **Mycelium inoculation: 5 mm-plug placed on top of the PDA plates;**
- **Spore suspension: 100 µL ($1x10^5$ spores/mL) spreaded on top of the plates.**

Plates were incubated at 25ºC. Mycelium diameter was measured at 5 days and spores were counted after 24h, resulting in % of growth inhibition and % of spore germination data, respectively.



CuO-NPs caused higher suppression of spore germination rather than impairing mycelium growth.

CuO-NPs can tightly contact the spores, comparing to the mycelium, thus inhibiting their germination.

Figure 2 - Influence of different exposure methods on mycelium growth (C) and spore germination (D).

Conclusions:

1. Fungal Cu toxicity depended on the formulations tested: $CuSO_4$ Toxicity > CuO-NPs Toxicity, and on the tested exposure method: Top media Toxicity > Embedded media Toxicity. This was associated with NPs and ions immobilization in the agar.

2. The variability observed in these results highlights the importance of considering the complex behavior, and chemical transformations of CuO-NPs along time when performing *in vitro* assays. Particularly, when toxicity responses are reported.

Future work will focus on the assessment of the copper extractable from the culture media as a proxy for the copper dose with an effective fungicide effect.
As the transformations that NPs undergo in the presence of pathogens and within the crop system remain unclear [1, 2], proper *in vitro* assays will help to predict the behavior of CuO-NPs within the plant system, aiming for more efficient *in vivo* experiments.

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