Physiological effects and mode of action of ZnO nanoparticles against postharvest fungal contaminants

Davide Sardella[a,b], Ruben Gatt[c], Vasilis P. Valdramidis[a,b,*]

[a] Department of Food Studies and Environmental Health, Faculty of Health Science, University of Malta, Malta
[b] Centre for Molecular Medicine and Biobanking, University of Malta, Malta
[c] Metamaterials Unit, Faculty of Science, University of Malta, Malta

ARTICLE INFO

ABSTRACT

Increasing concerns continue to be expressed about health hazards and environmental pollution resulting from the use of conventional fungicides for postharvest disease control. Nanoparticles represent an alternative solution for postharvest disease management. The objective of this work was to assess the physiological effects and the antifungal efficiency of ZnO nanoparticles (ZnO NPs) against a number of fungal contaminants. The efficacy of ZnO NPs was qualitatively and quantitatively assessed against: *Penicillium expansum*, *Alternaria alternata*, *Botrytis cinerea* and *Rhizopus stolonifer*. Mycelium growth diameters were measured onto Potato Dextrose Agar (PDA) plates loaded with different ZnO NPs concentrations (from 0 mM to 15 mM). Hereafter, the rate of the fungal diameter increase was quantified by linear regression modelling. Microscopic analysis was performed by scanning electron microscopy (SEM) images of agar plugs excised from plates with 0 mM and 12 mM ZnO. All the fungi were inhibited by ZnO NPs at concentrations higher than 6 mM. SEM images showed clear morphological aberrations in the fungal structures of all the isolates grown in presence of ZnO. Additionally, knowing that the chelating agent EDTA sequesters metal ions, it was added to fungal inoculated PDA plates with ZnO to study the NPs' mode of action. Cultures where ZnO was mixed with EDTA showed a decrease in the antifungal effect of the nanoparticles. In conclusion, ZnO NPs are therefore a good candidate as an effective postharvest disease control antifungal agent.

1. Introduction

Fruit losses due to fungal diseases may occur at any time during postharvest handling. Postharvest disease losses cause important reductions in fruit quantity and quality thus rendering products unsaleable or decreasing their value. Besides the economic considerations, consumption of diseased fruit also implies potential risks for human health. Among the fungal genera responsible for postharvest diseases, some of the most common and important ones are: *Penicillium*, *Rhizopus*, *Botrytis* and *Alternaria* (Sardella et al., 2016; Snowdon, 1990; Sutton, Aldwinckle, Agnello, & Walgenbach, 2014). Postharvest diseases usually generate from either latent infections which remain quiescent until fruit ripening or from infections initiated during and after harvest through surface wounds created by mechanical or insect injury (Sutton et al., 2014). An example of postharvest disease arising from quiescent infection is grey mold, caused by *Botrytis cinerea* (Coates, Johnson, & Dale, 1997). Common wound infections include blue mold, caused mainly by *Penicillium expansum*, and wet rot caused by *Rhizopus stolonifer* also known just as "Rhizopus rot"(Siefkes-Boer, Boyd-Wilson,

Petley, & Walter, 2009). *Penicillium expansum* is one of the oldest described *Penicillium* species and it has been established as the main cause of spoilage of pome fruits (Pitt & Hocking, 2009) while *R. stolonifer* is a common wound pathogen of a very wide range of fruits and vegetables causing a rapidly spreading rot (Sardella et al., 2016). In the case of *Alternaria* spp., fruit may be infected before or after harvest, although fruit inoculated up to 7 weeks before harvest do not develop symptoms within two months of storage at 0 °C (Sutton et al., 2014).

Traditional strategies for postharvest disease control and prevention include mainly the use of fungicides. In the postharvest phase, fungicides are often applied to control infections already established on the surface tissues or to protect against infections which may occur during storage and handling (Coates et al., 1997). Increasing concerns about the use of fungicides continue to be expressed regarding their health hazards, their impact on environmental pollution and the proliferation of resistant biotypes of fungal pathogens (Palou, Ali, Fallik, & Romanazzi, 2015). It is therefore important to explore novel approaches which may replace current synthetic fungicides (He, Liu, Mustapha, & Lin, 2011).

The advent of nanotechnology, which involves the manufacture and use of materials with size up to 100 nm, has brought great opportunities for the development of new antimicrobial agents (Espitia et al., 2012). In recent years, nanoparticles (NPs) have received increased interest thanks to their unique physical and chemical properties (He et al., 2011; Rai, Yadav, & Gade, 2009) and their strong antimicrobial properties (Pati et al., 2014). Among the different available types of nanoparticles, silver (Ag), gold (Au) and zinc oxide (ZnO) have demonstrated pronounced antibacterial activity (Pati et al., 2014). Since the use of Ag and Au on industrial scale is limited due to their high cost, current research is mainly focused on ZnO as a novel antimicrobial agent (Pati et al., 2014). Although some forms of zinc, including zinc oxide, are listed in the generally recognised as safe substances (GRAS) by the U.S. Food and Drug Administration (FDA, 2016; Savi, Bortoluzzi, & Scussel, 2013) concern is still expressed about potential hazardous effects of metal nanoparticles, especially in the case of silver (Ag) nanoparticles which seem to be the most toxic ones among other metals (*i.e.* copper or zinc) (Bondarenko et al., 2013). Nevertheless, ZnO NPs seem also to be hazardous in experimental animals mainly by inhalation, while skin penetration by ZnO NPs contained in sunscreens, currently the major source of human exposure, is negligible (Vandebriel & De Jong, 2012). Hitherto, the production and use of nanoparticle-containing products is currently not internationally regulated (Bondarenko et al., 2013) and more experimental data are needed in order to establish safety limits based on the different commercial applications.

The exact mechanism of the antimicrobial activity of nanoparticles still needs to be elucidated (Moritz & Geszke-Moritz, 2013). One of the possible cytotoxic mechanisms may result from the uptake of nanoparticles by bacterial cells since they are able to penetrate their cell wall (Martínez-Gutierrez et al., 2012). Other cytotoxicity mechanisms may derive from the release of ions from the NPs surface as it happens in the case of AgNPs (Rai et al., 2009) where the exposure of bacterial cells to silver ions induces changes in cell membrane leading to the enhancement of its permeability and consequent damage (Barani, Montazer, Samadi, & Toliyat, 2012). Nanoparticles may also generate reactive oxygen species (ROS) inducing membrane lipid peroxidation and consequent cell damage. These mechanisms are also connected to the NPs' toxicity to biological systems, including eukaryotic cells (Bondarenko et al., 2013), therefore the extent of the *in vivo* use of nanoparticles as antimicrobial agent needs to be limited in a way to still preserve the antimicrobial activity but also to avoid undesired damage to non-target eukaryotic systems (Seil & Webster, 2012).

In the case of fungi, Savi et al. (2013) investigated the antifungal properties of Zn-compounds against the mycotoxin-producing fungi *Fusarium graminearum*, *Penicillium citrinum* and *Aspergillus flavus* with their possible mechanism of action by scanning electron microscopy (SEM) and detection of the produced ROS. In their study, they observed deformed fungal hyphae with ruptures and unusual bulges after Zn-compounds treatment. Such morphological aberrations were associated with cell damage due to an increase in the production of ROS, thus leading fungal cells to death (Savi et al., 2013). ZnO NPs, with an average diameter of 70 ± 15 nm, were also found to successfully inhibit the grow of *B. cinerea* and *P. expansum* by affecting their cellular functions and inducing fungal hyphae distortion (He et al., 2011). Other relevant studies had focused on qualitatively analyzing selected fungi (Gunalan, Sivaraj, & Rajendran, 2012; Kairyte, Kadys, & Luksiene, 2013). Nevertheless, no information is available in literature about the morphological aberrations occurring on fungal spores, which are the main cause of cross-contamination during postharvest storage. Furthermore, to the knowledge of the authors, there are no morphological studies investigating the effect of ZnO NPs treatment against *Rhizopus stolonifer* and *A. alternata*, despite the former being of the most rampant and fast-growing fruit rot fungus (Pitt & Hocking, 2009) and the latter a fungus producing toxigenic metabolites that can accumulate in food thus becoming a hazard to human health (Pose, Patriarca, Kyanko,

Pardo, & Fernández Pinto, 2009).

Evidently, more studies are required in order to assess the responses of a series of fungal contaminants in presence of low size ZnO nanoparticles and the role of Zn^{2+} ions in their efficacy. These studies will need to be carried *in vitro* before considering further *in vivo* or *in situ* assessments.

The objective of the current study is to investigate the antifungal activity of ZnO nanoparticles against the postharvest fungi of *P. expansum*. *B. cinerea*, *R. stolonifer* and *A. alternata*. The responses of these fungi, in the presence of ZnO, are quantitatively and qualitatively assessed while ions chelation studies are performed in order to elucidate the possible mechanisms responsible for antifungal activity.

2. Materials and methods

2.1. Inoculum preparation

Postharvest disease fungi *Penicillium expansum*, *Botrytis cinerea*, *Rhizopus stolonifer* and *Alternaria alternata* used in this study were kindly provided by the fungal collection of the Postharvest Pathology group of IRTA (Spain). Fungal spores were harvested from 7-day old Malt Extract Agar (MEA) (Biolife, Milano, Italy) cultures by flooding the plates with a 0.05% Tween-80 solution and by scraping off the plates' surface with a sterile bent rod. The resulting suspension was aseptically filtered through a 4-layer sterile gauze to remove any mycelial contamination. Final spores' concentration was adjusted to 10^5 spores/mL with a haemocytometer for all the fungi.

2.2. Nanoparticles' suspensions preparation

ZnO nanopowder (< 50 nm particles size, > 97%, Sigma Aldrich, U.S.A., formula weight 81.39 g/mol, product code 677450) was suspended into 100 mL of sterile water. The nanoparticles' suspensions were then placed into an ultrasonicating bath for 30 min at 37 kHz sonicating frequency (Elmasonic S60, Elma, Singen, Germany) in order to break nanoparticles' aggregations. Resulting working concentrations were between 0 to 15 mM. The different levels were chosen such as the obtained fungal responses could be then used to estimate precise and accurate parameter estimates of the model presented in Section 2.4.

2.3. Medium preparation

This study was carried out on Potato Dextrose Agar (PDA)prepared from raw potatoes following the procedure shown by Pitt and Hocking (2009) in order to get a more satisfactory potato infusion than commercially dehydrated forms (Pitt & Hocking, 2009; Rinaldi, 1982). The refraction index of the potato infusion was measured with a refractometer (Atago, Japan) and was found to be $n_D = 1.3340 \pm 0.0001$ at 25 °C. The autoclaved PDA medium, was cooled down to ≈ 55 °C, poured into sterile 9-cm Petri dishes and then mixed, by an orbital shaker, with ZnO NPs suspensions in order to reach the final concentrations of 3, 6, 12 and 15 mM in a final volume of 20 mL per each plate. An additional batch of plates filled with PDA without ZnO NPs was also prepared as control. Finally, four perpendicular diameters were drawn onto the plates' surface for performing the fungal growth testing.

2.4. Diameter extension measurement

All the PDA plates were inoculated in the center with 10 μL of the previously obtained spores' suspension of each fungus. After drying, plates were enclosed into sterile polyethylene bags to prevent medium desiccation and incubated at 25 °C. Growth diameters were measured twice a day. Results (collected at last in triplicate) were plotted into graphs of diameters against time and then fitted to a linear model in order to estimate the fungal growth rate for to the different