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# Antifungal effect of engineered silver nanoparticles on phytopathogenic and toxigenic *Fusarium* spp. and their impact on mycotoxin accumulation

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ABSTRACT

Cereal grains are essential ingredient in food, feed and industrial processing. One of the major causes of cereal spoilage and mycotoxin contamination is the presence of toxigenic *Fusarium* spp. Nanoparticles have immense applications in agriculture, nutrition, medicine or health but their possible impact on the management of toxigenic fungi and mycotoxins have been very little explored. In this report, the potential of silver nanoparticles (AgNPs) (size 14–100 nm) against the major toxigenic *Fusarium* spp. affecting crops and their effect on mycotoxin accumulation is evaluated for the first time. The studied *Fusarium* spp. (and associated mycotoxins) were *F. graminearum* and *F. culmorum* (deoxynivalenol, 3-acetyldeoxynivalenol and zearalenone), *F. sporotrichioides* and *F. langsethiae* (T–2 and HT–2 toxins), *F. poae* (nivalenol), *F. verticillioides* and *F. proliferatum* (fumonisins $B_1$ and $B_2$) and *F. oxysporum* (mycotoxins no detected). The factors fungal species, AgNP dose (range 2–45 μg/mL), exposure time (range 2–30 h) and their interactions significantly influence spore viability, lag period and growth rate (GR) in subsequent cultures in maize-based medium (MBM) of all the studied species. The effective lethal doses ($ED_{50}$, $ED_{90}$ and $ED_{100}$) to control spore viability and GR were in the range 1–>45 μg/mL depending on the remaining factors. At high exposure times (20 − 30 h), the three effective doses ranged 1–30 μg/mL for all the studied species. At the end of the incubation period (10 days) mycotoxin levels in MBM cultures inoculated with fungal spores from treatments were strongly related with the size reached by the colony at that time. None of the treatments produced stimulation in conidia germination, GR or mycotoxin biosynthesis with respect to controls. Thus, the antifungal effect of the assayed AgNPs against the tested *Fusarium* spp. suggests that AgNPs could be a new antifungal ingredient in bioactive polymers (paints, films or coating) likely to be implemented in the agro-food sector for controlling these important toxigenic *Fusarium* spp. and their main associated mycotoxins.

## 1. Introduction

Mycotoxins are secondary fungal metabolites mainly produced by toxigenic species of *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria*. Infections of cereal grains by toxigenic fungi result in severe reductions in crop yield, mycotoxin contamination and economical losses thus limiting the marketability of grain supply worldwide (European Union, 2018). For a long time, the Food and Agriculture Organization has estimated that approximately 25% of the cereal-based foods produced in the world are contaminated with mycotoxins (FAO, 2004). Currently, this percentage could be higher due to climate change and to new estimations of mycotoxin occurrence in cereals and by-products by rapid methods that have become increasingly popular due to their high sample throughput and multi-analyte capabilities (Ogara et al., 2017; Romera et al., 2018).

Although fungi can collectively produce hundreds of mycotoxins, only few of them are subject to regulation and they are a serious global problem (Marasas et al., 2008). Most of the regulated mycotoxins in cereals and their by-products are produced by *Fusarium* spp. The distribution and growth of species belonging to the genus *Fusarium* and occurrence of the associated mycotoxins are closely related to climate factors, such as temperature and moisture (Desjardins, 2006; Xu et al., 2008), and their prevalence is consequently expected to increase as a result of present and future climate change (Medina et al., 2017; Moretti et al., 2019).

Fusarium head blight or scab in small-grain cereals (wheat, barley, oats, rye and triticale) and stalk and ear rot in maize are devastating and recurrent diseases affecting crops worldwide. They are caused by different *Fusarium* spp., especially, *F. graminearum* and *F. culmorum* (Bottalico and Perrone, 2002; Logrieco et al., 2002; Parry et al., 1995)

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and other phytopathogenic species, such as *F. oxysporum*, known to cause root rot in >100 relevant plant species (Agrios, 2005). In addition, various mycotoxins can accumulate in the seeds, among them, deoxynivalenol (DON), their 3– and 15–acetyl-derivatives (3–AcDON and 15–AcDON) and zearalenone (ZEA) (Eckard et al., 2011; Fredlund et al., 2013; Ogara et al., 2017; Oliveira et al., 2017). Generally, these fungi and secondary metabolites are accompanied by other relevant fungal species and their major mycotoxins. They are *F. sporotrichioides* and *F. langsethiae* and type-A trichothecenes, including T–2 and HT–2 toxins (Fredlund et al., 2013; Mateo et al., 2011, 2013; Thrane et al., 2004), *F. poae* and nivalenol (NIV) (Martin et al., 2018; Thrane et al., 2004), *F. verticillioides* and *F. proliferatum* and fumonisin $B_1$ ($FB_1$) and fumonisin $B_2$ ($FB_2$) (Cendoya et al., 2018; Choi et al., 2018; Oliveira et al., 2017).

All these mycotoxins pose significant hazards to human and animal health but only DON, ZEA and $FB_1$ + $FB_2$ are currently regulated in cereals and cereal derivatives in the European Union (European Commission, 2006, 2007). For T–2 + HT–2, recommendations about permissible levels have been published (European Commission, 2013). The International Agency for Research on Cancer (IARC, 1993) has classified DON, ZEA, $FB_1$ + $FB_2$, and T–2 + HT–2 into groups, 3, 3, 2B and 3, respectively. These mycotoxins are compounds with nephrotoxic, hepatotoxic, immunosuppressive and mutagenic properties, among others (Escrivá et al., 2015). Thus, prevention and control of these toxigenic *Fusarium* spp. and mycotoxin biosynthesis are priority objectives in food security and food safety.

Currently, there are several approaches to reduce these risks such as good agricultural and manufacturing practices (Luo et al., 2018), biocontrol (Nguyen et al., 2017) or the use of chemical antifungal agents (Mateo et al., 2011, 2013). However, the problems associated with fungi and mycotoxins persist, and new and additional strategies for an integrated management of toxigenic fungi in food and feed are required.

The rapid development of nanotechnology has transformed many domains of food science, especially those that involve agricultural practices, processing, packaging, storage, transportation, functionality and other safety aspects of food (Bajpai et al., 2018; Duhan et al., 2017; Hamad et al., 2018; Hannon et al., 2015; Jo et al., 2015; King et al., 2018; Mishra et al., 2014). In this context, metal nanoparticles (NPs) (Ag, Cu, Zn, Au, Ti…) and metal-based composites (ZnO, $SiO_2$, $TiO_2$, TiN…), with antimicrobial properties are used and exploited in the food industry to improve the properties of food packaging and other active materials (Bumbudsanpharoke and Ko, 2015; Carbone et al., 2016; Hannon et al., 2015). Among them, engineered silver nanoparticles (AgNPs) are currently the most studied and the most effective against a wide range of bacteria, yeasts, molds and viruses (Khezerlou et al., 2018; Rai et al., 2009). AgNPs exhibit low volatility and stability at high temperatures, can be hosted in different matrices, polymers and stabilizing agents. They show better antimicrobial properties than metallic silver due to their extremely large surface area, which can provide a better contact with the microorganism (Carbone et al., 2016; Hannon et al., 2015).

The main drawback of NPs in food technology is its possible migration from the hosted polymer to the food, by contact. Therefore, this issue has been the focus of considerable attention (Carbone et al., 2016; Souza and Fernando, 2016; Störmer et al., 2017). For silver, EFSA have provided upper limits for Ag migration (EFSA, 2011). Recommendations are not to exceed 0.05 mg/L in water and 0.05 mg/kg in food. This implies that precise and urgent estimations of the minimum effective doses of AgNPs to control growth of target microorganisms in food are necessary. To date, very little research focused on the effect of NPs on toxigenic fungi growth and mycotoxin production has been carried out (Abd-Elsalam et al., 2017).

The aims of the present study were: i) to get effective engineered silver nanoparticles (AgNPs) for controlling phytopathogenic and toxigenic *Fusarium* spp. and mycotoxin production using $AgNO_3$, sodium borohydride and sodium citrate, and ii) to evaluate the effect of the synthesized AgNPs on the growth of target fungal species and on the biosynthesis of their main associated mycotoxins. The fungi and mycotoxins included in the study were *F. graminearum* and *F. culmorum* (DON, 3–AcDON, and ZEA), *F. sporotrichioides* and *F. langsethiae* (T–2 and HT–2), *F. poae* (NIV), *F. proliferatum* and *F. verticillioides* ($FB_1$ and $FB_2$) and *F. oxysporum* (the used strain was previously assayed did not produce any of the studied mycotoxins).

## 2. Materials and methods

### 2.1. Reagents and standards

Silver nitrate ($AgNO_3$) (>99.9% pure), and standards of mycotoxins DON, 3–AcDON, NIV, ZEA, $FB_1$, $FB_2$, T–2, and HT–2 were purchased from Sigma-Aldrich (Alcobendas, Spain). Yeast extract was from Panreac (Montcada i Reixac, Barcelona, Spain). Sodium borohydride ($NaBH_4$, >99% pure), trisodium citrate (TSC, 99% pure), sodium hydroxide, ammonium formate and Tween 80 were from Merck (Darmstadt, Germany). All reagents supplied were of analytical grade. Acetonitrile, formic acid, ethanol, methanol (all LC grade) were from J.T. Baker (Deventer, the Netherlands). Pure water was obtained from a Milli-Q Plus apparatus (Millipore, Billerica, MA, USA).

### 2.2. Synthesis of AgNPs

AgNPs were obtained according to the method reported by Agnihotri et al. (2014) for synthesizing nanoparticles with a mean size of 30 nm. $NaBH_4$ was as used as a primary reductant while TSC was both a secondary reductant and a stabilizing agent. The reduction processes were carried out at two temperatures: 60 °C first and 90 °C afterwards, mediated predominantly by $NaBH_4$ and TSC, respectively. One hundred and eighty mL of 0.001 M $NaBH_4$ and 0.0055 M TSC was heated at 60 °C for 30 min in the dark under stirring. Afterwards, 20 mL of a 0.004 M solution of $AgNO_3$ was added drop wise. The temperature was raised to 90 °C and the pH was set to 10.5 using 0.1 M solution of NaOH. Heating proceeded for 20 min under continuous stirring. The AgNP suspension was cooled at room temperature and was centrifuged (12,000 × *g*, 20 min). The supernatant was carefully removed to avoid taking the nanoparticles located on the bottom and discarded. Then, the NPs were washed with pure water and centrifuged under the same conditions six times to remove the unreacted reagents and most products of the reaction (borate, excess citrate, ionic silver, *etc.*). Ionic silver was not detected in the supernatant using the turbidity test with concentrated solution of NaCl. Finally, the AgNPs were suspended in pure water and the pH was 6.6 ± 0.1. The suspension was finally stored at 4 °C when not in use. A small aliquot of fresh suspension was separated for characterization.

### 2.3. AgNP characterization

#### 2.3.1. Characterization by single particle inductively coupled plasma-mass spectrometry (SP-ICP-MS)

The reagents used for SP-ICP-MS characterization were a certified standard of Ag of 1000 mg/L (High-Purity Standards, Charleston, SC, USA) and a standard of gold nanoparticles (50 nm diameter) and $3.51 \times 10^{10}$ AuNPs/mL (Sigma-Aldrich). A standard of 1 mg Ag/L was prepared by dilution of the certified AgNP standard. Standards of AuNPs (50 nm) were also prepared by successive dilution of the concentrate AuNP standard. The original standard and all dilutions were sonicated for 10 min. The samples were prepared as described for AuNP standards.

The conditions and parameters for ICP-MS characterization were: Instrument: ICP-MS Agilent model 7900, with concentric nebulizer type Micromist, a Scott-type spray camera, Pt-cone interphase, double lens off-axis, hyperbolic quadrupole as mass filter and octopole collision/

reaction cell using He and $H_2$, respectively. Plasma gas (Ar) flow rate (L/min) 15; auxiliary gas flow (L/min) 1.0; RF power (W) 1550; RF matching (V) 1.80; sampling depth (mm) 8.0; nebulizer pump (rps) 0.1; carrier gas (L/min) 1.07; sample uptake rate (mL/min) 0.35; plasma mode: Low matrix; spray chamber temperature (°C) 2.0; integration parameters: Acquisition mode TRA (Time Resolved Analysis Mode); integration time/mass (ms) 3; acquisition time (s) 60; cell parameters: Isotope monitored $^{107}$Ag; cell gas He; cell gas flow (mL/min) 4.0; energy discrimination (V) 2.0; octopole RF (V) 200.

#### 2.3.2. Characterization by transmission electron microscopy (TEM)

The suspension of AgNPs was examined by TEM to obtain the distribution of nanoparticle sizes by a complementary method. A JEOL JEM-1010 (JEOL Co., Ltd., Tokyo, Japan) electron microscope of accelerating voltage 40–100 kV coupled to a digital camera AMT RX80 (8 Mpx) was used. A drop of the AgNP suspension in pure water was deposited onto a Formvar/carbon film 300-mesh copper grid. The solvent was allowed to evaporate at room temperature.

### 2.4. Fungal strains and inocula preparation

Isolates of the species *F. graminearum* (Fg22), *F. culmorum* (Fc15), *F. sporotrichioides* (Fs4), *F. langsethiae* (Fl021), *F. poae* (Fp11), *F. oxysporum* (Fo11), *F. proliferatum* (Fp16) and *F. verticillioides* (Fv27) were previously isolated from cereals grown in Spain except *F. langsethiae* that was isolated from oats grown in Germany (Schleswig-Holstein region) and marketed in Spain. Identification of fungi was carried out by conventional and PCR protocols following the methodology described by Gil-Serna et al. (2013). All strains are held in the Mycology and Mycotoxins Group Culture Collection (Valencia University, Spain). Before carrying out the study, the strains were grown in Yeast Extract Sucrose (YES) medium (20 g agar, 20 g yeast extract, 150 g sucrose, 1 g $MgSO_4{\cdot}7H_2O$, 1000 mL water). The medium was autoclaved at 115 °C for 30 min and poured into Petri dishes. The strains were inoculated on the center of the plates and incubated at 28 °C for 7 days. Spores of these fresh cultures were used to prepare inocula for further experiments on the efficacy of AgNPs to control fungal growth and mycotoxin production.

### 2.5. Impact of AgNPs on spore viability and colony growth

#### 2.5.1. Preparation of culture medium

To determine the effect of AgNPs on fungal growth, maize–based medium (MBM) (3% w/v of milled maize grains + 2% w/v agar in pure water), a representative matrix based on cereal was used (Tarazona et al., 2018). Previous analysis of maize grains ensured they had undetectable mycotoxin levels. MBM was autoclaved in Erlenmeyer flasks at 115 °C for 30 min. Water activity ($a_w$) level in the sterile medium was measured in a flask control and adjusted in all flasks to 0.99 by addition of sterile glycerol/water using a moisture adsorption curve for MBM previously determined. A RTD-502 unit (Novasina GmbH, Pfäffikon, Switzerland) was used to perform all $a_w$ measurements. Then, the medium was poured into Petri dishes (25 mL/dish).

#### 2.5.2. Treatments

Contact assays between fungal spores and AgNPs at different doses and exposure times were carried out. To this purpose, tubes containing 5 mL of sterile pure water and Tween 80 (0.005%) without AgNPs (controls) and tubes containing 5 mL of water and non-ionic surfactant Tween 80 (0.005%) and supplemented with 2, 5, 10, 15, 30 and 45 μg AgNPs/mL (treatments) were prepared. Controls and AgNP suspensions were inoculated with $1 \times 10^5$ spores/mL of fresh fungal cultures (7-day old) and vortexed for 30 s by a Stuart vortex mixer. Controls and treatments were placed in an orbital shaker (Infors-HT Aerotron, Bottminghen, Switzerland) at 60 rpm and 25 °C for 30 h. To evaluate the stability of the suspension during the exposure period of the spores to AgNPs the spectrum of a diluted suspension in water containing Tween 80 (0.005%) was recorded over a 30-h period in a quartz cuvette (1.0 cm) from 300 to 800 nm. An Agilent 8453 diode array spectrophotometer was used to this purpose.

#### 2.5.3. Evaluation of individual spore viability after treatments

To assess the number of viable spores of the different fungal species in controls and treatments, aliquots of 500 μL (Section 2.5.2) were removed from test tubes at 0, 2, 4, 20 and 30 h of contact between spores and AgNPs and transferred to tubes containing 4.5 mL of sterile saline solution (9 g NaCl/L pure water). The diluted sample was vortexed for 30 s and used to prepare successive tenfold serial dilutions ($10^{-2}$–$10^{-5}$) in sterile saline solution. From each dilution, a 100-μL aliquot was spread on the surface of MBM-containing Petri dishes. Inoculated plates were incubated at 28 °C, except *F. langsethiae* cultures, which were incubated at 25 °C (Mateo et al., 2011, 2013). Cultures were incubated for 5 days in the dark, enclosed in sealed plastic containers together with beakers of a glycerol–water solution matching the same $a_w$ (0.99) to maintain a constant equilibrium relative humidity inside the boxes. After the incubation period, fungal colony forming units/mL (CFU/mL), effective doses to reduce the number of viable spores to 50% ($ED_{50}$), 90% ($ED_{90}$) and 100% ($ED_{100}$) or minimal lethal concentration (MLC) were estimated by interpolation in the graphs of CFU/mL *versus* AgNP dose at each exposure time. Three independent tests were performed and they were repeated twice.

#### 2.5.4. Evaluation of colony growth after treatments

For monitoring colony growth, 3-μL aliquots of spore suspensions (control and treatments) (Section 2.5.2) were removed just after 2, 4, 20 and 30 h of contact (fungal spores-AgNPs) and centrally inoculated on the surface of MBM plates. Dishes were incubated at 28 °C, except *F. langsethiae* (25 °C), in the dark for 10 days, enclosed in sealed plastic containers together with beakers of a glycerol–water solution matching the same $a_w$ (0.99). The treatment effects (AgNP dose and exposure time) on colony lag period and colony radial growth rate (GR) of the different species was determined in MBM cultures. Lag phase was considered as the time (days) to reach a colony 5 mm diameter. Then, two perpendicular colony diameters were daily measured with the help of a ruler and a magnifying glass and the mean radius was calculated. Colony GR (mm/day) was calculated in all cultures as the slope of the line obtained by linear regression of mean radius *vs* time. The AgNP doses (μg/mL) necessary for 50%, 90%, and 100% growth reduction ($ED_{50}$, $ED_{90}$ and $ED_{100}$) in MBM were determined, when possible, by graphical interpolation in the plots of GR *vs* AgNP dose in treatments. Three independent tests were performed and they were repeated twice.

### 2.6. Determination of mycotoxins in MBM

To determine the effect of AgNPs on mycotoxin production, at the end of incubation period (10 days), the cultures in MBM used for GR evaluation were removed from Petri dishes, homogenized and analyzed for DON, 3–AcDON, NIV, ZEA, $FB_1$, $FB_2$, T–2 and HT–2 depending of the fungal species. For analysis, the UPLC–MS/MS method described by Romera et al. (2018) was followed with minor modifications concerning differences in matrix nature, the mycotoxins to be determined and their levels (as described in Sections 2.6.1, 2.6.2 and 2.6.3). Before determination of mycotoxin concentrations in MBM cultures, calibration and validation assays were carried out.

#### 2.6.1. Calibration

Solid mycotoxin standards were dissolved in acetonitrile to give stock concentrated solutions. They were further diluted and added to extracts from non-inoculated (blank) solid MBM to perform matrix-matched calibrations. Blank MBM were homogenized in stomacher. Two g of the homogenate was mixed with 8 mL of acetonitrile/water/formic acid (80:19:1, v/v/v) in a Falcon tube and shaken in orbital

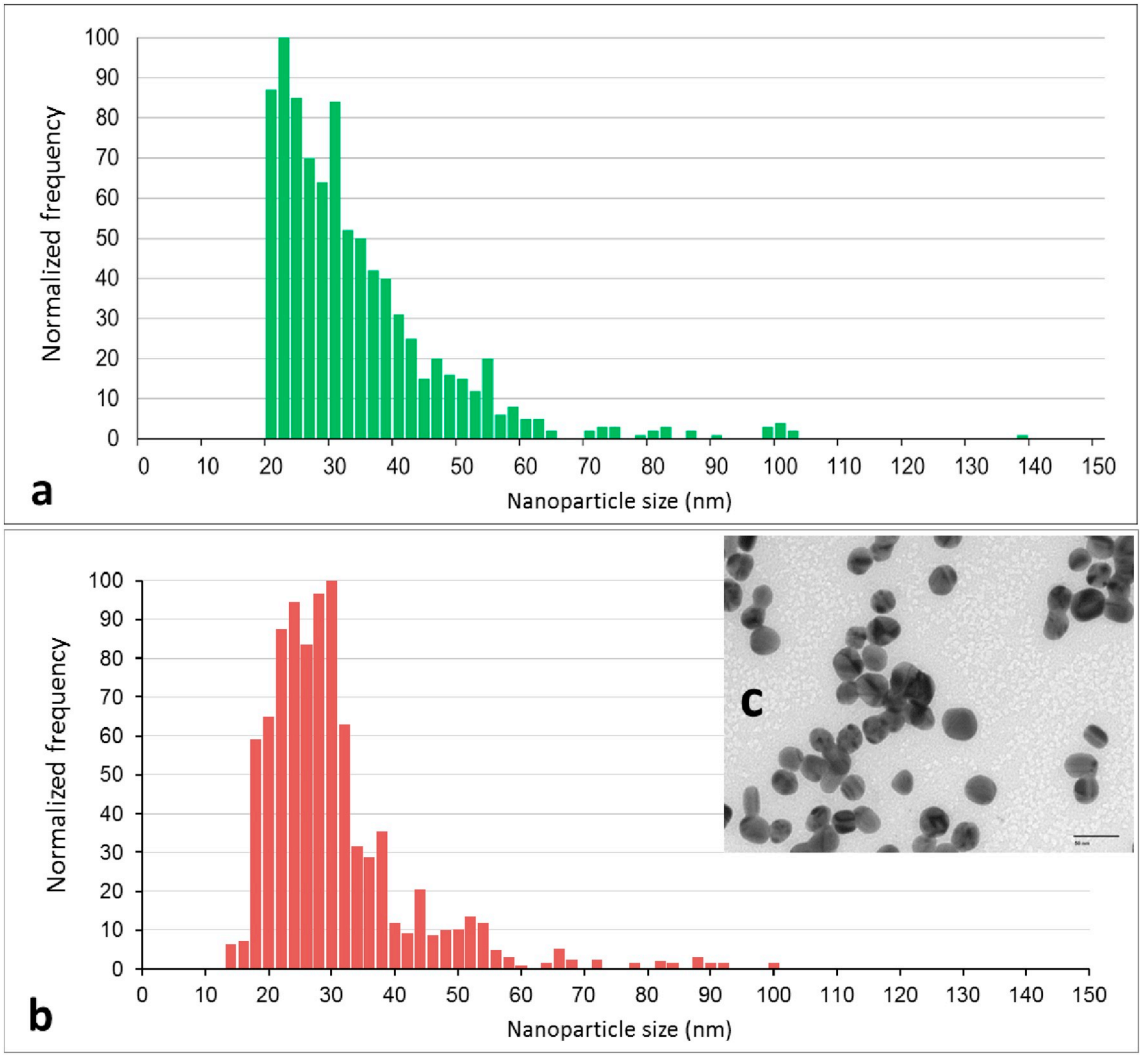


**Fig. 1.** (a) Histogram showing the distribution of nanoparticle size obtained by SP–ICP–MS; (b) histogram showing the distribution of nanoparticle sizes obtained by TEM; (c) micrograph of AgNPs obtained by TEM. Scale bar: 50 nm.

shaker for 60 min. The mixture was centrifuged at 4260 ×*g* for 5 min. Then, 2 mL of the supernatant was evaporated to dryness under a slight stream of $N_2$. The residue was added with the appropriate volumes of standards and then diluted (if necessary) with acetonitrile/water/formic acid (80:19:1, v/v/v) up to a volume of 2.0 mL to attain the desired concentrations. Calibration standards were filtered to injection vials using a syringe filter (0.22 µm, PTFE) and injected into the UPLC–MS/MS system. For each mycotoxin, calibration lines were obtained by linear regression (weighting by 1 / x) of the peak areas from the quantifier ion *vs* concentration. The concentration ranges (µg/mL) were DON (0.031–0.625); 3–AcDON (0.0125–0.250); NIV (0.031–0.625); ZEA (0.0125–0.200); $FB_1$ (0.03–0.940); $FB_2$ (0.03–0.313); T–2 and HT–2 (0.003–0.063).

### 2.6.2. Mycotoxin recovery

Method validation was carried out by analysis of blank MBM spiked with standards of ZEA, DON, 3–AcDON, NIV, $FB_1$, $FB_2$, T–2 and HT–2 (n = 5) at different concentrations, which was achieved by addition of aliquots of mycotoxin standard solutions to Erlenmeyer flasks containing 10 g of autoclaved MBM allowed to cool to around 45 °C. The level range (µg/g MBM) for recovery studies was 1.0–20.0 for DON, 0.1–0.80 for NIV, 1.0–3.0 for ZEA, 1.0–20.0 for 3–AcDON, 0.10–0.25 for T–2 and HT–2, 0.15–3.0 for $FB_1$, and 0.15–1.0 for $FB_2$. Once homogenized, the spiked MBM was poured in Petri dishes and let to cool at room temperature. After solvent evaporation, the solid medium was cut into small pieces and was homogenized using a stomacher. The spiked homogenate (2.0 ± 0.1 g) was extracted in a capped Falcon tube with 8 mL acetonitrile/water/formic acid (80:19:1, v/v/v) in orbital shaker for 1 h. After centrifugation at 4260 ×*g*, during 5 min, 2.0 mL of the supernatant was filtered through 0.22–µm PTFE syringe filter for injection into the UPLC–MS/MS system. Concentrations were determined by interpolation of the signals in the calibrations lines (Section 2.6.1). Suitable dilution was necessary with high mycotoxin concentrations to maintain them within the linear calibration range. Then, mean recovery rates and their relative standard deviations were calculated.

### 2.6.3. Determination of mycotoxins in MBM cultures

At the end of the incubation period, the mycotoxins accumulated in MBM by the *Fusarium* spp. strains were determined. The whole culture in Petri dishes (substrate plus fungal biomass) was cut into small pieces, removed, weighed, homogenized in a stomacher and analyzed as described in Section 2.6.2 for spiked media. Extracts filtered using 0.22-µm filters were injected into the UPLC–MS/MS system. When mycotoxin levels in cultures were too high for the linear calibration range, extracts were appropriately diluted with the same solvent and injected again. Concentrations were determined by interpolation in the calibrations lines obtained with standards (Section 2.6.1).

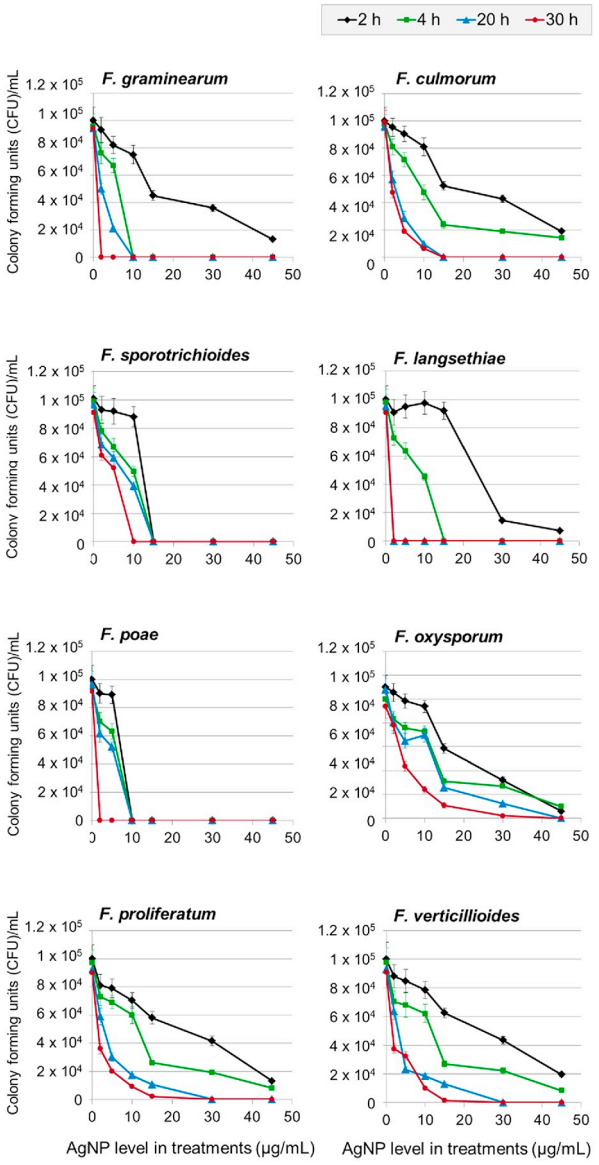


**Fig. 2.** Spore viability of *Fusarium* species on Maize-Based Medium (MBM) after different AgNP treatments. Error bars represent standard deviations.

#### 2.6.4. Instrumental conditions

The UPLC–MS/MS system consisted of an ACQUITY UPLC™ system (Waters, Manchester, UK), provided with an electrospray ionization interface (ESI). An ACQUITY UPLC BEH C18 column (50 × 2.1 mm, 1.7 µm particle size) (Waters) set at 25 °C was used for separation. The injection volume was 30 µL. The mobile phase was a time-programmed mix of a) an aqueous buffer of formic acid (0.1%)/ammonium formate (0.15 mmol/L) and b) methanol. The flow-rate was 0.35 mL/min. MS/MS detection was accomplished using an ACQUITY TQD tandem quadrupole mass spectrometer (Waters Co., Milford, MA, USA). The ESI interface was used in positive ion mode (ESI+). A detailed set of instrumental conditions are reported in Romera et al. (2018).

### 2.7. Statistics

Data were analyzed by multifactor analysis of variance (ANOVA) and linear regression using Statgraphics Centurion XV.II statistical package (StatPoint, Inc., VA, USA). *Post-hoc* Duncan's multiple range test ($\alpha = 0.05$) was used to find homogenous groups when significant differences between factors were revealed by ANOVA. For calculation purposes, undetectable mycotoxin levels were considered to be zero.

## 3. Results and discussion

### 3.1. AgNP characterization

The study of AgNP size distribution and concentration obtained by SP-ICP-MS gave the following results: average size (30 nm), range (20–100 nm), number of particles/mL ($6.6 \times 10^{10}$) and silver concentration (60.26 mg/L). The minimum particle diameter measured by using this technique was 20 nm due to sample background and the dwell time utilized (1500 cps, 3 ms). The size distributions obtained by SP-ICP-MS and by TEM appear in Fig. 1a and Fig. 1b, respectively. Overall, both agree although particles with sizes < 20 nm could be observed using TEM. A sample micrograph obtained by TEM for these AgNPs appears in Fig. 1c. The mean diameter was 30 nm. Differences in the level of nanoparticles with respect to the report of Agnihotri et al. (2014) may be due to small differences in the rates of heating, stirring and $AgNO_3$ addition, way of pH measurement or in centrifugation/washing steps.

The spectra of synthesized AgNPs in water containing Tween 80, medium used for fungal spore treatment, recorded between 300 and 800 nm displayed a surface plasmon resonance band with maximum absorbance at 405–409 nm and there were not substantial differences among them from 0 to 30 h. The absorbance at the maximum was constant and there was not red shift. The synthesized AgNPs were practically stable in the medium used for fungal spore treatment during the exposure time although some agglomeration of AgNPs seems of occur because the spectrum line changed slightly in the 450–550 nm zone in spite of the stability expected, since Kvítek et al. (2008) and Li et al. (2012) reported that Tween 80 aids to prevent AgNP de-stabilization. No turbidity that could be attributed to ion $Ag^+$ was apparent when an aliquot of treatments was centrifuged and part of the supernatant was mixed with a concentrated solution of NaCl. Moreover, continuous shaking assures homogeneity of the suspension during spore treatment.

### 3.2. Effectiveness of AgNP treatments on fungal growth

Some previous studies on the effect of AgNPs on the growth of phytopathogenic fungi have been published but very little is known about the impact of AgNPs on the growth of toxigenic fungi and, in the few cases where such impact was studied, the methodologies used were very different. The present work focuses on the study of the impact of AgNPs on the growth of the main toxigenic *Fusarium* spp. in food as well as on the study of the effects of these nanoparticles in the production and accumulation of their major associated mycotoxins in the substrate. A non-toxigenic phytopathogenic *F. oxysporum* strain was included in the study. In this report, treatments of spore suspensions with different AgNP doses at different exposure times were carried out. After treatments we assessed: a) spore viability level by monitoring the number of CFU/mL and effective doses of AgNPs to reduce the number of viable spores to 50%, 90%, and 100% ($ED_{50}$, $ED_{90}$ and $ED_{100}$ = MLC) as compared to untreated controls; b) colony development of the exposed spore populations by monitoring of lag period, radial GR of the colonies and effective doses of AgNPs to reduce GR to 50%, 90%, and 100% ($ED_{50}$, $ED_{90}$, and $ED_{100}$ = MLC); and c) mycotoxin accumulation in MBM cultures inoculated with spores from controls and treatments. To the best of our knowledge, this is the first report where the effect of AgNPs on these important parameters (spore viability, lag period, colony GR and mycotoxin production) related to toxigenic *Fusarium* spp. is simultaneously studied.

#### 3.2.1. Effect of AgNPs on individual spore viability

The combined effects of AgNP concentration and exposure time on conidia viability of toxigenic *Fusarium* spp. appear in Fig. 2 and Table 1.

**Table 1**
$ED_{50}$[a], $ED_{90}$[b] and $ED_{100}$[c] of AgNPs (μg/mL spore suspension) against spores of phytopathogenic or toxigenic *Fusarium* spp. at different exposure times. Evaluation of viable spores after treatments was carried out by monitoring the number of CFU/mL in MBM. Incubation temperature: 28 °C, except for *F. langsethiae* (25 °C).

| Fungal species | Contact time AgNPs-fungal spores | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 h | | | 4 h | | | 20 h | | | 30 h | | |
| | $ED_{50}$ | $ED_{90}$ | $ED_{100}$ | $ED_{50}$ | $ED_{90}$ | $ED_{100}$ | $ED_{50}$ | $ED_{90}$ | $ED_{100}$ | $ED_{50}$ | $ED_{90}$ | $ED_{100}$ |
| *F. graminearum* | 14.2 | 40.8 | 45 | 6.4 | 9.3 | 10 | 2.3 | 7.7 | 10 | 1.0 | 1.8 | 2 |
| *F. culmorum* | 18.7 | >45 | >45 | 9.8 | >45 | >45 | 3.0 | 10.1 | 15 | 1.9 | 8.5 | 15 |
| *F. sporotrichioides* | 12.2 | 14.4 | 15 | 10.0 | 14.0 | 15 | 7.7 | 13.8 | 15 | 5.6 | 9.1 | 10 |
| *F. langsethiae* | 23.2 | >45 | >45 | 11.2 | 14.2 | 15 | 1.0 | 1.8 | 2 | 1.0 | 1.8 | 2 |
| *F. poae* | 7.2 | 9.4 | 10 | 6.3 | 8.6 | 10 | 5.4 | 9.1 | 10 | 1.0 | 1.8 | 2 |
| *F. oxysporum* | 17 | 42.1 | >45 | 12.7 | 44.8 | >45 | 12.2 | 31.7 | 45 | 4.7 | 17.7 | 45 |
| *F. proliferatum* | 22.3 | >45 | >45 | 11.7 | 42.5 | >45 | 3.4 | 16.9 | 30 | 1.7 | 10.0 | 30 |
| *F. verticillioides* | 24.8 | >45 | >45 | 11.9 | 43.3 | >45 | 3.3 | 19.0 | 30 | 1.7 | 10.5 | 30 |

[a, b, and c]: AgNP dose required to reduce the number of viable spores by 50%, 90%, and 100% compared to untreated controls under identical conditions.

**Table 2**
Arrangement of homogeneous groups within AgNP dose, *Fusarium* species and exposure time, with regard to their effects on spore viability, lag phase and growth rate in MBM cultures according to Duncan's multiple comparison procedure ($\alpha = 0.05$).

| Factor | Level | Homogeneous groups | | |
|---|---|---|---|---|
| | | Spore viability | Lag phase | Growth rate |
| | | High → low | High → low | High → low |
| AgNP dose (μg/mL) | 0 | ● | ● | ● |
| | 2 | ● | ● | ● |
| | 5 | ● | ● | ● |
| | 10 | ● | ● | ● |
| | 15 | ● | ● | ● |
| | 30 | ● | ● | ● |
| | 45 | ● | ● | ● |
| *Fusarium* spp. | *F. graminearum* | ● | ● | ● |
| | *F. culmorum* | ● | ● | ● |
| | *F. sporotrichioides* | ● | ● | ● |
| | *F. langsethiae* | ● | ● | ● |
| | *F. poae* | ● | ● | ● |
| | *F. oxysporum* | ● | ● | ● |
| | *F. proliferatum* | ● | ● | ● |
| | *F. verticillioides* | ● | ● | ● |
| Exposure time (h) | 2 | ● | ● | ● |
| | 4 | ● | ● | ● |
| | 20 | ● | ● | ● |
| | 30 | ● | ● | ● |

Within each column, the levels containing a black circle form a group of means within which there are no statistically significant differences. Black circles in the same row indicate overlapping.

Overall, a decrease in the number of CFU/mL with increasing AgNP dose and contact time can be observed for all the assayed species (Fig. 2). *F. poae* was the most sensitive species as treatments of 10 μg AgNPs/mL for 2 h completely inhibited germination of the exposed spore population. By the contrary, doses of 45 μg AgNPs/mL during >4 h were required to achieve this result in treatments of *F. culmorum*, *F. proliferatum, F. verticillioides* and *F. oxysporum* spores. ANOVA showed that the factors fungal species, AgNP dose, exposure time and the three possible two-way interactions between these factors significantly influence spore viability ($p < 0.01$). With regard to AgNP concentration and exposure time, Duncan's test found seven and four homogeneous non-overlapping groups, respectively (Table 2). However, the eight species were grouped in three homogeneous groups. These groups, listed in the order of increasing spore susceptibility (decreasing spore viability) to AgNPs, were 1) *F. oxysporum*; 2) *F. culmorum*, *F. sporotrichioides*, *F. proliferatum*, and *F. verticillioides*; 3) *F. graminearum*, *F. poae* and *F. langsethiae*. Multiple linear regression indicates that there is a negative relationship with AgNP dose and exposure time and that the effect of both factors can explain about 59% of the whole variance concerning spore viability among the tested species.

The $ED_{50}$, $ED_{90}$, and $ED_{100}$ of AgNPs against fungal spores in each treatment and in each species are listed in Table 1. The $ED_{50}$ values were estimated for all the species at all exposure times (≤45 μg/mL). However, at exposure times of 2 and 4 h, in some cases, the $ED_{90}$ and $ED_{100}$ values exceeded 45 μg/mL depending on the species. After 20 h exposure time, the three effective doses could be estimated for all the species. Overall, when the contact time between AgNPs and spores increased these doses decreased.

### 3.2.2. Effect of AgNPs on lag period and colony growth rate

The mean lag period values of fungal colonies growing in MBM inoculated with spore populations from controls and treatments are shown in Fig. 3. For statistical calculations, when fungal growth was not observed at the end of incubation time (10 days), a lag-value of 10 days was arbitrarily assumed. The measurable lag phases ranged between 0.7 and 5.1 days, depending on the species and the treatment. Mycelial growth was not observed in cultures inoculated with spores previously exposed to ≥30 μg AgNPs/mL for ≥20 h, regardless of the species, except for *F. oxysporum*. The longest measurable lag phase (5.1 days) was found in cultures of *F. langsethiae* inoculated with spores treated with 10 μg AgNPs/mL during 4 h. Spores treated with 10 μg AgNPs/mL during 4 h produced undefined lag periods (>10 days)

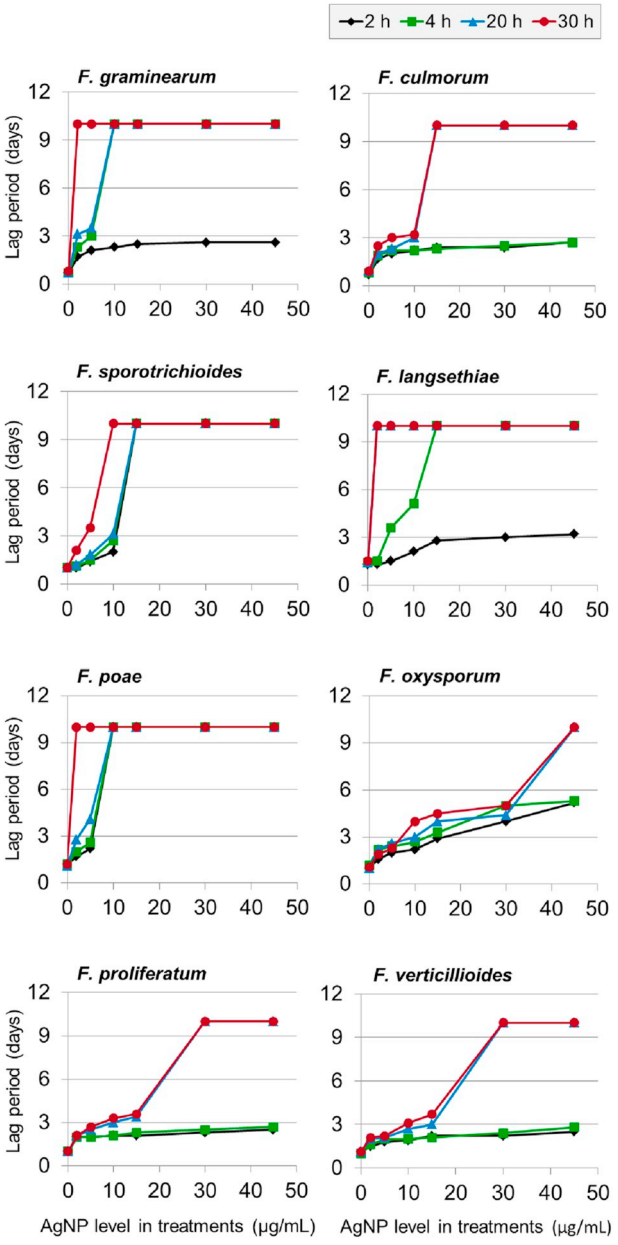


**Fig. 3.** Lag periods in colonies of *Fusarium* species on MBM after different AgNP treatments. Standard deviations ranged 0–5 h. Error bars are not shown because of poor visibility.

in *F. poae* and *F. graminearum* cultures. Fig. 4 shows the colony GR of the assayed species on MBM *vs* the AgNP dose applied in treatments. The $ED_{50}$, $ED_{90}$, and $ED_{100}$ regarding GR (Table 3) were estimated by graphical interpolation in the graphs displayed in Fig. 4. In short-time treatments (2–4 h), no ED could be estimated for *F. culmorum*, *F. proliferatum*, *F. oxysporum*, and *F. verticillioides* (all exceeded 45 µg/mL). However, at 20–30 h of exposure these parameters could be estimated for all the assayed species. At these exposure times, the most susceptible and the most resistant fungi were *F. langsethiae* and *F. oxysporum*, respectively (Table 3).

ANOVA showed that AgNP dose, exposure time, fungal species and all the two-way interactions significantly influenced ($p < 0.01$) the lag period and GR. Arrangement of the three main factors into homogeneous groups according to Duncan's test ($\alpha = 0.05$) appears in Table 2. Overall, there was a moderate negative correlation between GR and the lag period and between the lag period and the number of viable spores in inocula after AgNP treatments, which seems logical considering that high AgNP doses significantly decrease the number of viable spores in inocula (Table 2). Such high doses could produce alterations in the viable spores surviving the treatments, although this last hypothesis was not confirmed. Regarding lag-phase duration and GR, Duncan's test grouped all AgNP doses into five and four homogeneous non-overlapping groups, respectively. The fungal species were clustered into five homogeneous groups. Concerning the lag phase the fastest growing group was formed by *F. culmorum*, *F. verticillioides*, and *F. proliferatum* and the slowest growing mold was *F. poae* while there was one homogeneous group for each exposure time (Table 2).

Although lack of previous data makes comparative analysis difficult, some general conclusions can be drawn. The available results suggest that the susceptibility of phytopathogenic and/or toxigenic fungi to AgNPs depends on the size and species. Thus, it has been reported that smaller and well-dispersed AgNPs increase the reactive surface area increasing antimicrobial activity with respect to the larger ones (Hannon et al., 2015; Xiu et al., 2012). This is probably due to the high intensity at which the AgNPs are capable to saturate and adhere to the fungal hyphae and conidia. Bahrami-Teimoori et al. (2017) found that minimal inhibitory concentrations of AgNPs (10–32 nm in diameter) to control 50% of mycelial growth of *Macrophomina phaseolina*, *Alternaria alternata*, *Fusarium oxysporum*, *Trichoderma harzianum* and *Geotrichum candidum* in solid medium were 159.80, 337.09, 328.05, 400 and 400 µg/mL, respectively. Jo et al. (2015) showed that AgNPs (approx. 7.5 nm in diameter) reduce viability of *Gibberella fujikuroi* conidia by 50% when directly exposed to concentrations ranging from 0.015 to 1.5 µg/mL for 1 to 20 min, and that doses of 150 µg/mL, for 10 min and up to 24 h, significantly decreased colony forming units (CFU) of *G. fujikuroi* on rice seed. In addition, these authors found that seed treatment with 150 µg AgNPs/mL for 12 or 24 h significantly improved seedling emergence and height of *G. fujikuroi*-infested seeds. Adverse effects on germination rate and seedling growth were not observed with any of the AgNP treatments ($\leq$150 µg/mL for up to 48 h exposure) tested in the study. Elgorban et al. (2016) examined the inhibition effect of AgNPs (40–60 nm in diameter) in cultures of *Rhizoctonia solani* anastomosis groups (AGs) infecting cotton plants and found that the $ED_{50}$ and $ED_{90}$ of AgNPs in two solid cultures media ranged between 0.0002 and 0.0022 mol/L and between 0.0061 and 0.0671 mol/L, respectively. Kasprowicz et al. (2010) studied the impact of AgNPs on plant pathogenic spores of *F. culmorum* reporting a significant reduction in mycelial growth after a previous incubation of fungal spores with AgNPs (5–65 nm in diameter) at levels 0.12–10 ppm for 2–24 h. The antifungal effect of AgNPs was dependent on the exposure time. Suspensions of *F. culmorum* spores incubated with 2.5 ppm of AgNPs for 24 h had a significantly lower percentage of germinating spores compared to the control. Dananjaya et al. (2017) found that doses of 100 µg/mL of chitosan silver nanocomposites (373 nm size) were effective (CMI) to inhibit growth of *F. oxysporum* species complex, and caused morphological and ultrastructural negative changes in the pathogen, suggesting its usage as an antifungal agent.

The size of the AgNPs seems to be decisive in their possible transfer and application in the agri-food sector. In cytotoxicity assays measuring membrane damage with human lung cells, 20 and 50 µg/mL of AgNPs (10 nm in diameter), independent of the surface coating (citrate and polyvinylpyrrolidone), were toxic, whereas 40 and 75 nm NPs were not (Gliga et al., 2014). Although the mass concentration (mg/mL) was the same, 10 nm AgNPs have surface areas and particle concentrations (number/mL) that are over 10- and 1000-times larger than those of 100 nm AgNPs, respectively, which may increase the chance of interaction with surrounding biomolecules and trigger adverse responses (Cho et al., 2018). Similarly, Park et al. (2011) found that 4 nm AgNPs induce much higher levels of reactive oxygen species (ROS) production and interleukin-8 secretion from macrophage immune cells than 20 and 70 nm AgNPs, suggesting that particle size may be the main factor affecting AgNP toxicity. Silver in food additive E 174 is present in its elemental form (silver-colored powder or tiny sheets). In the EU,

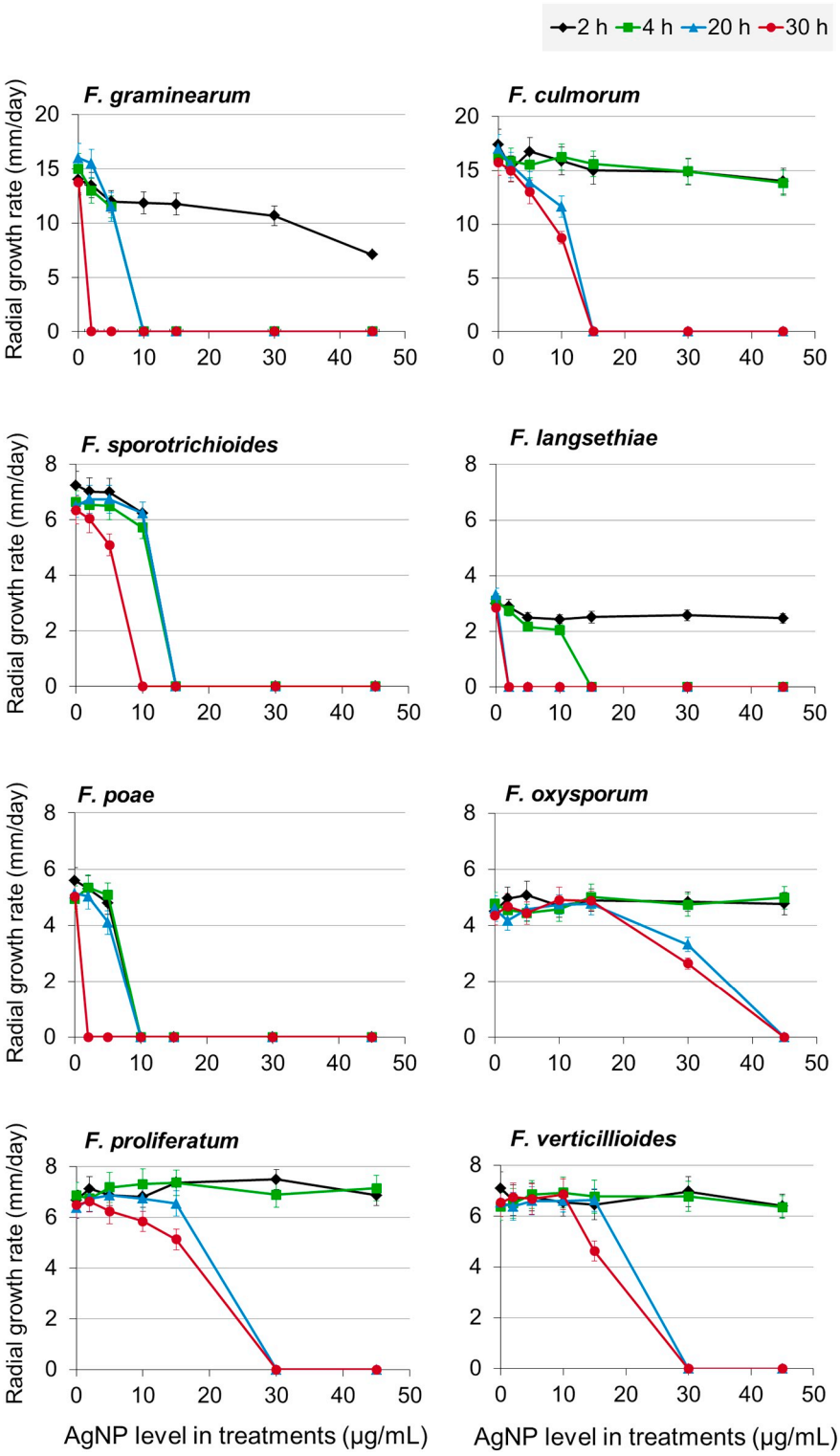


**Fig. 4.** Radial growth rates of *Fusarium* spp. colonies on MBM after different AgNP treatments. Error bars represent standard deviations.

specifications for silver have been defined (European Commission, 2012). According to the Panel, characterization of the particle size in the powder of E 174 should be included in the specifications.

In our study, the eight *Fusarium* spp. were grouped in three homogeneous groups according to their spore susceptibility to AgNPs and it was possible to determine the $ED_{50}$, $ED_{90}$, and $ED_{100}$ for all of them using AgNPs with an average size of 30 nm (range 14–100 nm by TEM) at doses of 2–45 µg/mL and exposure times in the range 2–30 h in liquid medium. In general, AgNP effectiveness increased with increasing dose, regardless of the fungal species. These results are in agreement with those obtained by the authors cited above and those reported by other researchers (Jo et al., 2009; Kim et al., 2009; Min et al., 2009; Park et al., 2006) concerning the control of different phytopathogenic fungal species.

**Table 3**
Effective doses ($ED_{50}$[a], $ED_{90}$[b] and $ED_{100}$[c]) of AgNPs (μg/mL) in treatments of phytopathogenic or toxigenic *Fusarium* species able to reduce/inhibit colony growth on MBM cultures. Incubation temperature: 28 °C, except for *F. langsethiae* (25 °C).

| Fungal species | Contact time AgNPs-fungal spores | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 h | | | 4 h | | | 20 h | | | 30 h | | |
| | $ED_{50}$ | $ED_{90}$ | $ED_{100}$ | $ED_{50}$ | $ED_{90}$ | $ED_{100}$ | $ED_{50}$ | $ED_{90}$ | $ED_{100}$ | $ED_{50}$ | $ED_{90}$ | $ED_{100}$ |
| *F. graminearum* | 35.2 | 43 | 45 | 6.8 | 9.3 | 10 | 7.3 | 9.5 | 10 | 1.0 | 1.8 | 2.0 |
| *F. culmorum* | >45 | >45 | >45 | >45 | >45 | >45 | 11.3 | 14.3 | 15 | 10.5 | 14.1 | 15 |
| *F. sporotrichioides* | 12.1 | 13.8 | 15 | 12.1 | 14.4 | 15 | 12.4 | 14.5 | 15 | 6.9 | 9.4 | 10 |
| *F. langsethiae* | >45 | >45 | >45 | 11.2 | 14.2 | 15 | 1.0 | 1.8 | 2.0 | 1.0 | 1.8 | 2.0 |
| *F. poae* | 7.1 | 9.4 | 10 | 7.5 | 9.5 | 10 | 7.4 | 9.5 | 10 | 1.0 | 1.8 | 2.0 |
| *F. oxysporum* | >45 | >45 | >45 | >45 | >45 | >45 | 34.6 | 42.9 | 45 | 32.7 | 42.9 | 45 |
| *F. proliferatum* | >45 | >45 | >45 | >45 | >45 | >45 | 22.7 | 28.5 | 30 | 20.9 | 28.2 | 30 |
| *F. verticillioides* | >45 | >45 | >45 | >45 | >45 | >45 | 22.6 | 28.5 | 30 | 19.4 | 27.9 | 30 |

[a, b and c]: AgNP dose required to reduce colony radial growth rate by 50%, 90% and 100% compared to untreated controls under identical conditions.

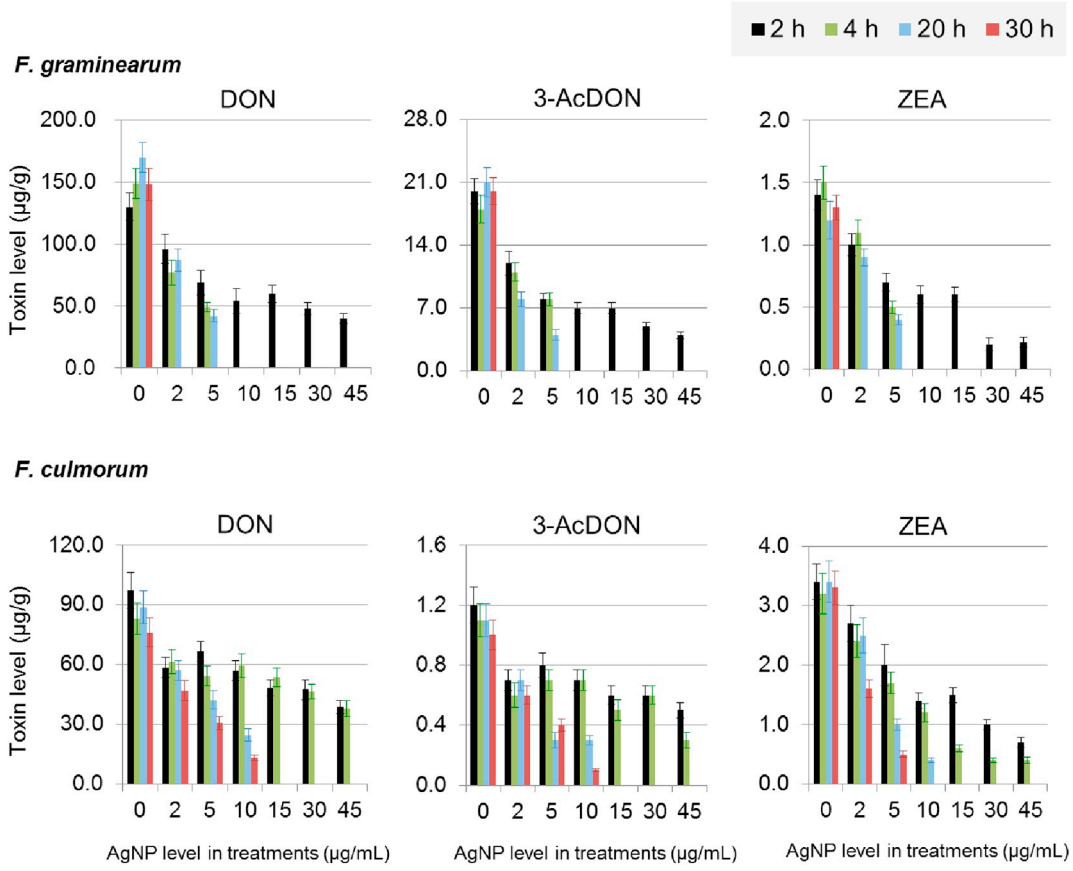


**Fig. 5.** Accumulation of DON, 3–AcDON and ZEA by *F. graminearum* and *F. culmorum* in MBM cultures after different AgNP treatments. Incubation time: 10 days. Incubation temperature: 28 °C. Error bars represent standard deviations.

### 3.3. Effect of AgNP treatments on mycotoxin production in MBM cultures

The method used for determination of the mycotoxins produced in MBM cultures using UPLC-MS/MS was validated on the basis of recovery percentages and their relative standard deviations (RSD). In the tested ranges (Section 2.6.2), mean recoveries and their RSD for the different mycotoxins were as follows: DON (97% ± 5%), NIV (97% ± 6%), ZEA (98% ± 6%), 3–AcDON (94% ± 7%), T–2 (99% ± 7%), HT–2 (96% ± 8%), $FB_1$ (102% ± 5%), and $FB_2$ (98% ± 6%). Some sample extracts had to be diluted because mycotoxin levels were too high for the linear calibration ranges. The limits of detection (LOD) (μg/mL) estimated as 3 × ratio signal/noise, were as follows: DON (0.02); NIV (0.02); ZEA (0.005), 3–AcDON (0.012), T–2 (0.002), HT–2 (0.002), $FB_1$ (0.015), and $FB_2$ (0.015). The limits of quantification (LOQ) were estimated as 3.3 times the above LOD values.

In general, mycotoxin levels in MBM cultures decreased when both AgNP dose and contact period increased (Figs. 5–7). Mycotoxin levels at the end of the incubation period (ten days) were closely correlated with the radius reached by the colony at that time. Fig. 5 shows the mycotoxin levels in cultures of *F. graminearum* and *F. culmorum* inoculated with spores previously treated (DON, 3–AcDON and ZEA). Toxins were undetectable in most cultures at AgNP doses > 10 μg/mL and 20–30 h of exposure, which was associated to lack of fungal growth. Fig. 6 shows the concentrations of T–2 and HT–2 in cultures of *F. sporotrichioides* and *F. langsethiae* and the levels of NIV in cultures of *F. poae* at the 10th incubation day. Doses ≥ 15 μg/mL combined with exposure

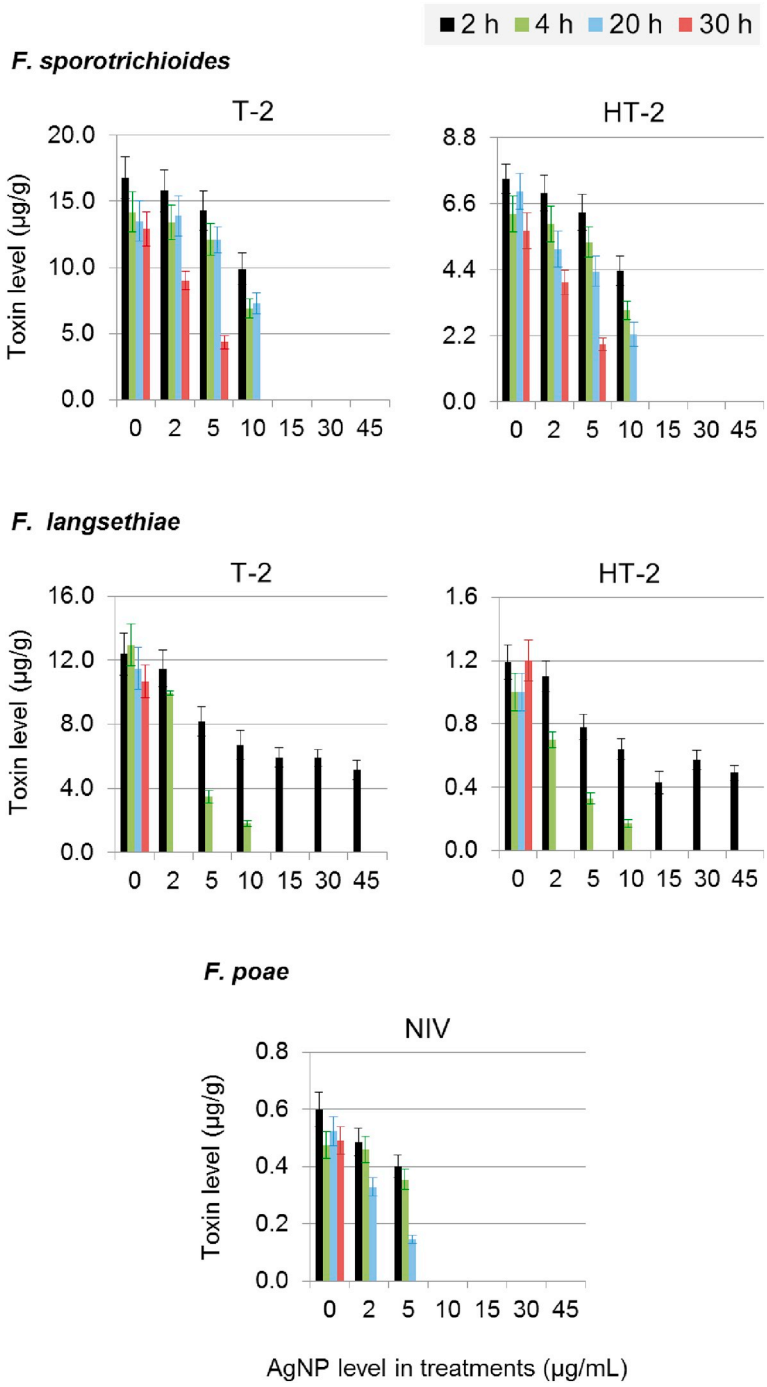


**Fig. 6.** Accumulation of T–2 and HT–2 toxins (*F. sporotrichioides* and *F. langsethiae*) and NIV (*F. poae*) in MBM cultures after different AgNP treatments. Incubation time: 10 days. Incubation temperature: 28 °C, except in *F. langsethiae* cultures (25 °C). Error bars represent standard deviations.

times ≥ 4 h inhibited T–2 and HT–2 production. Fig. 7 displays the concentrations of $FB_1$ and $FB_2$ in the cultures of *F. proliferatum* and *F. verticillioides*.

ANOVA found that AgNP dose and exposure time significantly influence ($p < 0.005$) the concentrations of DON, 3-AcDON and ZEA in the cultures of *F. graminearum* and *F. culmorum*. The strains of both species gave statistically comparable DON levels. However, *F. graminearum* produced significantly more 3–AcDON but less ZEA than *F. culmorum* produced. Table 4 shows the arrangement of homogeneous groups devised by the Duncan's multiple range test concerning mycotoxin levels. Concerning dose there were four homogeneous overlapping groups for DON and 3–AcDON and five for ZEA. With regard to exposure time, there were three, two and four homogeneous groups for DON, 3–AcDON, and ZEA, respectively.

ANOVA found that AgNP dose, exposure and species significantly influence the concentrations of T-2 and HT-2 in cultures of *F. sporotrichioides* and *F. langsethiae* ($p < 0.001$). The two-way interactions between the factors species, AgNP dose and exposure time was also significant ($p < 0.05$). The Duncan's test clustered the AgNP doses (0–45 µg/mL) into five and four homogeneous groups for T–2 and HT–2, respectively, and the contact period in three homogenous groups (Table 4). In *F. poae* cultures, the AgNP dose significantly influenced the NIV levels but, although such levels decreased with exposure time, this factor was not significant. Fumonisin levels were significantly affected

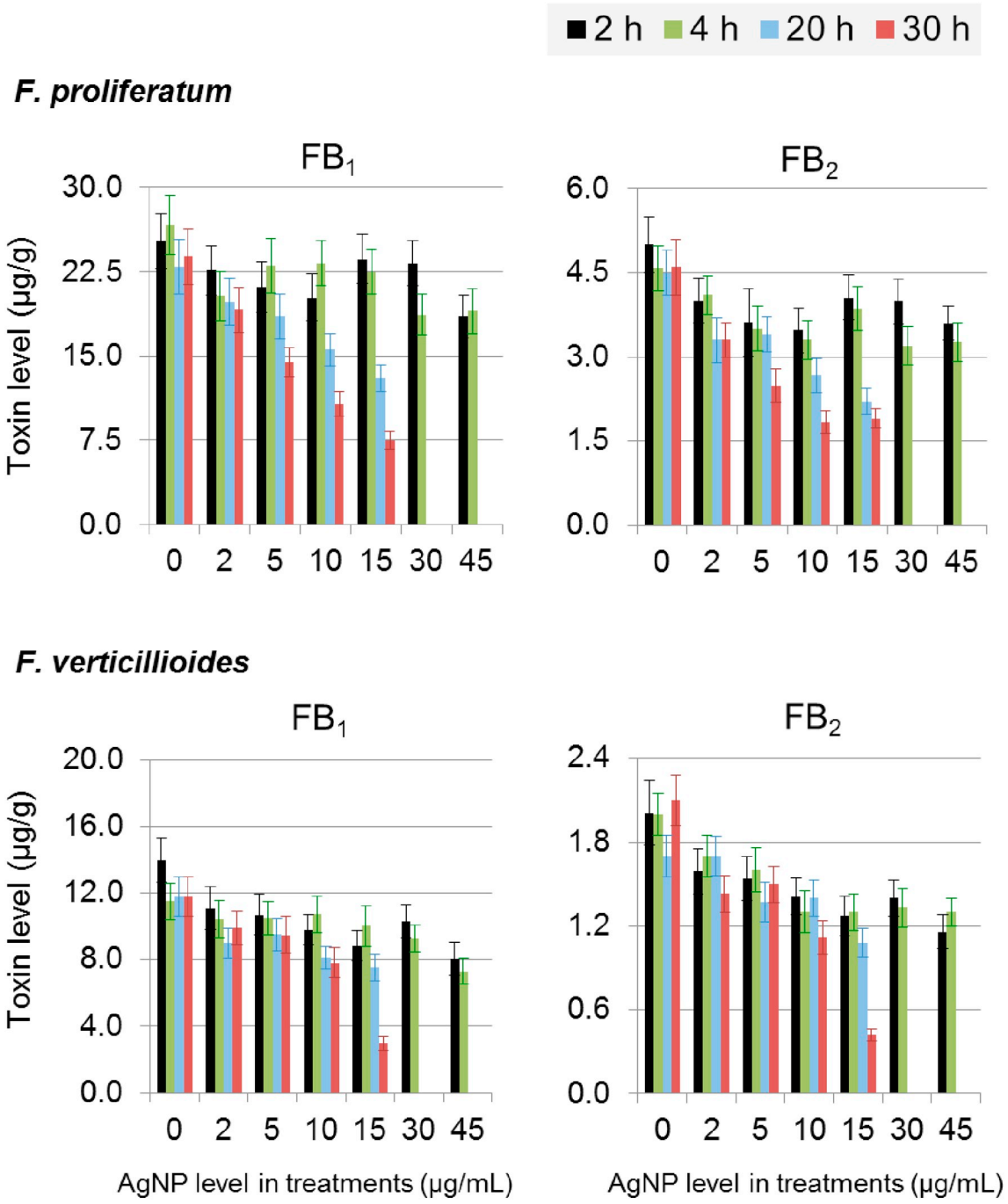


**Fig. 7.** Accumulation of fumonisin B1 and B2 by *F. proliferatum* and *F. verticillioides* in MBM cultures after different AgNP treatments. Incubation time: 10 days. Incubation temperature: 28 °C. Error bars represent standard deviations.

($p < 0.001$) by AgNP dose and contact time and *F. proliferatum* produced significantly more fumonisins than *F. verticillioides*. AgNP doses ≥ 30 µg/mL applied during ≥ 20 h were effective to inhibit fumonisin production. Duncan's arrangements of homogeneous groups for both fumonisins appear in Table 4.

According to our knowledge, there are no previous available data on the effect of AgNPs in the production of mycotoxins by toxigenic *Fusarium* spp. Some researchers have reported that AgNPs doses of 50–150 ppm can inhibit aflatoxin $B_1$ production at levels of 46.1–100% (Al-Othman et al., 2014; Mousavi and Pourtalebi, 2015). The partial or total inhibition of aflatoxin $B_1$ production in cultures was a direct consequence of the AgNP effect on fungal growth. This conclusion agrees with the data found in our study for the *Fusarium* spp. and mycotoxins studied.

In summary, the results obtained in the present study permit to extract general and important conclusions. The factors fungal species, AgNP dose, exposure time and their interactions significantly influence spore viability, lag period and growth rate of *F. graminearum* (Fg22), *F. culmorum* (Fc15), *F. sporotrichioides* (Fs4), *F. langsethiae* (Fl021), *F. poae* (Fp11), *F. oxysporum* (Fo11), *F. proliferatum* (Fp16) and *F. verticillioides* (Fv27). It is important to note that under the assayed conditions the $ED_{50}$, $ED_{90}$ and $ED_{100}$ for all fungal species were estimated using AgNPs of an acceptable size (average 30 nm, range 14–100 nm by TEM) and within an acceptable concentration range (2–45 µg/mL). The mycotoxin levels in the cultures inoculated with fungal spores from treatments at the end of the incubation period were strongly related with the

**Table 4**
Arrangement of homogeneous groups within AgNP dose, exposure time and *Fusarium* species with regard to their effects on mycotoxin accumulation in MBM cultures of various *Fusarium* species according to Duncan's multiple comparison procedure (α = 0.05).

| | | Homogeneous groups | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *F. graminearum* and *F. culmorum* | | | *F. sporotrichioides* and *F. langsethiae* | | *F. poae* | *F. proliferatum* and *F. verticillioides* | | |
| | | DON[a] | 3–AcDON[b] | ZEA[b] | T–2[b] | HT–2[b] | NIV | $FB_1$[b] | $FB_2$[b] | |
| Factor | Level | High → Low | High → Low | High → Low | High → Low | High → Low | High → Low | High → Low | High → Low | |
| AgNP dose (μg/mL) | 0 | ● | ● | ● | ● | ● | ● | ● | ● | |
| | 2 | ● | ● | ● | ● | ● ● | ● | ● | ● | |
| | 5 | ● ● | ● ● | ● | ● | ● | ● | ● | ● | |
| | 10 | ● ● | ● ● | ● | ● | ● | ● | ● ● | ● ● | |
| | 15 | ● | ● ● | ● ● | ● | ● | ● | ● | ● | |
| | 30 | ● | ● ● | ● | ● | ● | ● | ● | ● | |
| | 45 | ● | ● | ● | ● | ● | ● | ● | ● | |
| Exposure time (h) | 2 | ● | ● | ● | ● | ● | ● | ● | ● | |
| | 4 | ● | ● | ● | ● | ● | ● | ● | ● | |
| | 20 | ● | ● | ● | ● | ● | ● | ● | ● | |
| | 30 | ● | ● | ● | ● | ● | ● | ● | ● | |
| *Fusarium* species | *F. graminearum* | ● | ● | ● | | | | | | |
| | *F. culmorum* | ● | ● | ● | | | | | | |
| | *F. sporotrichioides* | | | | ● | ● | | | | |
| | *F. langsethiae* | | | | ● | ● | | | | |
| | *F. poae* | | | | | | ● | | | |
| | *F. proliferatum* | | | | | | | ● | ● | |
| | *F. verticillioides* | | | | | | | ● | ● | |

Within each column, the levels containing a black circle form a group of means within which there are no statistically significant differences. Black circles in the same row indicate overlapping between groups.
[a] There were not significant differences between the two species regarding mycotoxin accumulation.
[b] There were significant differences between the two species regarding mycotoxin accumulation.

diameter reached by the colony at that time. Obviously, the colony size depended on the number of viable spores in the inoculum, lag period and mycelial GR. None of the tested treatments produced stimulation of fungal growth or stimulation of mycotoxin biosynthesis with respect to controls. Thus, the antifungal effect of assayed AgNPs against the tested *Fusarium* spp. suggests that AgNPs could be a new antifungal ingredient likely to be implemented in the agro-food sector for management of these important toxigenic fungi and their main associated mycotoxins. In order to find effective concentrations of AgNPs in high-risk foods and strategies to block excessive migration of nanoparticles to food (cleaning solutions, paints, films…), further research will be necessary to perform in the future.

## Declaration of interests

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.

## Acknowledgments

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