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Inhibition of three toxigenic fungal strains and their toxins production using selenium nanoparticles

MARWA MAHMOUD ABDEL-KAREEM[1], ABDEL-NASER AHMED ZOHRI[2]

[1] Botany & Microbiology Department, Faculty of Science, Sohag University, EG-82524 Sohag, Egypt; marwaabdelkareem7@gmail.com

[2] Botany & Microbiology Department, Faculty of Science, Assiut University, EG-71516 Assiut, Egypt

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Spoilage and poisoning of foods by microfungi are a major problem, especially in developing countries. While selenium nanoparticles (Se-NPs) have been used for a wide range of applications including antibacterial, antioxidant and anticancer applications, the effects of Se-NPs on fungal strains remain for the most part unknown to date. Our research is a pioneering attempt to evaluate the antifungal activity and antimycotoxin properties of Se-NPs (32 nm). Se-NPs at different concentrations were evaluated against the growth and mycotoxins production of three toxigenic fungal strains.

The growth of *Aspergillus parasiticus*, *A. ochraceus* and *A. nidulans* was completely inhibited using 7000, 9000 and 3000 µg/ml of Se-NPs, respectively, while the complete inhibition in aflatoxins, ochratoxin A and sterigmatocystin production was reported by addition of 2000, 2000, and 800 µg/ml of Se-NPs, respectively.

Results of this study show that Se-NPs were effective against the fungal strains tested and their toxin production. These results suggest that Se-NPs could be used as an effective microfungicide in agricultural and food safety applications against toxigenic microfungi.

Key words: Se-NPs, mycotoxigenic moulds, mycotoxins, aflatoxins, ochratoxin A, sterigmatocystin.

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Zejména v rozvojových zemích představuje značný problém plesnivění potravin spojené s produkcí toxinů. Jako účinný prostředek k omezení výskytu plísní by mohly být využity selenové nanočástice, již hojně využívané pro své antibakteriální, antioxidační a protirakovinné účinky, ale jejich působení na houby je zatím z velké části neznámé. Tato studie představuje pionýrský pokus o zhodnocení účinnosti nanočástic (32 nm Se-NP) proti růstu a produkci mykotoxinů u tří kmenů toxigenních hub.

Růst druhu *Aspergillus parasiticus* byl zcela inhibován přidáním Se-NP v koncentraci 7000 µg/ml, růst *A. ochraceus* při koncentraci 9000 µg/ml a k inhibici růstu *A. nidulans* postačilo 3000 µg/ml. Úplná inhibice produkce aflatoxinů a ochratoxinu A nastala shodně při 7000 µg/ml, pro inhibici produkce sterigmatocystinu je postačující koncentrace 800 µg/ml.

Výsledky této studie ukazují, že selenové nanočástice jsou efektivním prostředkem proti testovaným kmenům hub a produkci jejich toxinů. Na jejich základě lze usuzovat, že Se-NP by mohly být účinným mikrofungicidem, využitelným v zemědělství a ochraně potravin proti toxigenním mikromycetům.

INTRODUCTION

Mycotoxins are formed by certain fungal species, whenever environmental factors are suitable. They are products of secondary metabolism of these microfungi, frequently occurring on foodstuffs and animal feeds. Unlike many bacterial toxins, several mycotoxins are highly stable during conventional heating processes of foodstuffs for human consumption. These microfungi and their mycotoxins have serious effects upon the growth rate and health of human beings as well as animals (FDA 1979, Ciegler & Vesonder 1983, Bahtnagar et al. 2002). Some mycotoxins, especially aflatoxins, ochratoxins and sterigmatocystin, have been recorded to cause a public health hazard and found to be carcinogenic and tremorgenic (Beuchat 1978: 63–84; Wray 1981, Bhatnagar et al. 2002).

Aflatoxins (AFs) are secondary metabolites with toxic and carcinogenic effects, which are produced by species of *Aspergillus*, particularly *Aspergillus flavus* and *A. parasiticus* (Razzaghi-Abyaneh et al. 2008). Corn and cottonseed have suitable conditions for growth of these microfungi, so contamination of these commodities with AFs often makes them unfit for consumption (Moschini et al. 2008, El-Nagerabi et al. 2012). AFs are considered the most carcinogenic, mutagenic, and teratogenic compounds found naturally in foods and feeds (Sanchez et al. 2005).

Ochratoxin A (OTA) is produced by microfungi of the genera *Aspergillus* and *Penicillium*. The major species involved in OTA production include *Aspergillus ochraceus*, *A. carbonarius*, *A. melleus*, *A. sclerotiorum* and *A. sulphureus*. OTA is reported to be nephrotoxic, teratogenic, immunotoxic, and probably carcinogenic. It is usually demonstrated that the main contributors to OTA intake are cereals, peanut and their derivative products (Zinedine 2010, Toffa et al. 2013).

Sterigmatocystin (ST) is a carcinogenic polyketide (Versilovskis & De Saeger 2010) produced by species in several fungal genera. Microfungi capable of producing ST are common food, feed and indoor contaminants, and may also be plant and mammalian pathogens, thus having a large economic impact in the biotechnological, agricultural and food industry (Wagacha & Muthomi 2008, Versilovskis & De Saeger 2010).

Novel antimicrobial properties were attested by different particles fabricated with nanotechnology. Shahverdi et al. (2010) showed that antifungal formulations in the form of nanoparticles can be used as potential fungicidal material.

While compared with microparticles, nanoparticles have increased surface areas and therefore have increased interactions with microbial cells. In addition, nanoparticles can be more easily entered into the cells than microparticles (Tran & Webster 2011). Due to this ability, nanoparticles have been synthesised from different sources and may be used as an antimicrobial agent. It could be proposed that the application of nanoparticles e.g. as spray-disinfectant could be a successful opportunity to prevent contaminations of food and feed caused by mycotoxigenic microfungi. Thus, it may be feasible to use nanoparticle-based formulations as alternative treatment against fungal infections in humans, animals and plants.

Selenium is one of the essential trace elements in the body due to its anti-oxidative as well as pro-oxidative effect and has great importance in nourishment and medicine (Zhang et al. 2004). Selenium is a key player in cellular metabolism, an essential component of enzymes protecting the body against free radical species, and has important roles in metabolism of the thyroid, human fertility and many other vital functions. All aspects of Se in biology have advanced in various fields such as genetic, biochemical, molecular, and health areas. Many stable organic selenium compounds have been successfully synthesised and are used as antioxidants, enzyme inhibitors, anti-tumour, anti-infective agents, cytokine inducers and immunomodulators (Parnham & Graf 1991, Sies & Masumoto 1997).

Many papers have attributed the antibacterial effects of different selenium compounds to the formation of free radicals (Tran et al. 2009). Moreover, selenium has also been found to trigger the generation of ROS (Reactive Oxygen Species), with both elements capable of reacting with intracellular thiols and forming intermediates causing oxidative stress as a consequence of the formation of superoxide radicals (Zannoni et al. 2008, Zonaro et al. 2015). Nanoparticles can contribute to functional damage of the cell membrane or wall by disrupting the integrity of these important envelopes (Pi et al. 2013). According to the World Health Organization (WHO), a recommended Acceptable Daily Intake (ADI) value for selenium is 40 µg/day (Neve 2002). However, selenium is not toxic at the high level of 3200 µg/day (Reid et al. 2004). He et al. (2014) reported that supranutritional levels of Se-NPs had no obvious toxic effects on rats, and could be used as potential candidates for cancer chemoprevention, although doses greater than 2.0 mg Se/kg-bw induced chronic toxicity. Selenium nanoparticles have significantly lower toxicity than other inorganic and organic forms of supplemental selenium (Kojouri et al. 2012, Shakibaie et al. 2013). Se-NPs have a variety of biological properties and might be candidates for a range of applications (Chen et al. 2008, Yazdi et al. 2012).

Selenium nanoparticles (Se-NPs) have become the focus of intensive research owing to their wide range of applications in areas such as antioxidants (Gao et al. 2002, Wang et al. 2007, Dhanjal & Cameotra 2010, Shahverdi et al. 2010), antibac-

terial activity (Tran & Webster 2011) and anticancer applications (Wang et al. 2010, Tran & Webster 2011). It has been found that Se-NPs exhibit low cytotoxicity compared with selenium compounds and possess excellent anticancer and therapeutic activities, making them apt for medical applications (Hatfield et al. 2014, Wadhwani et al. 2016). Se-NPs exhibit anticancer activity against kidney, breast and lung cancers (Ali et al. 2013, Ramamurthy et al. 2013) and hence can be used as chemopreventive and chemotherapeutic agents. The mechanisms of anticancer activity are not fully understood; however, several hypotheses have been proposed: (i) enhanced oxidative stress, carcinogen detoxification, and immune surveillance; (ii) induction of cellular and mitochondria-mediated apoptosis; (iii) inhibition of tumour cell invasion and angiogenesis; (iv) cell cycle arrest at S phase; (v) metastasis prevention by inhibition of matrix metalloproteinases expression; and (vi) mobilisation of endogenous copper (Chen et al. 2008, Shakibaie et al. 2010, Luo et al. 2012, Ahmad et al. 2015, Wadhwani et al. 2016).

However to date, not much has been reported on the assessment of antifungal activity of Se-NPs. Studies by Shahverdi et al. (2010) have shown that antifungal formulations in the form of nanoparticles could be used as potential fungicidal material, and Hariharan et al. (2012) showed that Se-NPs synthesised from a biological source possess a significant antimicrobial activity against pathogenic bacteria, microfungi and yeasts.

In recent years, applications of nanoparticle-based fungicides have been increasing gradually because of the many advantages over conventional chemical fungicides (Ismail et al. 2016). Selenium nanoparticles may be less toxic to humans and animals than synthetic fungicides.

The aim of this study was to investigate the inhibition of three toxigenic microfungi and their toxin production using different concentrations of Se-NPs.

MATERIAL AND METHODS

Mycotoxigenic strains. Three highly toxigenic fungal strains were utilised in the assays: two were purchased from CBS (Centraalbureau voor Schimmelcultures), Fungal Biodiversity Centre (the Netherlands) (*Aspergillus parasiticus* CBS 571.65 as aflatoxin B_1, B_2, G_1 & G_2 producer and *A. ochraceus* CBS 589.68 as ochratoxin A producer), and one local strigmatocystin producer strain (*Aspergillus nidulans* 69) was isolated from orange in a previous study (Zohri et al. 2014). These toxins were selected as model target analytes due to their presence in common foodstuffs and their highly toxic and potential carcinogenic effects.

Selenium nanoparticles. Se-NPs of 32 nm (diameter as measured by TEM) were commercially purchased from the Materials Science and Nanotechnology Dept., Faculty of postgraduate studies for advanced science, Beni Suef University, Egypt. Pure NPs without any stabiliser or additive or residues from their production were used; the NPs are stable during sterilisation and incubation. Amounts of 1.0, 0.9, 0.8, … etc. grams of Se-NPs were individually added to 100 ml sterilised distilled water to obtain concentrations of 10,000, 9000, 8000, etc. µg/ml, finally rendering a serial of Se-NP concentrations (from 10,000 to 200 µg/ml).

Evaluation of the inhibitory effect of Se-NPs on the growth of toxigenic strains. Gradual concentrations (200, 400, 600, 800, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000 and 10,000 µg/ml) of Se-NPs were added, individually, to potato dextrose liquid medium (sliced washed unpeeled potatoes 200 g/l and dextrose 20 g/l). Erlenmeyer flasks of 250 ml capacity were used. Each flask contained 50 ml medium. The flasks were sterilised at 121 °C for 20 minutes and inoculated after cooling with 2 ml of the toxigenic fungal spore suspension. Spore suspensions of microfungi were prepared by collecting conidia from five-day-old colonies grown on potato dextrose agar (sliced washed unpeeled potatoes 200 g/l, dextrose 20 g/l and agar agar 20 g/l) at 28 °C in 0.2% Tween solution. The amount of spores per ml was determined by using haemocytometer and brought to a standard concentration of spores of 10^5/ml. At the same time other flasks containing potato dextrose liquid medium without Se-NPs were inoculated with the toxigenic microfungi as a control. The cultures were incubated at 28 ± 2 °C as static cultivation for 7 days. At the end of the incubation period, the dry weight of fungal growth in each Se-NP concentration was recorded and compared with the control. This method was modified after Hili et al. (1997). All experiments were repeated three times.

Evaluation of the inhibitory effect of Se-NPs on mycotoxin production. The previous steps were repeated with cultivation for 15 days for mycotoxin detection. At the end of the incubation period, the content of each flask (medium + mycelia) were homogenised with 100 ml chloroform for five minutes in a high speed (16,000 rpm) MPW-309 blender with a stainless steel container of 50–250 ml in capacity (Mechanika Precyzyjna, Warszawa, Poland). The extraction procedure was repeated three times. The combined chloroform extracts were washed with an equal volume of distilled water, dried over anhydrous sodium sulphate, filtered, and then concentrated to near dryness. Mycotoxin levels were detected using the semiquantitative determination thin-layer chromatographic technique (TLC). TLC is sometimes a more eligible way to visualise the biosynthesis of a fluorescence-active compound in a sample than absolute quantification by e.g. HPLC especially in the case of Se-NPs for two reasons. First, the selenium nanoparticles which remain in the mycelial extracts disturb an exact

and reproducible detection by the fluorescence detector of the HPLC system. Second, the way of the toxin biosynthesis in relation to the concentration of selenium nanoparticles can be well visualised by using TLC plates (Kotzybik et al. 2016). The residual extracts were dissolved in 1 ml chloroform and 15 µl of the solution was dotted onto pre-coated TLC-sheets. As a solvent system, chloroform : methyl alcohol (97 : 3, v/v) was used for different mycotoxins. The developed plates were detected under short-wave (254 nm) and long-wave (356 nm) ultra- violet light.

RESULTS

The antifungal activity of gradual concentrations (µg/ml) of Se-NPs (32 nm) to inhibit the growth and mycotoxin production of three toxigenic fungal strains (*Aspergillus parasiticus*, *A. ochraceus* and *A. nidulans*) was examined and the results recorded in Tabs. 1–3.

The findings of the present study revealed that the effect of Se-NPs (32 nm) on the growth and mycotoxin production by mycotoxigenic moulds was dependent on the concentration used. The levels of produced mycotoxins decreased when the concentration of Se-NPs increased. The concentrations for complete inhibition of mycotoxins were less than those necessary for fungal growth complete inhibition.

The growth of *A. parasiticus* and aflatoxins production were completely inhibited by addition of 7000 and 2000 µg/ml of Se-NPs, respectively, while the lowest concentration for partial inhibition of aflatoxins formation (20%) was 1000 µg/ml (fungal growth was inhibited by 52% in this concentration) (Tab. 1). The ochratoxin A producing fungus (*A. ochraceus*) and ochratoxin A production were completely inhibited by addition of 9000 and 2000 µg/ml of Se-NPs, respectively, and the lowest concentration exhibiting fungal growth and ochratoxin A inhibition by 33% and 60%, respectively, was 600 µg/ml (Tab. 2). The growth of *A. nidulans* and sterigmatocystin production were completely inhibited using 3000 and 800 µg/ml of Se-NPs, respectively, while at the lowest concentration (200 µg/ml) both fungal growth and mycotoxin production were inhibited by 15 and 40%, respectively (Tab. 3).

DISCUSSION

The present study reported the ability of Se-NPs to extend a gradual inhibiting effect on the growth of three toxigenic fungal strains and their toxins production. In similar studies, Ismail et al. (2016) reported that selenium nanoparticles exert

Tab. 1. Inhibitory effects of Se-NPs on the growth of *Aspergillus parasiticus* and aflatoxins production.

Se-NPs concentration (µg/ml)	*A. parasiticus* CBS 571.65 (aflatoxins producer)		Aflatoxins inhibition	
	Fungal growth (dry weight ± SD*)	Inhibition of fungal growth (%)	Toxin level**	Inhibition of toxin production (%***)
Control	0.347 ± 0.057	0	+5	0
200	0.347 ± 0.057	0	+5	0
400	0.311 ± 0.001	10	+5	0
600	0.236 ± 0.042	32	+5	0
800	0.177 ± 0.002	49	+5	0
1000	0.168 ± 0.002	52	+4	20
2000	0.061 ± 0.002	82	0	100
3000	0.023 ± 0.001	93	0	100
4000	0.020 ± 0.002	94	0	100
5000	0.007 ± 0.012	98	0	100
6000	0.002 ± 0.001	99	0	100
7000	0.000 ± 0.000	100	0	100

* Each value represents the mean of three values (in grams) ± standard deviation.
** Detection of toxin level: +5 = toxin level 500 µg/l or more; +4 = toxin level 400 to less than 500 µg/l; 0 = toxin level 0 to less than 100 µg/l.
*** Approximate percentages assigned to the particular toxin levels.

Tab. 2. Inhibitory effects of Se-NPs on the growth of *Aspergillus ochraceus* and ochratoxin A production.

Se-NPs concentration (µg/ml)	*A. ochraceus* CBS 589.68 (ochratoxin A producer)		Ochratoxin A inhibition	
	Fungal growth (dry weight ± SD*)	Inhibition of fungal growth (%)	Toxin level**	Inhibition of toxin production (%***)
Control	0.407 ± 0.013	0	+5	0
200	0.407 ± 0.015	0	+5	0
400	0.407 ± 0.016	0	+5	0
600	0.272 ± 0.004	33	+2	60
800	0.209 ± 0.003	49	+2	60
1000	0.188 ± 0.004	54	+1	80
2000	0.130 ± 0.014	68	0	100
3000	0.112 ± 0.007	72	0	100
4000	0.101 ± 0.005	75	0	100
5000	0.088 ± 0.005	78	0	100
6000	0.068 ± 0.007	83	0	100
7000	0.068 ± 0.002	83	0	100
8000	0.060 ± 0.001	85	0	100
9000	0.000 ± 0.000	100	0	100

* Each value represents the mean of three values (in grams) ± standard deviation.
** Detection of toxin level: +5 = toxin level 500 µg/l or more; +2 = toxin level 200 to less than 300 µg/l; +1 = toxin level 100 to less than 200 µg/l; 0 = toxin level 0 to less than 100 µg/l.
*** Approximate percentages assigned to the particular toxin levels.

Tab. 3. Inhibitory effects of Se-NPs on the growth of *Aspergillus nidulans* and sterigmatocystin production.

Se-NPs concentration (µg/ml)	*A. nidulans* 69 (sterigmatocystin producer)		Sterigmatocystin inhibition	
	Fungal growth (dry weight ± SD*)	Inhibition of fungal growth (%)	Toxin level**	Inhibition of toxin production (%***)
Control	0.375 ± 0.029	0	+5	0
200	0.318 ± 0.016	15	+3	40
400	0.277 ± 0.001	26	+2	60
600	0.198 ± 0.004	47	+1	80
800	0.178 ± 0.008	53	0	100
1000	0.063 ± 0.001	83	0	100
2000	0.003 ± 0.001	99	0	100
3000	0.000 ± 0.000	100	0	100

* Each value represents the mean of three values (in grams) ± standard deviation.

** Detection of toxin level: +5 = toxin level 500 µg/l or more; +3 = toxin level 300 to less than 400 µg/l; +2 = toxin level 200 to less than 300 µg/l; +1 = toxin level 100 to less than 200 µg/l; 0 = toxin level 0 to less than 100 µg/l.

*** Approximate percentages assigned to the particular toxin levels.

a gradual inhibiting effect on the growth of the pathogenic fungus *Alternaria solani*. They reported that the lowest concentration of 50 µg/ml rendered 8.33% inhibition, while at 800 µg/ml of selenium nanoparticles completely inhibited the fungus growth. Kazempour et al. (2013) reported that the MICs (Minimum inhibitory concentrations) of Se-NPs (90–320 nm) biosynthesised with a two-phase system by using *Klebsiella pneumoniae* and without purification against *Aspergillus niger* and *Candida albicans* were 250 µg/ml and 2000 µg/ml, respectively. Shakibaie et al. (2015) reported the antifungal activity of biogenic Se-NPs (120–140 nm, synthesised by *Bacillus* sp. MSh-1) against *Aspergillus fumigatus* and *Candida albicans*. Minimum inhibitory concentration (MIC) measurements of the antifungal activity of Se-NPs against *C. albicans* (70 µg/ml) and *A. fumigatus* (100 µg/ml) suggested that biogenic Se-NPs are useful antifungal agents. Se-NPs have also been reported to be a potent anti-microbial agent against *Staphylococcus aureus* (Tran & Webster 2011).

In this respect Zohri et al. (1997) examined the effect of sodium selenite at concentrations of 0.052–4% on the highly aflatoxigenic *Aspergillus parasiticus* var. *globosus* IMI 120920 and reported a high inhibition of both fungal biomass and aflatoxin formation at high concentrations of selenite with obvious malformations in the fungus morphology in presence of different levels of selenite. Mousavi & Pourtalebi (2015) demonstrated that the MIC value of silver nanoparticles (Ag-NPs) against *A. parasiticus* was 180 µg/ml and Ag-NPs inhibited

aflatoxin B_1 production at 50% of the MIC (90 µg/ml). Khalifa & Sameer (2014) also indicated that selenium started to inhibit the fungal growth of *Penicillium digitatum*, causing green mould disease of orange fruit growth, at 100 µg/ml with 5.56% inhibition. However, at 500 µg/ml, selenium showed a fungal growth inhibition rate of 85.22%.

Mittal et al. (2015) described that under certain conditions nanoparticles are able to penetrate the epidermis and serve as a shuttle for human and animal vaccines. It should be considered that the same mechanism could be used to transport fungicides into living plants. Kotzybik et al. (2016) estimated a strong decrease in ochratoxin A biosynthesis as a function of the concentration of SiO_2 nanoparticles, which itself depended strongly on nanoparticle size. This observation makes sense as Schmidt-Heydt et al. (2015) described that mycotoxin biosynthesis, in this case citrinin biosynthesis, is induced when a fungal cell is subjected to oxidative stress. Kotzybik et al. (2016) reported that particles of 200 nm in size inhibited the growth of toxigenic *P. verrucosum* up to a concentration of 1000 µg/ml. In this case the potential contact area of nanoparticles with microorganisms like bacteria or fungi is substantially increased as well as the chance and the ability to penetrate into cells increased. Therefore the effectivity of using nanoparticles in order to inhibit fungal growth and mycotoxin biosynthesis depends strongly on the chemical composition and the concentration of the nanomaterial (Kotzybik et al. 2016).

The mechanism behind our results may explain some biological responses like the activation of alternative pathways which result after exceeding a defined physiological beginning value. Thus, different concentrations of supplemented Se-NPs may result in the activation of diverse stress-compensation pathways. Another factor may be the interaction between the surface of the respective Se-NPs and the cell wall which has an influence on the diffusion of nutrients and therefore also affects the growth rate and mycotoxin production.

Conclusion

The results of the present study suggest that Se-NPs have antifungal activity against aflatoxins, ochratoxin A and sterigmatocystin-producing microfungi and their respective mycotoxin production. The mechanism of this antifungal effect is unknown and deserves further in vivo and in vitro studies, so this is a challenge for us to do a follow-up study. The application of nanoparticles should be carefully reconsidered for each case, especially for food products.

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