

The Effect of Nanosilver on Pigments Production by *Fusarium culmorum* (W. G. Sm.) Sacc.

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Abstract

A disk-diffusion method experiment assessed the impact of nanosilver on production of secondary metabolites (pigments) by the *Fusarium culmorum* fungus. Nanosilver colloidal particles in water have been obtained by the use of a method based on high voltage electric arcs between silver electrodes. The silver nanoparticles size in colloid ranged between 15 and 100 nm and 7, 35 and 70 ppm concentration. Nanosilver modifies the metabolism of the researched *F. culmorum* strain. Coming into contact with nanosilver colloids induces more intensive mycelia pigmentation correlated with nanosilver concentration levels. The performed analysis of metabolites indicates that under the influence of nanosilver fungi biosynthesize aurofusarin more intensively and the conversion of rubrofusarin to aurofusarin is intensified as compared to the control culture. Under the influence of nanosilver *F. culmorum* intensively biosynthesizes an unidentified dye which shares structural features with aurofusarin but which is not produced by fungi in standard cultures.

Key words: *Fusarium culmorum*, pigment, silver nanoparticles

Introduction

Filamentous fungi are renowned for their ability to produce bioactive secondary metabolites that can affect organisms inhabiting the same ecological niche. This is also true for the genus *Fusarium* which has been reported to produce ecotoxins, phytotoxins, growth inhibitors and antibiotics (Chełkowski, 1989; Kwaśna and Chełkowski, 1991). An especially interesting group of *Fusarium* metabolites are the mycelium pigments aurofusarin, bikaverin, fusarubins and intermediates in their corresponding biosynthetic pathways. Several of these metabolites have been shown to have antibiotic properties and may, in future, be put to practical use. However the physiological basis for the synthesis of these compounds, the role of the pigments for the producing organism, the genetic background for the process as well as the effects on living organisms other than bacteria should be comprehensively researched. However, the majority of *Fusarium* sp. are plant pathogens that pose a huge problem in agricultural production, as infections of cereals crops by these fungi results in reduced harvest yields and the accumulation of toxic secondary metabolites dangerous for humans and ani-

mals (Chełkowski, 1989; Kwaśna and Chełkowski, 1991; Parry *et al.*, 1995; Schaafsma *et al.*, 2001; Bottalico and Perrone, 2002; Logrieco *et al.*, 2002; Pirgozliev *et al.*, 2003; Snijders, 2004; Desjardins, 2006; Rep and Kistler, 2010). Members of the *Fusarium* genus are typically identified by their red or brown mycelium coloration and banana shaped conidia (Kwaśna *et al.*, 1991; Leslie *et al.*, 2006). The red coloration is due to the production of either the monomeric polyketide bikaverin (Cornforth *et al.*, 1971; Kjaer *et al.*, 1971; Linnemannstöns *et al.*, 2002), the monomeric polyketide fusarubin (Pariot *et al.*, 1990) or the homodimeric polyketide aurofusarin (Medentsev and Akimenko, 1998; Medentsev *et al.*, 2005). Aurofusarin is among others produced by species belonging to the *F. graminearum* species complex. The biosynthesis is dependent on a 25 kb gene cluster, consisting of ten genes (PKS12, aurR1, aurO, aurT, aurR2, aurZ, aurJ, aurF, GIP1 and aurS). Targeted gene replacement studies of these genes have facilitated the development of a theory for the biosynthetic pathway of aurofusarin (Medentsev *et al.*, 2005; Malz *et al.*, 2005; Frandsen *et al.*, 2011). The pathway includes at least six enzyme catalyzed steps and four stable intermediates have been identified and structurally

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characterized; YWA1, nor-rubrofusarin, rubrofusarin and fuscofusarin. The presence of signal peptides (export signals) in three of the involved enzymes suggest that the last steps of the biosynthetic pathway is catalyzed extracellularly. However the accumulation of rubrofusarin in several of the constructed strains, Δ aurF, Δ aurS and Δ GIP1, has hampered mapping of the last steps that converts rubrofusarin into aurofusarin (Frandsen *et al.*, 2006).

Apart from *F. graminearum* the most common pathogenic *Fusarium* sp. found in moderate climate cereals is *F. culmorum*. The *F. culmorum* species is characterized by a wider feeding spectrum and is deemed to be a pathogen of many vascular plants and edible mushrooms. It may also occur within potato stores and is commonly found in soil in the form of saprophytes. *F. culmorum*, similar to *F. graminearum*, is considered to be highly toxic and its mycelia is intensively coloured (the reverse side of mycelia in cultures is carmine red) (Kwaśna *et al.*, 1991; Kang and Buchenauer, 2002; Jackowiak *et al.*, 2005). The biosynthetic basis for aurofusarin formation in *F. culmorum* is identical to what has been described in *F. graminearum* (Malz *et al.*, 2005).

Nanosilver is characterized by its strong antibacterial activity which is owed to its large surface area to weight ratio and its effects therefore depends on the size of nanoparticles (Lee *et al.*, 2005; Morones *et al.*, 2005; Panacek *et al.*, 2006; Kim *et al.*, 2007; Pal *et al.*, 2007; Shrivastava *et al.*, 2007; Lewinski *et al.*, 2008; Martinez-Castanon *et al.*, 2008; Tien *et al.*, 2008; Kim *et al.*, 2012). The antibiotic effects of nanosilver has been linked to its interaction with thiol groups in proteins, affecting the replication of DNA, and its ability to uncouple the electron transport chain from oxidative phosphorylation in the mitochondrial membrane and by collapsing the proton-motive force across the cytoplasmic membrane (Holt and Bard, 2005; Marini *et al.*, 2007). Toxic effects have also been reported for eukaryotic cells and literature includes data for both human cell lines (T, neuron, liver, skin, lung epithelial cells, and macrophages) and fungi cells. For human or animal cells it is reported that silver nanoparticles exerts cytotoxicity through the generation of reactive oxygen species resulting in induction of programmed cell death (apoptosis). Apoptosis eliminates damaged or unwanted cells *via* nuclear condensation, membrane blebbing, and DNA fragmentation in the process of development or in response to infection or DNA damage. Nanosilver has also been shown to deplete the antioxidant glutathione pool, cause mitochondrial dysfunction, and induce the release of lactate dehydrogenase (Hussain *et al.*, 2005; Keuk-Jun *et al.*, 2008; Asharani *et al.*, 2009; Foldbjerg *et al.*, 2009; Kim *et al.*, 2009; Miura and Shinohara, 2009; Yen *et al.*, 2009; Eom and Choi, 2010; Samberg *et al.*, 2010; Yang *et al.*, 2010). Nevertheless, ongoing

projects aims to investigate a variety of clinical applications of nanosilver such as delivery vehicle for genes and drugs, biosensors, implantable materials, and bone prostheses, and as drugs against skin infecting fungi (dermophytes) (Ahmed *et al.*, 2010; Faunce and Watal, 2010). Nanosilver's current applications are mainly focused on its antibacterial properties and it is used in personal care products, household appliances, antiseptic materials (Margaret *et al.*, 2006; Rujitanaroja *et al.*, 2008; Dastjerdi *et al.*, 2009), water purification (Pradeep and Anshup, 2009) and food packaging (Tankhiwale and Bajpai, 2009).

The conducted research aimed for an initial cognitive evaluation of the *in vitro* effect of nanosilver on a Polish *F. culmorum* isolate as well as an analysis of metabolites in fungal cultures subjected to contact with silver nanoparticles. Preliminary tests carried out may indicate whether the well-known nano-antibacterial activity may be useful as inductor of bioproduct synthesis like fungal metabolites.

Experimental

Materials and Methods

Used nanosilver. Silver nanoparticles have been obtained by the use of a method based on high voltage electric arcs between silver electrodes. A detailed description of the method and the apparatus used to produce the nanoparticles has been described by Kasprowicz *et al.* (2010). The silver nanoparticles size ranged between 15 and 100 nm. Nanomaterial used is shown in Fig. 1.

Fungal strains and culture conditions. *F. culmorum* strain was isolated from red cabbage (*Brassica oleracea* subsp. *capitata* f. *rubra*) in 1997 and purchased from the collection of the Plant Protection Institute in

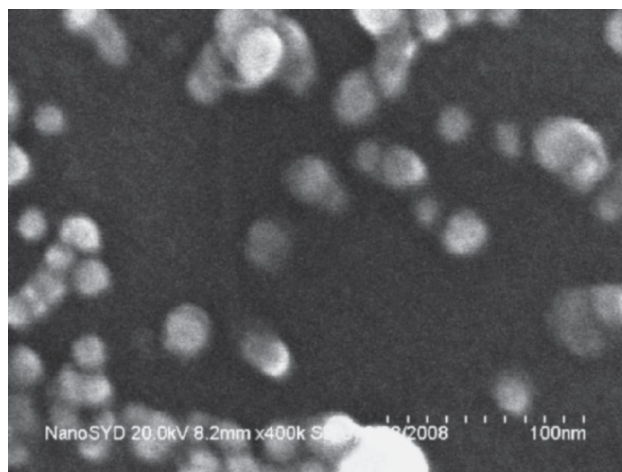


Fig. 1. TEM image of silver nanoparticles.

Poznań (Poland). The fungus was cultured on solid SNA medium in natural photoperiod conditions until intensively sporulating mycelium developed. Synthetic nutrient agar (SNA) medium – a base for obtaining abundant sporulation of the fungus *Fusarium* sp. composed of: 1 g KH_2PO_4 ; 1 g KNO_3 ; 0.5 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$; 0.5 g KCl; 0.2 g glucose; 0.2 g saccharose; 0.6 ml NaOH (1 M) and 13.2 g agar in 1 l of distilled water. A concentrated sterile water suspension of the fungus spores (about $5 \cdot 10^6$ pcs conidia $\cdot \text{ml}^{-1}$) was prepared. The number of spores was estimated using a Bürker chamber. Smears of fresh spore suspension were placed on solid culture media in sterile 90 mm diameter Petri dishes using sterile swabs. Immediately after smearing, 4 sterile 10 mm diameter tissue paper discs were placed in the Petri dish saturated with silver nanoparticles of 7, 35 and 70 ppm concentration. The control group consisted of dishes containing spore smears with sterile distilled water saturated discs placed on top. The resulting cultures were incubated at 21°C in an incubation chamber for 14 days and a natural photophase. Experiments were carried out on standardized solid PDA medium (Biocorp company, Warszawa, Poland) comprising of potato extract $4 \text{ g} \cdot \text{l}^{-1}$, glucose $20 \text{ g} \cdot \text{l}^{-1}$ and agar $15 \text{ g} \cdot \text{l}^{-1}$. Strained medium was also used, containing half of the values per litre of the above mentioned ingredients. The plate based experiment was conducted with seven replicates. Following incubation the pigmentation on the reverse side of the mycelium was established using an Agfa Duoscan T 1200 (Agfa – Gevaert N.V., Belgium) scanner and computer analysis. The pictures were processed using the UTHSCA Image Tool program v. 3.0 (The University of Texas Health Science Center at San Antonio, Texas, USA). Picture processing comprised of separating the RGB components and selecting the green hue which was exhibited the strongest contrast between the background and the areas where the dye occurred, using median filters, opening and closing, histogram equalization, limiting grey hues, single step binarization, labelling objects and measuring the number of pixels in a given object (Fig. 2).

The F-Snedecor single factor analysis of variance test for the area of occurrence of dye in experiments was used to a statistical analysis.

Analysis of metabolites. Five mm agar plugs were cut out of the agar plates and metabolites were extracted and prepared for HPLC-DAD analysis as described in Smedsgaard (1997). The samples were analysed on a Hewlett-Packard Model 1100 HPLC equipped with a diode array detector (200–500 nm) and a GROM-SIL 120 ODS-5ST, 3 nm, 60×4.6 mm column (Grom Analytik +HPLC GmbH). Ten microliter samples were introduced onto the column with an isocratic flow of 20% acetonitril and then eluted on a linear gradient to 80% acetonitril over an 8 minutes period. The eluents

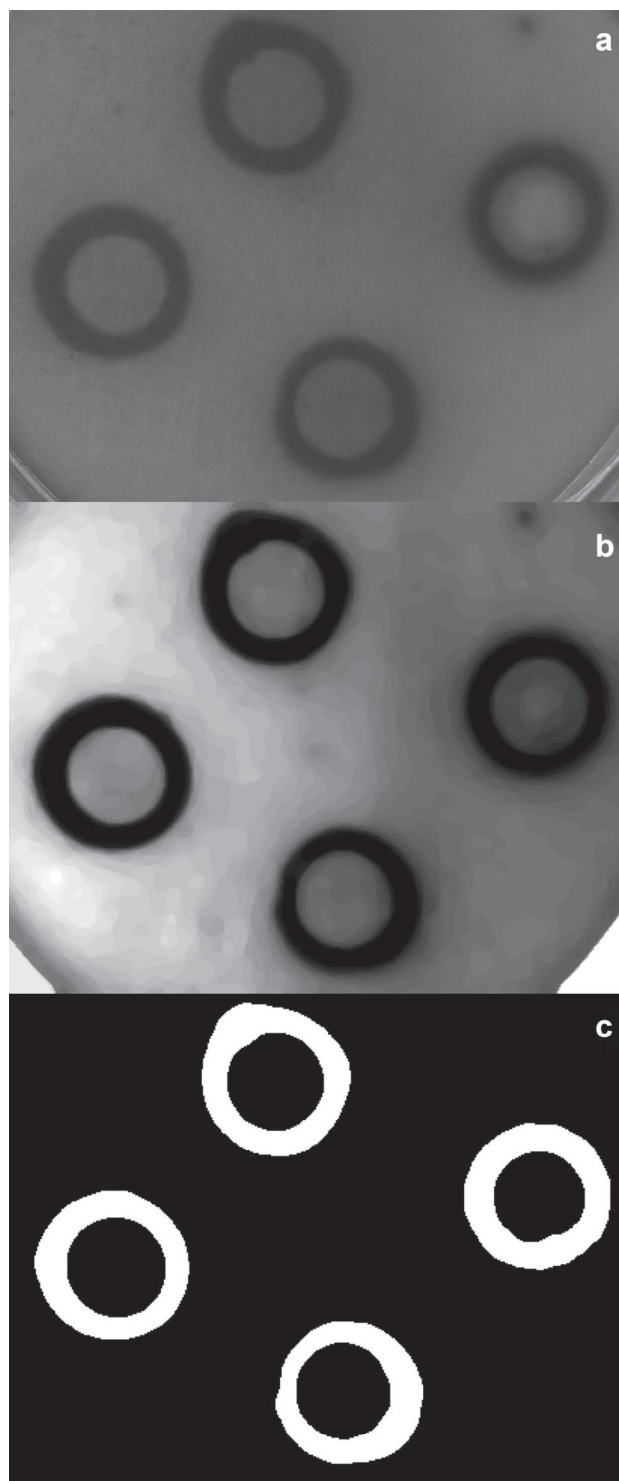


Fig. 2. a) reverse side of the *F. culmorum* cultures with nanosilver soaked paper disks; b) image after selecting green component and median, opening and closing filtering; c) binarized image of the dish reverse side.

were adjusted to 0.1% O-phosphoric acid. Aurofusarin, rubrofusarin and nor-rubrofusarin were identified by comparing with published UV-spectra (Frandsen *et al.*, 2006; Shoji *et al.*, 1967; Eisaku *et al.*, 1968) and metabolites extracted from *F. graminearum* PH-1 (wild type), Δ aurF and Δ aurJ mutants published in Frandsen

et al. (2006; 2011). The concentration of the extracted metabolites was normalized to the fungal biomass, by measuring the ergosterol concentration in the extracts. The ergosterol concentration was determined by injecting 10 μl of sample on the same column as described above, but elude with an isocratic flow of 100% acetonitril + 0.1% O-phosphoric acid, as described in Frandsen *et al.* (2006). Ergosterol was quantified by comparing to 100, 200, 500, 750 and 1000 $\mu\text{g} \cdot \text{ml}^{-1}$ ergosterol standards (Invitrogen). The incubations, extractions and LC-DAD analysis were performed in triplicates.

Results and Discussion

The disk-diffusion method is a research test commonly employed in order to establish the sensitivity of bacteria or fungi to a given compound. This method has previously been used in other studies to evaluate the effect of nanosilver on fungal growth. Petica *et al.* (2008) as well as Gajbhiye *et al.* (2009) have both reported a clear growth inhibition of several fungal species. We have previously reported (Kasprowicz *et al.*, 2010) the effects of nanosilver colloids on concentrated water solutions of the same *F. culmorum* isolate as used in the current study, where we observed an intensive inhibition of mycelia surface growth cultured from spores after contact with nanosilver. The vegetative mycelia obtained from spores, that had been in contact with nanosilver was also characterised by a significantly more intensive sporulating as compared to the control culture. The next modified characteristic of the fungus was the germination of spores. We concluded a decrease in the number of germinating spores as well as the length of germ tube for the nanosilver group as compared to the control group. The research was conducted for low concentrations levels of nanosilver (0.12–2.5 ppm). Panacek *et al.* (2006) in testing fungistatic effects of nanosilver colloids on *Candida albicans* observed an inhibition of fungal growth and development for very low nanosilver colloid concentrations, with no cytotoxicity for human fibroblasts, which provides a basis for further research on the practical uses of nanosilver in treating human mycosis. The results obtained to date permit one to conclude that nanosilver stem the life functions of fungi.

In the conducted experiment, a significant stimulation of pigment biosynthesis in *F. culmorum* was observed when the fungus was exposed to nanosilver in concentrations of 35 and 70 ppm. The heavily pigmented surface of the reverse side of the mycelium grown on full PDA media with the above mentioned concentrations was three times as large as that observed in the control group. At the lower concentration, 7 ppm nanosilver, the mycelia pigmentation was comparable

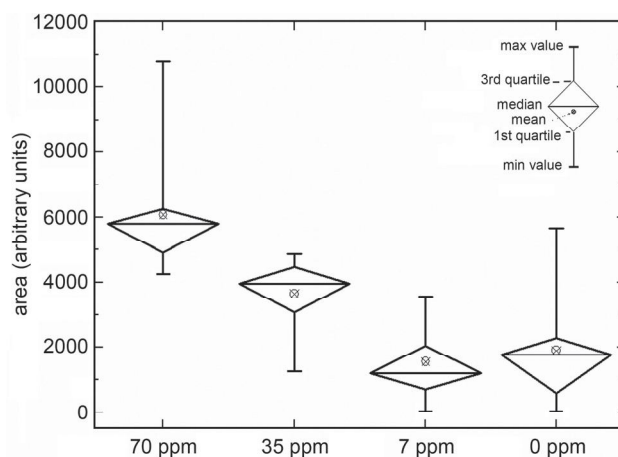


Fig. 3. Effects of nanosilver on the *F. culmorum* mycelia reverse side pigmentation in full PDA medium cultures as compared to the control group.

with that of the control group. The results obtained for the pigmented area of mycelia cultures on a standard PDA medium is shown in Fig. 3. It was concluded that together with the increase in nanosilver concentrations in saturated disks placed over the mycelia, the area of pigmentation on the reverse side of cultures increased significantly, which is attributed to a modification of the physiological processes in the fungus in the presence of nanosilver.

Table I depicts a statistical analysis of variance for the obtained results. The lack of statistically significant differences in the pigmentation of cultures as compared to the control group was only obtained for the lowest nanosilver concentration levels of 7 ppm. The higher concentration levels, which are 35 and 70 ppm, of nanosilver significantly, modified the culture pigmentation as compared to the control group.

Table I

The F-Snedecor single factor analysis of variance test (F-test = 4.17) for the area of occurrence of dye on solid PDA medium – shaded cells indicate no statistically significant difference.

	0 ppm	7 ppm	35 ppm
7 ppm	0.66	–	–
35 ppm	15.66	32.42	–
70 ppm	63.35	94.55	27.04

In cultures on strained PDA media, the control cultures displayed the smallest area of discolouration. The use of nanosilver stimulated the evaluated fungal strain to biosynthesise pigments at significantly elevated levels as compared to that of the control group, an effect that was dose dependent. The results obtained are illustrated in Figure 4. Statistical analysis of variance (Table II) indicated that only for nanosilver con-

Table II

The F-Snedecor single factor analysis of variance test (F-test = 4.22) for the area of occurrence of dye on solid poor PDA medium – shaded cells indicate no statistically significant difference.

	0 ppm	7 ppm	35 ppm
7 ppm	45.35	–	–
35 ppm	290.51	45.18	–
70 ppm	245.50	51.50	1.20

centration levels of 35 and 70 ppm were the obtained pigmented areas similar.

The effects of nanosilver on mycelium pigment production in *F. culmorum* was analysed by LC-DAD. The addition of 70 ppm nanosilver on a paper disk test changed the mycelium colour from yellow to red

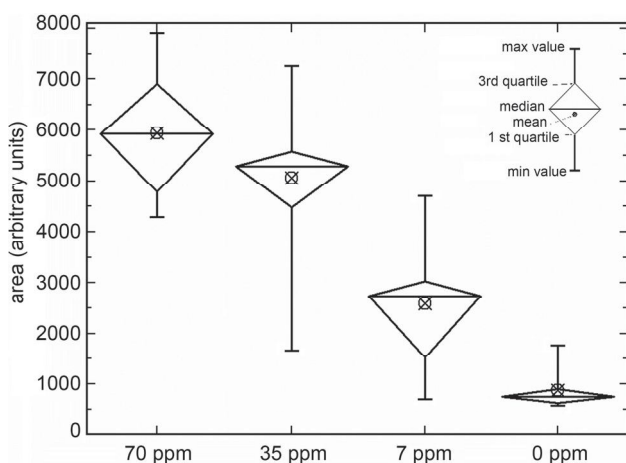


Fig. 4. Effects of nanosilver on the *F. culmorum* mycelia reverse side pigmentation in strained PDA medium cultures as compared to the control group.

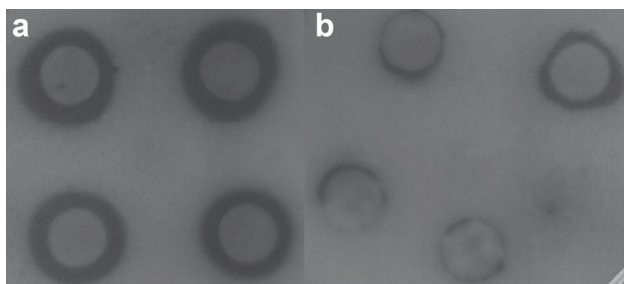


Fig. 5. Pictures of cultures with (A) and without (B) nanosilver.

(Fig. 5). Similar shifts due to pH changes have previously been reported for aurofusarin (Malz *et al.*, 2005). However the results presented in Fig. 6 show that incubation with nanosilver results in an increased accumulation of the red pigment aurofusarin and a decrease in the concentration of its precursor rubrofusarin (yellow), showing that the observed change in colour was not solely due to changes in pH. This suggested that

nanosilver stimulates the conversion of rubrofusarin into aurofusarin, but whether this conversion was due to a direct catalytic effect of nanosilver or due to stimulation of the natural enzyme system is unknown. However, the addition of nanosilver also resulted in the accumulation of a novel pigment (rt = 3.504 min) with a UV/VIS spectrum very similar to that of aurofusarin (Fig. 6 C and D). This compound has not been observed previously in the natural system, pointing towards both a biotic and abiotic effect of nanosilver. The novel compound eluted earlier than aurofusarin under the used reverse phase chromatography conditions suggesting that it is more hydrophilic than aurofusarin. This combined with the similarities in the UV/VIS spectrum suggest that the novel compound is a dimer of one of the previously described intermediates in the biosynthetic pathway, such as YWA1, nor-rubrofusarin, rubrofusarin or combinations of these. Definitive identification of this compound will rely on NMR and HR-MS studies. However there is no doubt that the unknown compound is structurally related to aurofusarin (dimeric), based on the high degree of similarity to the aurofusarin UV-spectrum. The UV spectrum is primarily the result of the extensive conjugated bond system within the molecule.

The toxic effects of nanosilver have been suggested to be mediated by their production of reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^*) (Gajbhiye *et al.*, 2009; Ivask *et al.*, 2010). An increased concentration of extracellular radicals might explain the formation of the novel aurofusarin related metabolites in the current experiments. Dimerization and polymerization of fungal pigments has been linked to radical mediated activation of monomers, known as oxidative coupling (Gill *et al.*, 1990), this is also the case for the aurofusarin biosynthetic pathways where the monooxygenases (AurF) or laccases (GIP1) are responsible for catalyzing the dimerization of two monomers. The biological function of polyaromatic pigments for fungi is in part to protect from damage caused by VIS/UV-radiation, by absorbing light, and secondly to act as an antioxidant defence mechanism that quenches radicals, formed by both abiotic and biological mechanisms. Future experiments will be directed towards understanding whether the effects of nanosilver is due to ROS that affects the biosynthetic apparatus, or whether the effect is more directly based on the formation of radical forms of the aurofusarin intermediate, which might facilitate premature dimerization of intermediates to form non-natural intermediates.

Conclusion. Nanosilver modifies the metabolism of the analyzed *F. culmorum* strain. Coming into contact with nanosilver colloids induces more intensive mycelia

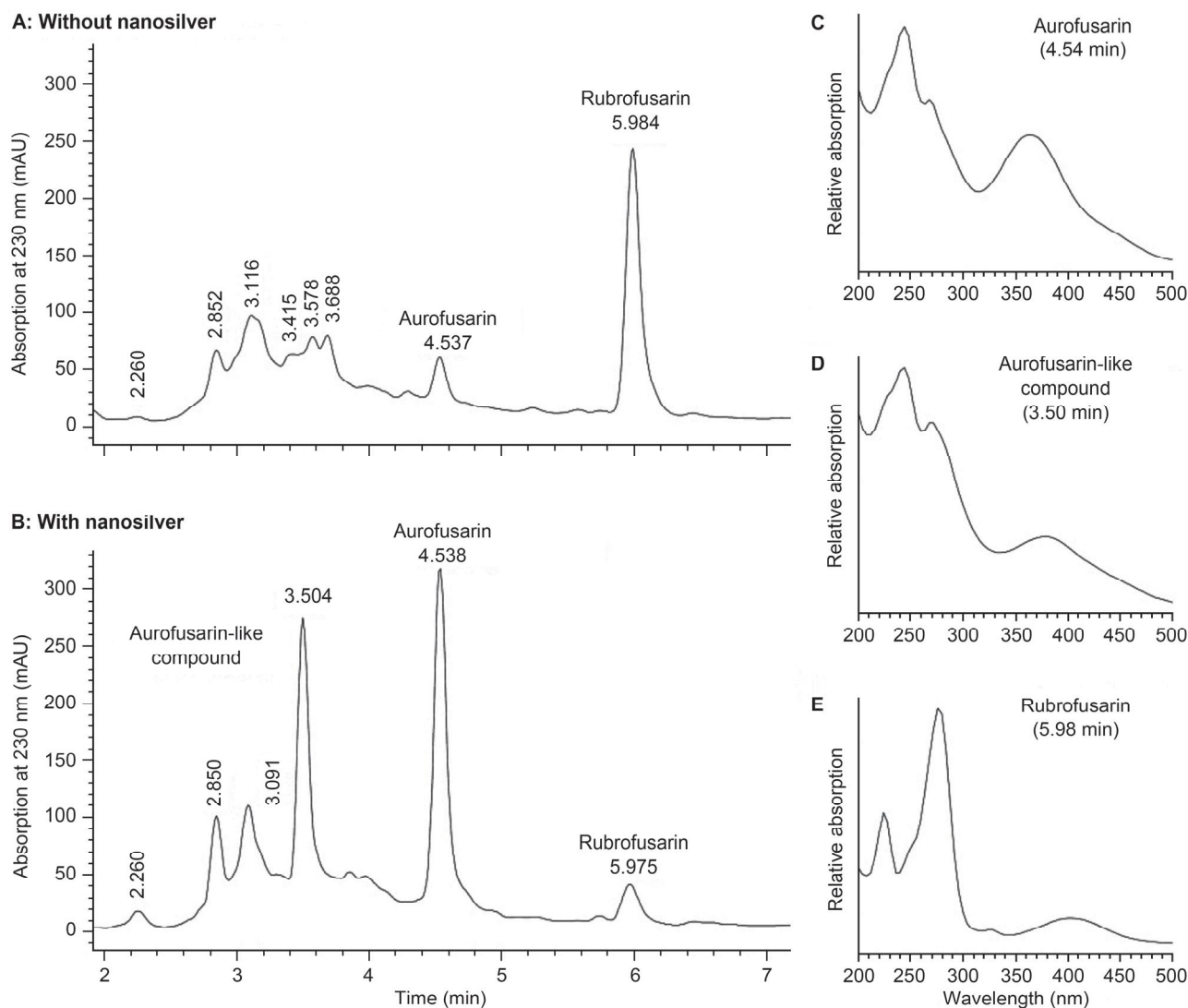


Fig. 6. LC-DAD analysis of extracted metabolites from *F. culmorum* cultures without (A) and with 35 ppm nanosilver (B). Growth on standard PDA medium resulted in the production of high concentrations of rubrofusarin and lower amounts of auofusarin. The addition of nanosilver to the growth medium resulted in the production of high concentrations of auofusarin and an unknown auofusarin-like compound, while the rubrofusarin concentration was reduced to 1/10 of that observed in the wild type. The auofusarin-like compound (D) display a UV/VIS spectrum very similar to that previously reported for auofusarin (C), however the compound was more hydrophilic than auofusarin and eluted app. 1 minute earlier than auofusarin.

pigmentation correlated with nanosilver concentration levels. The performed analysis of metabolites indicates that under the influence of nanosilver fungi biosynthesise auofusarin more intensively and the conversion of rubrofusarin to auofusarin is intensified as compared to the control culture. Under the influence of nanosilver *F. culmorum* intensively biosynthesises an un-identified dye with a UV/VIS spectrum that is similar to that of auofusarin, though not produced by fungi in standard cultures. Achieved preliminary results for Polish strain of *Fusarium* fungus indicate that in addition to its antibacterial and fungistatic nanosilver can be used for induction of biosynthesis specific dyes of fungi. However, this requires further detailed study in relation to other species and strains of *Fusarium* fungi, as well as recognition of the direction of changes in biosynthesis.

Acknowledgments

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