In vitro evaluation of the activity of thiosemicarbazone derivatives against mycotoxigenic fungi affecting cereals

Francesca Degola, Caterina Morcia, Franco Bisceglie, Francesca Mussi, Giorgio Tumino, Roberta Ghizzoni, Giorgio Pelosi, Valeria Terzi, Annamaria Buschini, Francesco Maria Restivo, Tiziana Lodi

PII: S0168-1605(15)00079-3
DOI: doi: 10.1016/j.ijfoodmicro.2015.02.009
Reference: FOOD 6806

To appear in: *International Journal of Food Microbiology*

Received date: 24 September 2014
Revised date: 26 January 2015
Accepted date: 5 February 2015

Please cite this article as: Degola, Francesca, Morcia, Caterina, Bisceglie, Franco, Mussi, Francesca, Tumino, Giorgio, Ghizzoni, Roberta, Pelosi, Giorgio, Terzi, Valeria, Buschini, Annamaria, Restivo, Francesco Maria, Lodi, Tiziana, *In vitro* evaluation of the activity of thiosemicarbazone derivatives against mycotoxigenic fungi affecting cereals, *International Journal of Food Microbiology* (2015), doi: 10.1016/j.ijfoodmicro.2015.02.009

In vitro evaluation of the activity of thiosemicarbazone derivatives against mycotoxigenic fungi affecting cereals.

Francesca Degola[a], Caterina Morcia[c], Franco Bisceglie[b], Francesca Mussi[a], Giorgio Tumino[c], Roberta Ghizzoni[c], Giorgio Pelosi[b], Valeria Terzi[c], Annamaria Buschini[a], Francesco Maria Restivo[a**], Tiziana Lodi[a]

a) Dipartimento di Bioscienze, Università di Parma, Parma, Italy

b) Dipartimento di Chimica, Università di Parma, Parma, Italy

c) Consiglio per la Ricerca e la sperimentazione in Agricoltura, CRA-GPG, Genomics Research Centre, Fiorenzuola d'Arda, Italy

** to whom correspondence should be addressed

Francesco Maria Restivo, Dipartimento di Bioscienze, Viale delle Scienze 11A, 43124, Parma , Italy

e-mail: francescomaria.restivo@unipr.it

phone +39-0521-905603

fax: +39-0521-905604

key words: thiosemicarbazone, *Fusarium*, *Aspergillus flavus*, mycotoxin, cytotoxicity, cereals

Highlights

- A panel of thiosemicarbazones derivatives were synthesized
- The compounds were tested for their effect on fungi (*Fusarium* and *Aspergillus*) affecting cereal crops
- Species-specific effects on fungal growth and mycotoxins biosynthesis were observed
- Two molecules were considered promising for fungal growth and mycotoxin containment
- Potential deleterious effects on seed germination and on human cells were tested

Abstract

With a steadily increasing world population, a more efficient system of food production is of paramount importance. One of the major causes of food spoilage is the presence of fungal pathogens and the production and accumulation of mycotoxins. In the present work we report a study on the activity of a series of functionalized thiosemicarbazones (namely cuminaldehyde, trans-cinnamaldehyde, quinoline-2-carboxyaldehyde, 5-fluoroisatin thiosemicarbazone and 5-fluoroisatin N^4-methylthiosemicarbazone), as antifungal and anti-mycotoxin agents, against the two major genera of cereal mycotoxigenic fungi, i.e. *Fusarium* and *Aspergillus*. These thiosemicarbazones display different patterns of efficacy on fungal growth and on mycotoxin accumulation depending on the fungal species. Some of the molecules display a greater effect on mycotoxin synthesis than on fungal growth.

1. Introduction

Plant diseases are probably the greatest obstacle to the increase of global crop production and also one of the major factors limiting crop quality. Crop protection plays a key role in ensuring food security and, in turn, is directly related to food preservation, a key step to increasing both food supply and food safety. In fact about 40% of the food produced worldwide is lost or spoiled (Oerke and Dehne, 2004) and this not only reduces its availability, but also has an impact on global climate change by causing excess consumption of fresh water and fossil fuels.

One of the major causes of food spoilage is the presence of micro-organisms. In particular, fungal pathogens associated with various crop diseases can lead not only to significant production losses, but can also result in the production and accumulation of secondary metabolites - mycotoxins - hazardous to human and animal health. In cereals, the main mycotoxin producers belong to the genera *Aspergillus*, *Penicillium* and *Fusarium*. *Fusarium* are "field" fungi with worldwide distribution that can colonize the cereal plant during flowering causing Fusarium Head Blight (FHB), a destructive disease that leads to severe yield losses and to the accumulation of mycotoxins in the grains (Goswam and Kistler, 2005). Types A and B trichothecenes, and zearalenone are the most important toxic products that can occur individually or in combination. In European environments, the most common species in the FHB complex are *F. graminearum, F. culmorum, F. sporotrichioides* and *F. langsethiae*. As far as *Aspergillus* is concerned, a few species belonging to this genus (mainly *A. flavus* and *A. parasiticus*) may colonise various crops intended for human and animal nutrition. As these fungi are well known mycotoxin producers (mainly

aflatoxins; AFs), they consitute a serious threat to humans and animals. In particular, *Aspergillus flavus*, can be found in corn fields throughout the world and is responsible of severe economic losses for the farmers and the stakeholders of the post-harvest phase (Abbas et al., 2009; Williams, 2008; Wu and Khlangwiset, 2010). In fact, the AF risk for human health comes not only from direct consumption of crop materials, but may persist in processed products (milk, cheese, etc) derived from animals fed with contaminated feed. Several and complementary strategies have been adopted to control FHB and AF, starting from the breeding of resistant cereal varieties, to the adoption of more suitable agronomic practices including the application of fungicides (for reviews see Abbas et al., 2009; Terzi et al., 2014). Currently, fungicides are required to increase food availability, to reduce food waste and to increase food safety (Cooper and Dobson, 2007). However the use of fungicides can lead, in the medium and long term, to the evolution of populations of pathogens resistant to the antimicrobial agents used against them (Anderson, 2005; Ma and Michailides, 2005). Hence, the identification of new molecular targets becomes of paramount importance for novel strategies in crop protection.

A secondary but no less important issue that should be considered when testing new fungicides for their activity on growth inhibition concerns a possible contradictory and unwanted effect on mycotoxin biosynthesis; i.e. inhibition of mycelium growth resulting in an increase of toxin production (Schmidt-Heydt et al., 2013). In some cases, such as *Aspergillus flavus* colonization of maize crops, the prevalent economic damage emerges from the sanitary risk deriving from toxin contamination of the food matrix rather than to a negative effect on plant yield (van Egmond and Jonker, 2004).

Thiosemicarbazones, an important class of nitrogen and sulfur containing compounds, have received great attention because of their chemical and biological activities, such as antibacterial, antivirus, antiamoebic, and antitumor activities in human pharmacology (Pelosi, 2010). Numerous thiosemicarbazone derivatives are also active for the control of some human pathogens, however there are only a limited number of studies concerning their applications [see patents: CN 102219769 (2011) CN 103694155 (2014)]. Recently, data on the effect of some thiosemicarbazone metal complexes on a subset of plant pathogens were published (Tyagi et al., 2014); however the study was mainly aimed at characterizing the coordination compounds and investigating the influence of the metal on the features of the compounds, and a very limited part of the work was dedicated to mycological studies.

The aim of the present work was therefore to evaluate the potential of a panel of thiosemicarbazones, differing in their functionality, for crop protection and food spoilage control,

with a particular focus on the biological activity of these compounds as antifungal and anti-mycotoxin agents against the two major genera of cereal mycotoxigenic fungi, i.e. *Fusarium* and *Aspergillus*.

2. Material and methods

2.1. Thiosemicarbazones synthesis

Figure 1 depicts the synthesized thiosemicarbazones for which the acronyms reported in parentheses are used: cuminaldehyde thiosemicarbazone (**Htcum)**, *trans*-cinnamaldehyde thiosemicarbazone (**Htcin**), quinoline-2-carboxyaldehyde thiosemicarbazone (**Htisq**), 5-fluoroisatin N^4-methylthiosemicarbazone (**HtmeFis**) and 5-fluoroisatin thiosemicarbazone (**HtFis**). All reagents used in the syntheses were purchased from Aldrich. C, H, N analyses were obtained with a Carlo-Erba 1108 instrument. IR spectra were recorded using KBr pellets on a Nicolet 5PC FTIR spectrophotometer, or using the compounds directly on the ATR accessory in the 4000-400 cm^{-1} range. The relative intensity of reported FT IR signals are defined as s = strong, br = broad, m = medium, and w = weak. ^1H NMR spectra were recorded on a Bruker Avance spectrometer at 300 MHz with TMS as the internal reference. The splitting of proton resonances in the reported ^1H NMR spectra are defined as s = singlet, br s = broad singlet, d = doublet, t = triplet, and m = multiplet. Melting points were determined with a Gallenkamp instrument (Weiss–Gallenkamp). UV measurements were performed on a Varian UV-vis Cary 50 spectrometer with quartz cuvettes. The ligands were prepared following a modified procedure based on the following refs (Bisceglie et al., 2014; Chiyanzu et al., 2003; Karali et al., 2007; Yi et al., 2011). **Htcum** and **Htcin**· were synthesized by respectively adding 0.39 mL (2.3 mmol) of cuminaldehyde (*p*-isopropyl benzaldehyde) or 0.29 mL (2.3 mmol) of *trans*-cinnamaldehyde (3-phenyl-2-propenal) to 20 mL of a hot stirred ethanolic solution of thiosemicarbazide (0.21 g, 2.3 mmol). The mixture was then refluxed for 7-9 h and left cooling down to room temperature. **Htisq** was obtained by dissolving thiosemicarbazide (337.6 mg, 3.7 mmol) in 80 mL of methanol at room temperature under stirring and adding an equimolar amount of quinoline-2-carboxaldehyde (582.1 mg, 3.7 mmol). After adding a few drops of glacial acetic acid to catalyze the reaction, the solution became clear and its color turned to orange. This reaction mixture was kept under stirring and placed in an ice bath and left standing for 20 hrs. The white precipitate was filtered on a Buchner and washed using ethanol. **HtmeFis** was synthesized by dissolving, in about 70 mL of methanol,350.6 mg (3.3 mmol) of 4-methyl-3-thiosemicarbazide together with an equimolar amount of 5-fluoro-isatin (389.6 mg).

Similarly, **HtFis** was obtained by reacting 110.6 mg (1.1 mmol) of thiosemicarbazide with an equimolar amount of 5-fluoro-isatin (200.3 mg) in about 60 mL of methanol. To the resulting orange solutions, few drops of glacial acetic acid were added and the resulting mixture was heated to reflux temperature under magnetic stirring for 24 h. A yellow powder was isolated by filtration using a Buchner funnel and washed 3 times with methanol.

2.1.1. *Cuminaldehyde thiosemicarbazone* (**Htcum**)

Yield: 86%. Mp: 144 °C. FT-IR (KBr, cm^{-1})3411, m, 3280, m, ν (NH); 3013, m, ν($CH_{aromatic}$);2957, mw, ν($CH_{aliphatic}$), 1586, s, ν (C=N); 820, s, ν (CS). Anal. Calc. for $C_{11}H_{15}N_3S$: C 59.64; H 6.78; N 18.98; S 14.48. Found: C 59.54; H 6.62; N 18.67; S 14.19 %. ^{1}H NMR data (δ, ppm; DMSO d_6): 1.23 (d, 6 H, CH_3); 2.89 (ept, 1H, CH *isopropyl*); 7.27, 7.30 (2s, 2H, aromatic); 7.70 (m, 2H, aromatic); 7.93 and 8.03 (2s, 2H, NH_2); 8.17 (s, 1H, CH=N);11.34 (br, 1H, NHCS). UV−visible (solvent MeOH): 312 nm (n → π*), 223 nm (n → σ*); (solvent DMSO): 311 nm (n → π*), 240 nm (n → σ*).

2.1.2. *trans-Cinnamaldehyde thiosemicarbazone* (**Htcin**)

Yield: 91%. Mp: 139 °C. FT-IR (KBr, cm^{-1})3416, s, 3260, m, 3154, m, ν (NH); 3042, mw, ν($CH_{aromatic}$); 1630, ms, ν (C=N); 1621, ms, ν (C=C); 820, w, ν (CS). Anal. Calc. for $C_{10}H_{13}N_3OS$: C 53.78; H 5.87; N 18.81; S 14.36. Found: C 53.65; H 5.35; N 19.12; S 14.53 %. ^{1}H NMR data (δ, ppm; DMSO d_6): 6.68 (m, 1 H, CH=C); 7.06 (d, 1 H, CH=C); 7.30-7.60 (m, 6 H, aromatic *o* and *m,* and NH_2); 7.90 (d, 1H, aromatic *p*); 8.17 (m, 1 H, CH=N); 11.38 (br, 1H, NHCS). UV−visible (solvent MeOH): 297 nm (n → π*), 246 nm (n → σ*); (solvent DMSO): 324 nm (π → π*), 241 nm (n → σ*).

2.1.3. *Quinoline-2-carboxyaldehyde thiosemicarbazone* (**Htisq**)

Yield: 77%. Mp: 180 °C. FT-IR (cm^{-1}): 3395, 3262 and 3152 ν N-H, 3066 ν C-H arom., 2986 ν C-H aliph., 1609 ν C=N, 1533 and 1503 ν C=C, 1110 and 839 ν C=S. Anal. Calc. for $C_{11}H_{10}N_4S$: C 57.37, H 4.38, N 24.33. Found: C 57.38, H 4.22, N 24.55 %. ^{1}H NMR data (δ, ppm; DMSO d_6) 11.79 ppm (1H, s, C=N-NH), 8.45 ppm (2H, d, J = 8.7 Hz, NH2), 8.35 ppm (2H, m, CH arom.), 8.23 ppm (1H, s, CH=N), 8.00 ppm (2H, t, J = 8.7 Hz, CH arom.), 7.78 ppm (1H, t, J = 8.4 Hz, CH arom.), 7.62 ppm (1H, t, J = 8.1 Hz, CH arom.).

2.1.4. *5-fluoroisatin N^4-methylthiosemicarbazone* (**HtmeFis**)

Yield: = 34%. Mp: 150 °C. FT-IR (cm^{-1}): 3196. ν (N-H), 1693 ν (C=O), 1670 ν (C=O), 1552 ν (C=N), 842 and 743 ν (C=S). Anal. Calc. for $C_{10}H_9FN_4OS$: C 47.6, H 3.6, N 22.2. Found: C 47.48, H 3.57, N 21.85 %.^{1}H NMR data (δ, ppm; DMSO d_6) 12.50 ppm (1H, s, C=N-NH), 9.35 ppm (1H, s, CH_3-N*H*), 7.48-6.95 ppm (3H, m, CH arom.), 3.10 ppm (3H, m, C*H*$_3$-NH).

2.1.5. *5-fluoroisatin thiosemicarbazone* (**HtFis**)

Yield: = 58%. Mp: 135 °C. FT-IR (cm^{-1}): 3435-3261 ν (N-H), 1685 ν (C=O), 1482 ν (C=N), 863 and 754 ν (C=S). Anal. Calc. for $C_9H_7FN_4OS$: C 45.37, H 2.96, N 23.52. Found: C 45.36, H 2.96, N 23.58 %.^{1}H NMR data (δ, ppm; DMSO d_6) 12.36 ppm (1H, s, C=N-NH), 11.21 ppm (1H, s, CH arom.), 9.13-8.77 ppm (2H, 2s, N*H*$_2$), 7.52-6.90 ppm (3H, m, CH arom.).

2.2. Fungi

Strains of *Fusarium graminearum* and *Fusarium sporotrichioides* (provided by the Università Cattolica del Sacro Cuore, Piacenza, Italy) isolated from bread wheat plants in Northern Italy in 2002 and 2010 respectively were used. Strains of *Aspergillus flavus* were isolated from corn kernels collected in the fields of the Po Valley and have been described previously (Degola et al., 2011). The toxigenic *A. flavus* strain Fri2+ and the atoxigenic strain Toϕ were mainly used in the experiments reported in this work**.**

2.3. Evaluation of the effects of thiosemicarbazones on *in vitro* mycelial growth

2.3.1. *Fusarium*

The mycelial growth of *F. graminearum* and *F. sporotrichioides* was evaluated on solid PDA medium (Liofilchem, Teramo, Italy), amended with 0.5% Tween 20 (Sigma, St Louis, MO, USA) and 100 μM of **Htcum**, **Htcin**, **Htisq**, **HtmeFis**, **HtFis** thiosemicarbazones. Moreover, **Htcum** and **Htcin** molecules were tested in the range from 0 to 300 μM. The experiments were conducted twice in triplicate in 60-mm Petri dishes, inoculated with 8-mm PDA plugs from actively growing cultures. Mycelial growth was evaluated as the mean diameter measured each day from fungal inoculation up to 6 days post-inoculation. The effect of different molecules and of different concentrations was expressed as percentage inhibition, calculated according to the formula:

$$I=[(C-T)/C] \times 100$$

where I is the percentage of inhibition, C is the control plate colony diameter in mm, T is the treated plate colony diameter in mm.

2.3.2. *Aspergillus*

Conidia suspensions were obtained from 10-day YES-agar [2% (w⁄v) yeast extract (Difco, Detroit, MI), 5% (w⁄v) sucrose (Sigma, St Louis, MO), 2% (w⁄v) agar (Difco)] cultures incubated at 28 °C; conidia concentration (quantified by OD600) and viability (>90%) were determined according to Degola et al. (2011). Determination of *A. flavus* radial growth was performed in YES-agar amended with 100 µM of B, C, I, M, O thiosemicarbazones: three single spots (5 µL of a 10^7 conidia/ml suspension each) of aflatoxigenic strain Fri2 were equidistant inoculated in 90-mm Petri dishes, plates were incubated for 4 days at 25 °C and the mycelial growth was evaluated daily by measuring the reverse of colonies along two orthogonal diameters. Radial growth was expressed as cm/d ± SD. Additionaly, conidial germination rate and post germination hyphal outgrowth were assessed analyzing changes in optical density of spore suspensions over time: in a 96 well microtiter plate (Sarstedt, Newton, NC, USA) 10^4 spores were inoculated in a final volume of 200 µL of YES liquid medium amended with **Htcum**, **Htcin**, **Htisq**, **HtmeFis**, **HtFis** thiosemicarbazones and incubated at 28 °C. The optical density at 620 nm (OD_{620}) was recorded for each well every 120 min with a microplate reader (MULTISKAN EX, Thermo Electron Corporation, Vantaa, Finland) without shaking. Samples were inoculated in triplicate. Preliminary experiments indicated that OD values, recorded between 38 and 46 h after inoculum, could reliably be used to compare growth rate of the different samples. Results obtained according to radial growth and optical density determinations were comparable, as far as the inhibitory effect of the thiosemicarbazones are concerned. Biomass variation was also determined in some experiments involving inoculation in CCM medium (see 2.4.2): fresh or dry mycelium weight were obtained, for each sample, withdrawing the mycelium from eight wells of the microplate and pooling them. The pooled mycelia were then blotted dry on clean paper and weighed (fresh weight determination) and then incubated at 80 °C for 48 h before dry weight determination. Samples were inoculated in triplicate.

2.4. Evaluation of the effect of molecules on mycotoxin production

2.4.1. *Fusarium*

The entire mycelium was collected from control and treated plates inoculated with *Fusarium graminearum* and *Fusarium sporotrichioides* six days after inoculation. The deoxynivalenol concentration produced by *F. graminearum* mycelium was determined using Ridascreen Fast DON ELISA kit (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions. The T2-HT2 concentration produced by *F. sporotrichioides* mycelium was determined using VERATOX ELISA kit (Diessechem srl, Milan, Italy), according to the manufacturer's instructions.

2.4.2. *Aspergillus*

A highthroughput procedure performed in multiwell plate was used to assess aflatoxin accumulation in a coconut milk-derived medium (CCM) (Degola et al., 2012, 2011). Briefly: 400 mL of commercial coconut cream was diluted to the final volume of 1.2 L with bidistilled water, sterilized by autoclaving (10 min, 120 °C), cooled at 4 °C overnight and clarified by centrifugation (15 min at 3200 g). The residual floating material and the pellet were discarded, and the intermediate phase was then recovered and used as culture medium in the aflatoxin inhibition assays. The effect on aflatoxin biosynthesis was assessed by the microplate fluoresescence-based procedure described in Degola et al. (2012). Standard flat-bottom 96-well microplates (Sarstedt, Newton, NC, USA) were used. Suspensions of conidia were diluted to the appropriate concentrations and brought to the final concentration of $5x10^2$ conidia/well; cultures were set in a final volume of 200 µL/well of CCM medium added with **Htcum**, **Htcin**, **Htisq**, **HtmeFis**, **HtFis** thiosemicarbazones. The plates were incubated in the dark under stationary conditions for up to 6 days at 25 °C. Aflatoxin accumulation was monitored by fluorescence emission determination: readings were performed directly from the bottom of wells of the culture plate with a microplate reader (TECAN SpectraFluor Plus, Männedorf, Switzerland) using the following parameters: λex = 360 nm; λem = 465 nm; manual gain = 83; lag time = 0 µs; number of flashes = 3; integration time = 200 µs).

2.5. Effects of thiosemicarbazones on seed germinability

2.5.1. Barley

Four hulled barley cultivars (Cometa, Martino, Nure and Tremois) and three hulless cultivars (Astartis, Fior7341 and Zacinto) were used for germination tests. Stocks of 100 seeds/cultivar were treated for 2 h with solutions of 0.05% Irol Plus in sterile distilled water containing 0 µM, 300 µM and 600 µM of **Htcum** and **Htcin** molecules. After the treatments, the seeds were placed on a wetted filter paper (2 mL sterile distilled water) in a Petri dish at room temperature and the percentage of germination was evaluated after 1 week. Two replicates of the experiment were done. A chi-squared analysis was performed to compare germination percentages of treated and non-treated seeds.

2.5.2. Corn

Effect of thiosemicarbazones on corn germination was assessed according to the International Seed Testing Association (ISTA) rules (http://seedtest.org/en/home.html). Maize kernels soaked for 5 min in a solution containing **Htcum** or **Htcin** thiosemicarbazone to the final concentration of 100

µM were placed and rolled in wet paper towels (that increases the surface contact area between the seeds and the moisture supply),which were then placed in an upright position (MacKay, 1972). Rolls were incubated up to 4-5 days at 25 °C. Seeds were considered to have germinated after the emergence and development from the embryo of dicots and root. Root number per seed and mean root lenght were also recorded.

2.6. DPPH radical scavenging activity assay

Measurement of scavenging activity of thiosemicarbazones against the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was performed in accordance with Choi et al. (Choi et al., 2002) measuring the bleaching of a purple colored methanol solution of the stable DPPH radical. Briefly, to a 1 mL aliquot of freshly prepared 85 µM DPPH was added a 4 mL solution containing the selected compound. The resulting mixture was stirred and then incubated for 30 min at room temperature The thiosemicarbazone concentrations were 10 µM, 100 µM and 1000 µM.The decrease in absorbance measured at 518 nm depicts the scavenging activity of the compound against DPPH. Ascorbic acid (30 mM) was used as positive control to determine the maximal decrease in DPPH absorbance (Choi et al., 2002). Values were expressed in percentage of inhibition of DPPH absorbance in relation to the control values without the thiosemicarbazone (ascorbic acid maximal inhibition was considered 100% of inhibition).

2.7. Effect of thiosemicarbazones on *Aspergillus flavus* growth and mycotoxin production on maize kernels

Autoclaved maize kernels (100 seeds, 30 g) were inoculated by soaking in a suspension (0.1% Tween in distilled water) containing 10^3 conidia of *A. flavus*, transferred wet to a sterile Petri plate (diam. = 15 cm) and incubated at 28 °C in the dark for 5-7 days. For the treatment with the thiosemicarbazones, the conidial suspension was amended with **Htcum** or **Htcin** molecules to the final concentration of 100 µM. Controls received an equivalent concentration of the thiosemicarbazone solvent (DMSO; 1% final concentration). The experiments were performed twice in triplicate. Data are reported as mean ± SD of the number of infected seeds as detected by visual inspection. For aflatoxin determination, infected maize kernels were added to 150 mL of a 80% MetOH solution and ground for 3 min in a blender. The maize methanol mixture was then passed through a paper filter, diluted 1:10 in bidistilled water and filtered with a microfiber filter (1.5 µm, VICAM, Watertown, MA, USA). Aflatoxin concentration was determined by HPLC as described by Degola et al. (2009) with minor modifications. The filtrate was diluted 1:100 in the mobile phase before HPLC injection (Spotti et al., 1999). The chromatographic analyses were

performed using a JascoModel PU-1580 pump, equipped with a Vertex column (150×4.6 mm, Eurospher 100-5C18, Kramer, Berlin, Germany), a Jasco Model AS-1555 autosampler (loop=0.1 ml), and a Jasco Model FP-1520 fluorescence detector (λ_{ex}=365 nm and λ_{em}=440 nm).

2.8. Toxicity of thiosemicarbazones on human cells

The growth inhibitory effect towards a human foetal lung fibroblast cell line was evaluated by the MTS assay (CellTiter96^{R}AQ$_{ueous}$One Solution Cell Proliferation Assay, Promega Corporation, Madison, WI, USA).

Briefly, 5×10^{3}cells/well were seeded in 96-well plates in 100 μL of DMEM medium supplemented with 1% glutamine, 0.5% penicillin/streptomycin and 5% foetal bovine serum and then incubated at 37 °C in a humidified (95 %) CO_2 (5 %) incubator. After seeding (24 h), cells were treated, in quadruplicate, with increasing concentrations of the compounds (**Htcum** and **Htcin**) for 24 h. The cytotoxicity assay was performed by adding 20 μL of the CellTiter96^{R}AQ$_{ueous}$One Solution Cell Proliferation Assay directly to the culture wells, incubating for 4 h and then recording the absorbance at 450 nm with a 96-well plate reader (MULTISKAN EX, Thermo Electron Corporation, Vantaa, Finland).

3. Results and Discussion

3.1. Chemistry

Antifungal activity associated to thiosemicarbazones has already been described in the literature (Beraldo and Gambino, 2004). However, in all previous reports, only MIC value against few fungal genera were determined (Kasuga et al., 2006; Rodríguez-Argüelles et al., 2009, 2007, 2005) and no data were shown on the effect on mycotoxin production. In this work, a panel of thiosemicarbazones differing in their derivatization were synthesized and tested for their antifungal and antimycotoxinogenic activity against strains of *Fusarium graminearum*, *Fusarium sporotrichioides* and *Aspergillus flavus*. In particular**,** we focused our attention on five molecules (**Htcum**, **Htcin**, **Htisq**, **HtmeFis** and **HtFis**) derived from natural aldehydes/ketones differing in shape and in the pattern of the potential donor-acceptor atoms, in order to gain information about possible structure-activity relationships. Their chemical structure and data concerning the synthesis

are reported in Fig.1 and in the Materials and Methods section. Compounds **Htcum** and **Htcin** are thiosemicarbazones containing aromatic fragments deriving from cuminaldehyde and cinnamaldehyde (essential oil components, known for their natural mycostatic properties), while **Htisq**, **HtmeFis** and **HtFis** are thiosemicarbazones of quinoline and fluoroisatine, compounds known for their antiproliferative properties (Adsule et al., 2006; Hall et al., 2011). Since thiosemicarbazones are known to be metal ion chelators and in many cases, their activity has been associated with their ability to sequester metal ions essential to life, such as iron, (Pelosi, 2010) we have chosen molecules **Htisq** and **HtmeFis** because they possess in the aromatic moiety a further donor atom, which increases the denticity to three donor atoms (N, N, S or O, N, S) favouring the chelating properties of these molecules. In addition, in order to understand if the amino group of the thiosemicarbazide plays a determinant role in the activity, we have also synthesised compound **HtFis**, which differs from **HtmeFis** for the presence of a methyl on the terminal nitrogen.

3.2. Antifungal and antimycotoxin activity

A preliminary assay was performed to assess the relative effect of the different thiosemicarbazones on growth and on mycotoxin accumulation of the different fungi. Using 100 μM as a starting concentration for testing, some of the thiosemicarbazones here analysed were very effective in inhibiting mycelium growth (Table 1). In particular, treatments with molecules **Htcum** and **Htcin** were more effective than those with **HtmeFis**, **Htisq** or **HtFis** (see also Fig. 2d which shows a visual comparison of the differential effect of **Htcum** and **Htisq** thiosemicarbazones on *A. flavus* mycelium development). These preliminary observations showed that the cuminaldehyde and trans-cinnamaldehyde moieties are essential in the activity of these compounds, while the chelating properties, which characterize molecules **HtmeFis**, **Htisq** and **HtFis**, do not play a key role in the activity mechanisms of these thiosemicarbazones. This fact allows us to suggest that the inhibitory activity is not due to a sequestering action on essential metal ions present in the medium. An increased sensitivity of *A. flavus* to both **Htcum** and **Htcin** thiosemicarbazones as compared to the two *Fusarium* species here analysed, was observed. For example, thiosemicarbazone **Htcin** exerted a much stronger inhibitory activity on *A. flavus* mycelium growth (58%) than on *F. sporotrichioides* or *F. graminearum* (around 25% for both species). Similarly, *A. flavus* growth was strongly affected (76%) by **Htcum**, whereas both Fusaria experienced a limited inhibition of mycelium biomass increase (25-39%). Next, we determined the effect of the various molecules on mycotoxin production. Here again, a different pattern of efficacy of the thiosemicarbazones on toxin production by the two genera of fungi was observed. Compounds **Htcum** and **Htcin** were found be

very effective in decreasing T2-HT2 and aflatoxin B accumulation by *F. sporotrichioides* and *A. flavus* respectively, whereas deoxynivalenol (DON) production by *F. graminearum* was nearly unaffected (Table1). On the other hand, **Htisq**, **HtmeFis** and **HtFis** molecules did not affect toxin accumulation in either *Fusarium* species (inhibition < 6%). Surprisingly this was not the case for *A. flavus*: aflatoxin accumulation in the culture medium decreased by 44%, 60% and 35% with **Htisq**, **HtmeFis** and **HtFis** thiosemicarbazones respectively. A favorable outcome of our survey is the absence of mycotoxin inducing activity by the majority of the tested thiosemicarbazones, either in the *Fusarium* or *Aspergillus* species here analysed.

Our analysis focused subsequently on the effect of the two thiosemicarbazones that provided the greatest efficacy in preventing fungal growth and/or mycotoxin accumulation by the three species analysed. Serial dilution of **Htcum** and **Htcin** molecules were performed to assess their range of activity. Increasing **Htcum** or **Htcin** concentration, up to 300 μM, in the culture medium affected the growth of both *Fusarium* species. However, the inhibition (40% - 65% for **Htcum** and 50% - 70% for **Htcin** for *F. sporotrichioides* and *F. graminearum* respectively) at the higher concentrations used indicated a different sensitivity of the *Fusarium* species to the two abovementioned thiosemicarbazones (Fig. 2a, 2b). Concentrations of thiosemicarbazones over 100 μM were not considered for *A. flavus* treatments, since an inhibitory effect of the solvent (DMSO, present in the stock solution) on mycelium growth was observed in this conditions. In particular *A. flavus,* and to a lesser extent *F. graminearum,* displayed a greater sensitivity to **Htcum** than to **Htcin** over the entire range of concentrations tested (Fig. 2c), whereas no differential effect of the relevant molecules was observed for *F. sporotrichioides*. When considering the effect of **Htcum** and **Htcin** molecules on mycotoxin production by the fungal strains, here again, *A. flavus* was the most responsive: treatment of conidial suspensions with as low as 25 μM concentrations of either thiosemicarbazone strongly inhibited aflatoxin accumulation in the medium (Fig. 2c). This was not the case for *F. sporotrichioides* (74% inhibition of T2-HT2 with both molecules) (Fig. 2a) and for *F. graminearum* (73% and 68 % inhibition of DON with **Htcum** and **Htcin** respectively) (Fig. 2b). To sum up, these findings indicate that the two *Fusarium* species are less susceptible than *A. flavus* to **Htcum** and **Htcin** thiosemicarbazones, *F. sporotrichioides* and *F. graminearum* being the most resistant, to mycotoxin biosynthesis inhibition and growth inhibition respectively. In Fig. 1S, the data concerning aflatoxin inhibition in *A. flavus* cultures grown in CCM medium are reported on a mycelium dry weight basis as an additional confirmation of the observed effect of **Htcum** and **Htcin.**

The results described above prompted us to verify whether they could be validated in an "in field" simulation. To this purpose, maize kernels previously treated with 100 μM of **Htcum** or **Htcin** molecules were inoculated with a conidial suspension of *A. flavus* and incubated at 28 °C for 7-10 days. As reported in Fig. 3, both thiosemicarbazones significantly lowered seed contamination by *A. flavus*: less than 30% of the seeds were infected when compared with the untreated control. No significant increase in the level of fungal infection was observed extending the time (10 days) of the incubation (data not shown). Aflatoxin accumulation (Fig. 3) was also drasticaly reduced, as compared to the untreated control, when the seeds were treated either with **Htcum** (2%) or **Htcin** (36%). Analogous experiments were not performed on *Fusarium* species as inflorescence (and not kernels, as in maize) are the main infected organ in barley.

3.3. Antioxidant activity

It is well established that, in several fungal species, an oxidative imbalance may trigger mycotoxin biosynthesis (Reverberi et al., 2012, 2010). In contrast, the oxidative stress that may be experienced by fungi during their interaction with the host plant, lowers the production of toxin (Barberis et al., 2010; Chulze, 2010; Farnochi et al., 2005; Magan et al., 2010; Nesci et al., 2008, 2003; Torres et al., 2003). We were then prompted to analyse if the observed inhibitory effect of the synthesized thiosemicarbazones on toxin accumulation may, at least in part, be attributed to a possible antioxidant activity. In order to elucidate the ability of the compounds to act toward a reactive radical species, we carried out the DPPH radical scavenging assay. The **Htcum**, **Htcin**, **Htisq**, **HtmeFis** and **HtFis** compounds were tested at 10, 100, and 1000 μM concentrations. As reported in Fig. 4 a remarkable radical scavenging activity of the compounds was reached at 1000 μM where all the compounds inhibited DPPH by more than 90%. At the lowest concentrations tested **Htcin**, **Htisq**, **HtmeFis** and **HtFis** inhibited the radical already by more than 80%, whereas **Htcum** showed 20% of scavenging activity as compared to the control. It is apparent that no correlation between the antioxidant efficacy of the different molecules and their inhibitory activity on mycotoxin biosynthesis may be observed: all the molecules here tested exhibited antioxidant activity but differentially affected mycotoxin accumulation in the different fungal species. Since *in vitro* and *in vivo* assays are sensitive to different factors, it is not surprising that the antioxidant capacity determined by chemical analyses does not correlate well with cell-based assays (López-Alarcón and Denicola, 2013). In agreement with the modern concept of antioxidant, as "a compound able to modulate the redox state of the cell", we may speculate that *in vivo* more subtle mechanisms are involved in the oxidative stress defense network and at cascade in the regulation of the biosynthetic pathways of the various toxins.

3.4. Phytotoxicty and toxicity to human cells

The early identification of the toxicological properties of new chemical substances is a fundamental step in protecting human and environmental health. A rapid identification of toxicity and toxicological risk related to the possible release of new chemicals in the environment is a strategic tool to improve the chemical synthetic design. In addition, if the molecules are intended to be used to prevent fungal growth on seeds during storage, it is also important to ascertain the absence of negative effects on seed viability. In our case, no negative effects on seed germination, root and epicotyl elongation, as determined according to ISTA procedure, were observed when corn seeds were treated with 100 μM of **Htcum** or **Htcin** thiosemicarbazones (data not shown). Similarly, no inhibition of germination could be detected when the relevant molecules were tested at fungicidal concentrations on both hulless and hulled barley seeds. The different cultivars showed variability in the response to the highest concentrations (600 μM) of **Htcum** and **Htcin**, with a significant, albeit negligible inhibition of germination mainly of hulless seeds (Fig. 5). The cytotoxic activity of the selected thiosemicarbazones on human cells (HFL-1 cell line) was assessed by treating normal embryonic lung fibroblasts at different concentrations (0.5; 1.0; 5.0; 10.0; 50.0; 100.0 μM) of **Htcum** or **Htcin**. The two molecules presented a different toxicological behaviour (Fig. 6). **Htcum** induced a strong cytotoxicity with a dose-response relationship, its IC_{50} was 49 μM. On the contrary, in the range of the tested doses, **Htcin** showed only a slight antiproliferative activity starting from 50 μM with an IC_{50}> 100 μM

4. Conclusions

In this paper we have provided evidence that thiosemicarbazones depending on the functionalization of the molecules and on the fungal species considered display different patterns of activity against fungal growth and mycotoxin accumulation. With these molecules no negative effect was recorded against seed germination and low toxicity was observed against human cells (at least for one of the two molecules considered). These findings open up the way to the design of more specific molecules to be used according to the target of interest avoiding unwanted effects on biological processes and/or organisms that occupy the same environmental niche. Moreover we consider particularly interesting those molecules that display a prominent effect on mycotoxin synthesis rather than on fungal growth. These molecules could also be profitably used as probes to gain information on the regulatory mechanism affecting toxin biosynthesis, and, as such, providing insight into developing improved and more specific inhibitors or new strategies to fight mycotoxin contamination of food and feed commodities. As concerns possible structure/activity relationships, we can affirm that an increased capacity to sequester ions does not correspond to a more effective

inhibition of proliferation. We can therefore exclude that the activity of these compounds is based on, and limited to, essential metal ion sequestration in the growth medium. An additional benefit of the low cost and high throughput procedures described in this paper emerges from the possibility to test combinatorial treatments of different molecules in order to release new antifungals that may withstand a well known and problematic issue: the development of resistance in the population of treated fungi. In this way we may contribute to the goal of potentiating and/or extending the time of efficacy of the antifungal product**.**

In conclusion, with this work we have provided an experimental system potentially useful to study the molecular mechanisms which underlie the antifungal properties of derivatives of natural molecules. We have found that there is a remarkable variability both among different fungal genera and within single species. We have also demonstrated the possibility to evaluate the impact of molecular fragments and the possibility to modify them and evaluate the effect of the substitution. The marked inhibitory activity towards the mycotoxin biosynthesis shown by these compounds, which have exhibited antioxidant properties, can be useful for the identification and the characterization of regulatory genes involved in mycotoxin biosynthesis, paving the way to the development of targeted strategies for crop protection.

References

Abbas, H., Wilkinson, J., Zablotowicz, R., Accinelli, C., Abel, C., Bruns, H., Weaver, M., 2009. Ecology of Aspergillus flavus , regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn. Toxin Reviews 28, 142–153. doi:10.1080/15569540903081590

Adsule, S., Barve, V., Chen, D., Ahmed, F., Dou, Q.P., Padhye, S., Sarkar, F.H., 2006. Novel Schiff base copper complexes of quinoline-2 carboxaldehyde as proteasome inhibitors in human prostate cancer cells. Journal of medicinal chemistry 49, 7242– 7246.

Anderson, J.B., 2005. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. Nature reviews. Microbiology 3, 547–56. doi:10.1038/nrmicro1179

Barberis, C., Astoreca, A., Fernandez-Juri, M.G., Dalcero, A.M., Magnoli, C., 2010. Effect of antioxidant mixtures on growth and ochratoxin a production of Aspergillus section Nigri species under different water activity conditions on peanut meal extract agar. Toxins 2, 1399–413. doi:10.3390/toxins2061399

Beraldo, H., Gambino, D., 2004. The wide pharmacological versatility of semicarbazones, thiosemicarbazones and their metal complexes. Mini Rev. Med. Chem. 4, 31–9.

Bisceglie, F., Pinelli, S., Alinovi, R., Goldoni, M., Mutti, A., Camerini, A., Piola, L., Tarasconi, P., Pelosi, G., Zwack, P.J., 2014. Cinnamaldehyde and cuminaldehyde thiosemicarbazones and their copper(II) and nickel(II) complexes: A study to understand their biological activity. J. Inorg. Biochem. 140, 111–125.

Chiyanzu, I., Hansell, E., Gut, J., Rosenthal, P.J., McKerrowb, J.H., Chibale, K., 2003. Synthesis and Evaluation of Isatins and Thiosemicarbazone Derivatives against Cruzain, Falcipain-2 and Rhodesain. Bioorganic & Medicinal Chemistry Letters 13, 3527–3530.

Choi, C.W., Kim, S.C., Hwang, S.S., Choi, B.K., Ahn, H.J., Lee, M.Y., Park, S.H., Kim, S.K., 2002. Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. Plant Science 163, 1161–1168.

Chulze, S.N., 2010. Strategies to reduce mycotoxin levels in maize during storage: a review. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment 27, 651–7. doi:10.1080/19440040903573032

Cooper, J., Dobson, H., 2007. The benefits of pesticides to mankind and the environment. Crop Protection 26, 1337–1348.

Degola, F., Berni, E., Restivo, F.M., 2011. Laboratory tests for assessing efficacy of atoxigenic Aspergillus flavus strains as biocontrol agents. International journal of food microbiology 146, 235–43. doi:10.1016/j.ijfoodmicro.2011.02.020

Degola, F., Dall'Asta, C., Restivo, F.M., 2012. Development of a simple and high-throughput method for detecting aflatoxins production in culture media. Letters in applied microbiology 55, 82–9. doi:10.1111/j.1472-765X.2012.03264.x

Farnochi, M.C.., Torres, A.M.., Magan, N.., Chulze, S.N., 2005. Effect of antioxidants and competing mycoflora on Fusarium verticillioides and F. proliferatum populations and fumonisin production on maize grain. Journal of Stored Products Research 41, 211–219.

Goswam, R., Kistler, H., 2005. Pathogenicity and In Planta Mycotoxin Accumulation Among Members of the Fusarium graminearum Species Complex on Wheat and Rice. Phytopathology. 95., 1397–404. doi:doi: 10.1094/PHYTO-95-1397.

Hall, M.D., Brimacombe, K.R., Varonka, M.S., Pluchino, K.M., Monda, J.K., Li, J., Walsh, M.J., Boxer, M.B., Warren, T.H., Fales, H.M., Gottesman, M.M., 2011. Synthesis and structure-activity evaluation of isatin-β-thiosemicarbazones with improved selective activity toward multidrug-resistant cells expressing P-glycoprotein. Journal of medicinal chemistry 54, 5878–89. doi:10.1021/jm2006047

Karali, N., Gursoy, A., Kandemirli, F., Shvets, N., Kaynak, F.B., Ozbey, S., Kovalishyne, V., Dimoglo, A., 2007. Synthesis and structure–antituberculosis activity relationship of 1H-indole-2,3-dione derivatives. Bioorganic & Medicinal Chemistry 1 5, 5888–5904.

Kasuga, N.C., Onodera, K., Nakano, S., Hayashi, K., Nomiya, K., 2006. Syntheses, crystal structures and antimicrobial activities of 6-coordinate antimony(III) complexes with tridentate 2-acetylpyridine thiosemicarbazone, bis(thiosemicarbazone) and semicarbazone ligands. Journal of inorganic biochemistry 100, 1176–86. doi:10.1016/j.jinorgbio.2006.01.037

López-Alarcón, C., Denicola, A., 2013. Evaluating the antioxidant capacity of natural products: a review on chemical and cellular-based assays. Analytica chimica acta 763, 1–10. doi:10.1016/j.aca.2012.11.051

Ma, Z., Michailides, T., 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Protection (24, :853–863.

MacKay, D.B., 1972. The measurement of viability., in: Roberts, E.H. (Ed.), Seed Viability. Syracuse Univ. Press, Syracuse, N.Y.

Magan, N., Aldred, D., Mylona, K., Lambert, R.J.W., 2010. Limiting mycotoxins in stored wheat. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment 27, 644–50. doi:10.1080/19440040903514523

Nesci, A., Ferrari, L., Etcheverry, M., 2008. Effect of synthetic antioxidants on stored maize grain mycoflora in situ. J. Sci. Food Agric. 88, 797–804.

Nesci, A., Rodriguez, M., Etcheverry, M., 2003. Control of Aspergillus growth and aflatoxin production using antioxidants at different conditions of water activity and pH. Journal of Applied Microbiology 95, 279–287. doi:10.1046/j.1365-2672.2003.01973.x

Oerke, E.-C., Dehne, H.-W., 2004. Safeguarding production—losses in major crops and the role of crop protection. Crop Protection 23, 275–285.

Pelosi, G., 2010. Thiosemicarbazone Metal Complexes: From Structure to Activity. The Open Crystallography Journal 3, 16–28.

Reverberi, M., Punelli, M., Smith, C. a, Zjalic, S., Scarpari, M., Scala, V., Cardinali, G., Aspite, N., Pinzari, F., Payne, G. a, Fabbri, A. a, Fanelli, C., 2012. How peroxisomes affect aflatoxin biosynthesis in Aspergillus flavus. PloS one 7, 1–15. doi:10.1371/journal.pone.0048097

Reverberi, M., Ricelli, A., Zjalic, S., Fabbri, A. a, Fanelli, C., 2010. Natural functions of mycotoxins and control of their biosynthesis in fungi. Applied microbiology and biotechnology 87, 899–911. doi:10.1007/s00253-010-2657-5

Rodríguez-Argüelles, M.C., López-Silva, E.C., Sanmartín, J., Pelagatti, P., Zani, F., 2005. Copper complexes of imidazole-2-, pyrrole-2- and indol-3-carbaldehyde thiosemicarbazones: inhibitory activity against fungi and bacteria. Journal of inorganic biochemistry 99, 2231–9. doi:10.1016/j.jinorgbio.2005.07.018

Rodríguez-Argüelles, M.C., Mosquera-Vázquez, S., Tourón-Touceda, P., Sanmartín-Matalobos, J., García-Deibe, A.M., Belicchi-Ferrari, M., Pelosi, G., Pelizzi, C., Zani, F., 2007. Complexes of 2-thiophenecarbonyl and isonicotinoyl hydrazones of 3-(N-methyl)isatin. A study of their antimicrobial activity. Journal of inorganic biochemistry 101, 138–47. doi:10.1016/j.jinorgbio.2006.09.004

Rodríguez-Argüelles, M.C., Tourón-Touceda, P., Cao, R., García-Deibe, A.M., Pelagatti, P., Pelizzi, C., Zani, F., 2009. Complexes of 2-acetyl-gamma-butyrolactone and 2-furancarbaldehyde thiosemicarbazones: antibacterial and antifungal activity. Journal of inorganic biochemistry 103, 35–42. doi:10.1016/j.jinorgbio.2008.08.015

Schmidt-Heydt, M., Stoll, D., Geisen, R., 2013. Fungicides effectively used for growth inhibition of several fungi could induce mycotoxin biosynthesis in toxigenic species. International journal of food microbiology 166, 407–12. doi:10.1016/j.ijfoodmicro.2013.07.019

Terzi, V., Tumino, G., Stanca, A.M., Morcia, C., 2014. Reducing the incidence of cereal head infection and mycotoxins in small grain cereal species. Journal of Cereal Science 59, 284–293.

Torres, A.M., Ramirez, M.L., Arroyo, M., Chulze, S.N., Magan, N., 2003. Potential use of antioxidants for control of growth and fumonisin production by Fusarium verticillioides and Fusarium proliferatum on

whole maize grain. International Journal of Food Microbiology 83, 319–324. doi:10.1016/S0168-1605(02)00380-X

Tyagi, M., Chandra, S., Tyagi, P., 2014. Mn(II) and Cu(II) complexes of a bidentate Schiff's base ligand: spectral, thermal, molecular modelling and mycological studies. Spectrochimica acta. Part A, Molecular and biomolecular spectroscopy 117, 1–8. doi:10.1016/j.saa.2013.07.074

Van Egmond, H.P., Jonker, M.A., 2004. Worldwide regulation on aflatoxins: the situation in 2002. J Toxicol Toxin Rev 23, 273–293.

Williams, J.H., 2008. Institutional stakeholders in mycotoxin issues – past, present and future, in: Leslie, J., Bandyopadhyay, R., Visconti, A. (Eds.), Mycotoxins: Detection Methods, Management, Public Health and Agri- Cultural Trade. CAB Intern, Wallingford (UK), pp. 349–58.

Wu, F., Khlangwiset, P., 2010. Health economic impacts and cost-effectiveness of aflatoxin-reduction strategies in Africa: case studies in biocontrol and post-harvest interventions. Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment 27, 496–509. doi:10.1080/19440040903437865

Yi, W., Dubois, C., Yahiaoui, S., Haudecoeur, R., Belle, C., Song, H., Hardré, R.M., Réglier, R., Boumendjel, A., 2011. Refinement of arylthiosemicarbazone pharmacophore in inhibitionof mushroom tyrosinase. Eur. J. Med. Chem. 46, 4330–4335.

Acknowledgements

CRA-GPG has been supported by"SO.FI.A." (SOstenibilità della FIliera Agroalimentare) Cluster Agrifood project. We are also indebted with the Stazione Sperimentale per l'Industria delle Conserve Alimentari (SSICA) of Parma, and in particular to dr Elettra Berni, for using their facilities and for help in HPLC analysis and to Antonietta Cirasolo and Roberto Silva for their technical help. FB is grateful to dr Beatrice Bonati for her assistance in performing UV experiments.

Contribution

F.D. performed all the experiments in the corn-*Aspergillus flavus* system while C.M., R.G., G.T. and V.T. equally contributed to the experimental work done in the barley-*Fusarium* system. G.P. and F.B. synthesized the thiosemicarbazones and determined their antioxidant activity. F.M. and A.B. performed the human toxicity tests. T.L. and G.P. conceived the idea. T.L., G.P., V.T., A.B. and F.M.R. wrote the manuscript and all the authors contributed to editing the manuscript.

Legend to figures

Figure 1 Structures of the synthesized thiosemicarbazones. (**Htcum**) cuminaldehyde thiosemicarbazone; (**Htcin**) trans cinnamaldehyde thiosemicarbazone; (**Htisq**) quinoline-2-carboxyaldehyde thiosemicarbazone; (**HtmeFis**) 5-fluoroisatin N^4-methylthiosemicarbazone; (**HtFis**) 5-fluoroisatin thiosemicarbazone.

Figure 2 *In vitro* mycelium growth inhibition and mycotoxin production of *Fusarium graminearum* (A), *Fusarium sporotrichioides* (B) and *Aspergillus flavus* (C) treated with the indicated concentrations (μM) of **Htcum** and **Htcin** thiosemicarbazones. Mycotoxin released into the culture medium or accumulated by thiosemicarbazone **Htcum** (**Htcum**-M) or **Htcin** (**Htcin**-M) treated micelium, is expressed as ppm for *A. flavus* and as ppb for both *Fusaria* species. Growth inhibition induced by thiosemicarbazone **Htcum** (**Htcum**-GI) or **Htcin** (**Htcin**-GI) is expressed as percent of control. A microscopic visualisation (D) of the effect of different concentrations (0, 25, 50 and 100 μM) of two thiosemicarbazones (**Htcum** and **Htisq**) on *A. flavus* mycelium development is also reported.

Figure 3 *In vivo* mycelium growth inhibition and aflatoxin production of *Aspergillus flavus* treated with 100 μM of **Htcum** and **Htcin** thiosemicarbazones. Growth inhibition is expressed as % of infected corn seeds (means are presented; n = 100; three replicates). Aflatoxin detected on kernels is reported as ppm (means are presented; three replicates).

Figure 4 DPPH scavenging activity of thiosemicarbazones. The values are expressed in percentage of inhibition in relation to ascorbic acid scavenging activity (100%). Data for three thiosemicarbazones concentrations (10, 100 and 1000 μM) are presented as mean ± S.E.M. (n = 3).

Figure.5 Barley seed germination in presence of 300 μM and 600 μM of **Htcum** and **Htcin** molecules. Significative differences from the control are indicated with asterisk. $p<0.05$

Figure 6 Antiproliferative activity on Hfl-1 human cell line: growth percentage after 24 hours of treatment with increasing concentrations of **Htcum** and **Htcin** thiosemicarbazones.

Figure 1

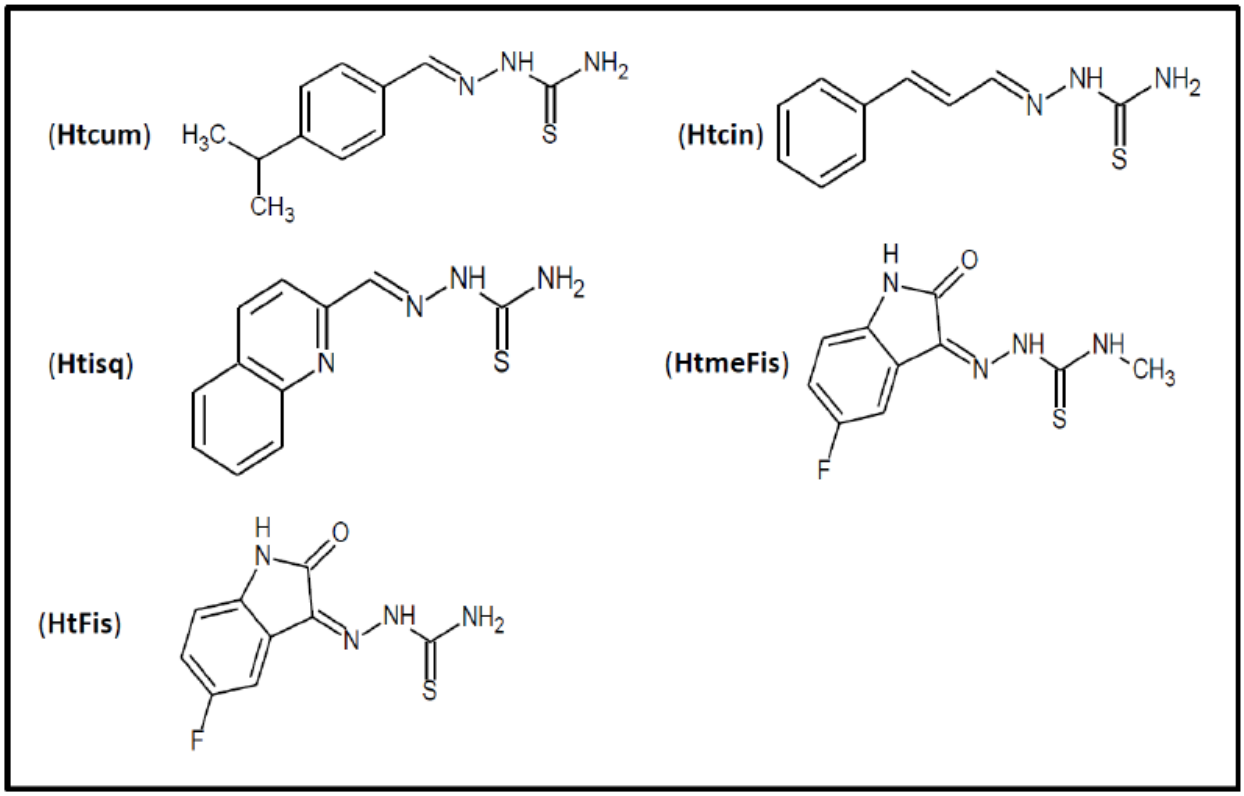


Figure 2

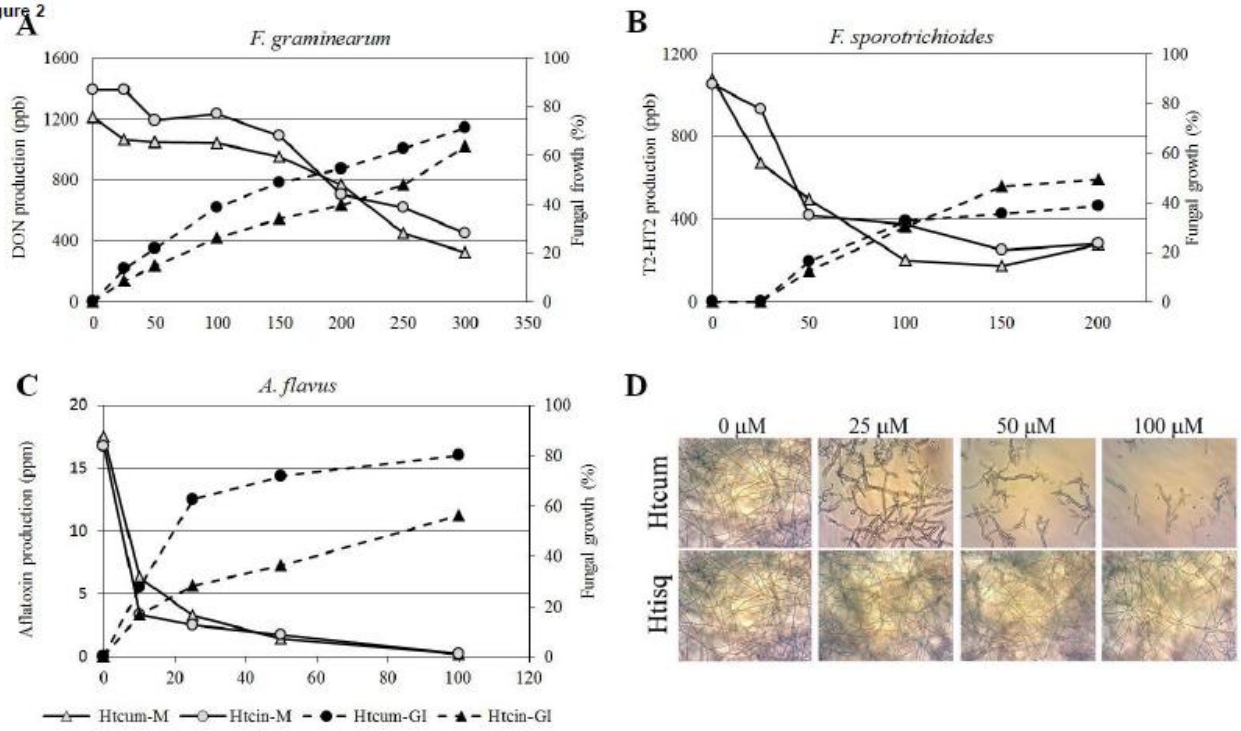


Figure 3

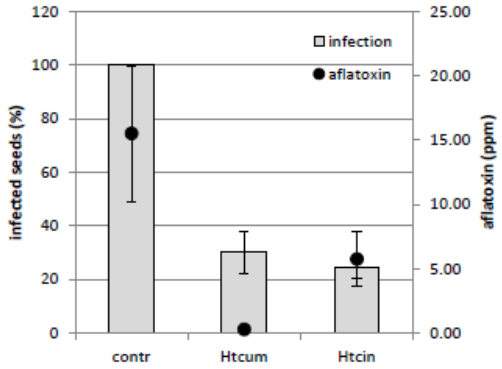


Figure 4

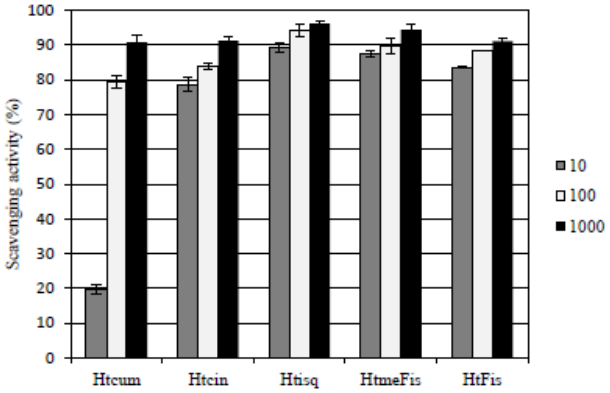


Figure 5

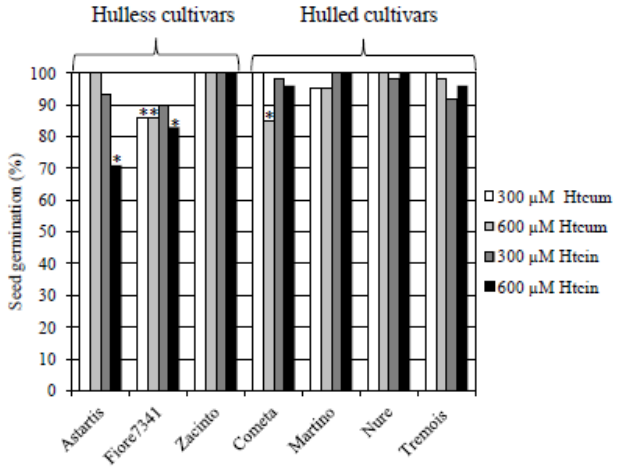
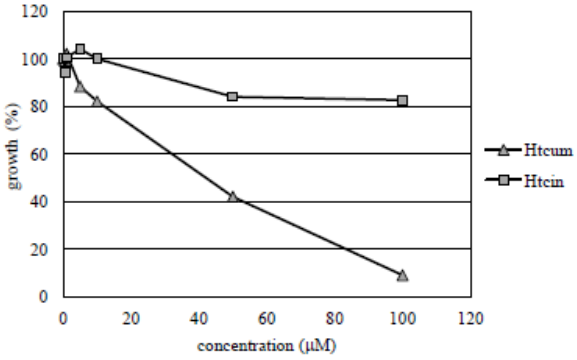


Figure 6



	inhibition of mycelium growth (%)			inhibition of mycotoxin[a] production (%)		
	F. sporot	***F. gramin***	***A. flavus***	***F. sporot***	***F. gramin***	***A. flavus***
Htcum	26.3	38.8	76.3	86.9	14.3	99.0
Htcin	24.9	26.2	58.1	91.4	11.4	96.5
Htisq	11.7	10.9	2.2	-	-	44.4
HtmeFis	9.8	7.5	-	-	5.3	67.9
HtFis	6.7	5.8	-	3.4	5.7	34.7

Table 1 Effects of 100 μM concentrations of **Htcum**, **Htcin**, **Htisq**, **HtmeFis** and **HtFis** molecules on mycelial growth (expressed as mean % inhibition in comparison with non-treated controls) and on mycotoxin production (expressed as mean % inhibition in comparison with non-treated controls) of *Fusarium sporotrichioides* (*F. sporot*), *Fusarium graminearum* (*F. gramin*) and *Aspergillus flavus* (*A. flavus*).

[a] mycotoxins: T2-HT2, DON and aflatoxin B for *Fusarium sporotrichioides*, *Fusarium graminearum* and *Aspergillus flavus* respectively.

Highlights

- A panel of thiosemicarbazones derivatives were synthesized
- The compounds were tested for their effect on crops (cereals) affecting fungi (*Fusaria* and *Aspergillus*)
- Species-specific effects on fungal growth and mycotoxins biosynthesis were observed
- Two molecules were considered promising for fungal growth and mycotoxin containment
- Potential deleterious effects on seed germination and on human cells were tested