Inhibition of species of the *Aspergillus* section *Nigri* and ochratoxin A production in grapes by fusapyrone

**Running Title:** Inhibition of black aspergilli & ochratoxin production

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ABSTRACT

Fusapyrone (FP), an antifungal natural compound, was tested against the three main ochtatoxigenic species of the Aspergillus section Nigri. The minimal inhibition concentrations (MIC) at 24 h were 6.0, 11.6, and 9.9 µg/ml for A. carbonarius, A. tubingensis, and A. niger, respectively. Strong inhibition of growth and morphological changes were still observed at half MIC after 7 days. The application of a 100 µg/ml FP solution in a laboratory assay on artificially inoculated grapes resulted in a significant reduction of A. carbonarius CFU counts, up to 6 orders of magnitude. Dramatic reductions of the OTA content were obtained with application of either 100 or 50 µg/ml of FP (average amount of OTA, n=3: 0.6 and 5.1 ng/g of grape, respectively), compared to positive control (112.5 ng/g).
Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, teratogenic, immunosuppressive and carcinogenic properties (38) that has been classified by the International Agency for Research on Cancer as a possible human carcinogen (Group 2B) (22). OTA was first isolated from moldy corn meal in South Africa (37). Subsequently, OTA has been found in a number of agricultural commodities and foodstuffs, including cereals, coffee beans and beer, as a byproduct of contamination with fungi of the genera Aspergillus and Penicillium, mainly A. ochraceus (a.k.a. A. alutaceus) and P. verrucosum. In recent years there has been a growing interest in the occurrence of OTA in grapes and grape derivatives. In particular, concern has been raised as for OTA contamination of wine grapes, due to the large and increasing consumption of wine and the economical relevance of wine industry (5, 11, 36, 41). Several reports have indicated that members of the Aspergillus section Nigri, the so-called black aspergilli, are the dominant ochratoxigenic species on wine grapes world-wide (7, 10, 13, 27). Among these, A. carbonarius seems to be the most important source of OTA because of high proportion of producing strains and high amounts produced (6, 30). A correlation has been found between occurrence of OTA in wines and both wine color and geographic area of production. The risk of OTA contamination and the concentration of toxin is higher in red wines and in wines made with grapes from Mediterranean and sub-tropical areas (29, 41). There are evidences that grapes are already contaminated with OTA before harvest (6, 9, 28, 34), although its concentration may increase substantially in the time between harvest and alcoholic fermentation (19, 41). OTA is a rather stable molecule (33) and it does not undergo substantial degradation in the course of the technological process of winemaking (1, 12). Nevertheless, experiments of microvinification aimed at studying the fate of OTA during winemaking have shown that the levels of OTA drop by around 90% from must to wine, mostly as a result of its adsorption onto biomass and pomace (solid parts residue) surfaces (1, 12, 26, 32). In spite of this, severe infestations of raw materials with black aspergilli may result in contamination of the final product with OTA at levels above the allowed limits (2 µg/liter in the European Union).
Therefore, management of the sanitary state of grapes is a critical point in a strategy aimed at prevention of OTA occurrence in wine. Unfortunately, very few chemical pesticides seem to be effective (27, 36). In addition, the intensive use of these compounds may cause different important drawbacks, such as lost of natural competitors, arising of resistant pathogen populations, and presence of residues in the products and in the environment. In this context the availability of alternative methods of control of black aspergilli would be highly desirable.

A relatively novel and promising field of study is the application of antimicrobial compounds of microbial origin as an alternative to synthetic pesticides (35). Fusapyrone (FP) is a bioactive metabolite produced by the fungus *Fusarium semitectum* (16). Structurally, FP consists of a highly functionalized aliphatic chain and a 4-deoxy-β-xylo-hexopyranosyl C-glycosyl moiety bound to the C-6 of the 2-pyrone ring (Fig. 1). FP exhibited considerable antifungal activity against several plant pathogenic, mycotoxigenic and human pathogenic filamentous fungi, including *A. flavus*, *A. parasiticus*, *A. niger*, and *A. fumigatus* (3). The inhibitory activity of FP to *A. flavus* and *A. parasiticus* was similar to that of the antibiotic nystatin in disk diffusion assays (3) and higher than that of the fungicides benomyl and dicloran on *A. parasiticus* in broth dilution assays (4). Interestingly, FP was found to be ineffective towards yeasts isolated from plants and fruits and not toxic to *Artemia salina* (brine shrimp), a common invertebrate model in ecotoxicological testing (3, 4). In consideration of these features, we examined the ability of FP to inhibit the growth of the main ochratoxigenic species of *Aspergillus* section *Nigri*, viz. *A. carbonarius*, *A. niger* and *A. tubingensis*, and prevent fungal colonization and OTA production in grapes.

**Fusapyrone.** FP was purified from crude corn culture extracts of *F. semitectum* by SiO$_2$ medium pressure (20 bar) column chromatography and preparative SiO$_2$ TLC, following the method described by Evidente *et al.* (17). Purity of FP as determined by HPLC was higher than 97%.
Inhibitory activity of FP to ochratoxigenic Aspergillus spp. Antifungal activity of FP was tested against 16 Aspergillus isolates, belonging to 3 different ochratoxigenic species, viz. A. carbonarius, A. niger, and A. tubingensis. The test isolates were obtained from the collection of the Institute of Sciences of Food Production (ISPA), Bari, Italy, and were originally isolated from grapes in several countries of the Mediterranean basin (table 1). The minimal inhibitory concentration (MIC) of FP to ochratoxigenic aspergilli was determined by a broth dilution method (18). Sterile Czapek Dox broth (Difco, Detroit, MI), at pH 7.0 (35 g of lyophilized medium dissolved in 1 liter of 0.05 M phosphate buffer pH 7.0 and sterilized in the autoclave for 20 min at 110°C) was used for preparation of serial dilutions of FP. Eleven twofold dilutions (range from 50 to 0.05 µg/ml) were prepared; 180 µl of each dilution and medium control were then transferred in duplicate into wells of 96-well microtitration plates. Inoculum of the fungi was obtained from fresh (7-day old) cultures on PDA. Each microwell was inoculated with 20 µl of a conidial suspension of the test isolates at the concentration of 10^5 conidia/ml in sterile Czapek Dox broth pH 7.0. Plates were sealed with Parafilm (Pechiney, Chicago, IL) in order to prevent evaporation, and incubated at 26 ± 1°C for 7 days. Growth was observed 1, 2, 3 and 7 days after inoculation under a reverse microscope. Conidia were assumed as not germinated if the germ tube was not longer than the conidium diameter. The MIC was identified as the lowest concentration that resulted in complete inhibition of germination of the test fungus. Antifungal tests were performed three times.

The in vitro inhibitory activity of FP to OTA-producing aspergilli is summarized in table 1. The MIC method is subject to inherent variability and therefore the procedure is generally considered accurate within ±1 twofold dilution (39). The data are presented in table 1 in the form of MIC range for each isolate tested. In addition, average MICs of different species were calculated to allow for comparison of susceptibility. Different susceptibility to FP of the species tested was found. Aspergillus carbonarius was the most sensitive, whereas A. niger and A. tubingensis were less susceptible. Inhibitory effects (reduction of percentage of germinated conidia and germ tube
length) were found also at concentrations lower than MICs. Strong inhibition of growth and
morphological changes were still observed at half the MIC (sub-MIC) after 7 days (Fig. 2). Under
these conditions, germ tubes of *A. niger* and *A. tubingensis* exhibited severe thickening and
irregular growth. The phenotype of these conidia resembled the phenotype described by Ram et al.
(31) in *A. niger* transformants lacking the glutamine : fructose-6-phosphate amidotransferase gene
(*gfaA*), encoding for the enzyme responsible of the first step in chitin synthesis. Abnormal swelling
of cells and conidia was observed in all of the *A. tubingensis* isolates, but not in isolates of the other
two species. The swollen cells were up to 10 times bigger than normal cells (Fig. 2). Katoh et al.
(23) reported an abnormal swelling of *A. niger* conidia that germinated in the presence of the
antibiotic tunicamycin, similar to that we observed in *A. tubingensis*. Tunicamycin is a non-specific
inhibitor of chitin synthesis that acts by blocking the synthesis of a lipid-linked saccharide
intermediate involved in protein-chitin complexes (21). Based on its amphiphilic nature, it was
hypothesized that FP has an effect on plasma membrane function and integrity, similar to antifungal
biosurfactants (4). The results of the present work suggest that FP also interferes with chitin
synthesis and cell wall structural integrity. This mechanistic hypothesis is supported by the relative
insensitivity of yeasts to FP (3). Chitin is a major component in filamentous fungi cell walls,
accounting up to 10-30 % of the cell wall dry weight (15), while it is only 1-2 % in yeast cell walls
(25). Should this multiple mechanism of action be confirmed by further studies, the probability that
resistance to FP arises in populations of black aspergilli can be predicted to be low. In fact, in this
case the development of resistance might require major structural change of the plasma membrane
and cell wall. Variation in sensitivity exhibited by different species in the *Aspergillus* section *Nigri*
is likely due to diversity in cell wall structure, composition and permeability to FP, as well as to a
variable fatty acid composition of plasma membranes (21). Differences in the cell wall composition
of species in the *Aspergillus* section *Nigri*, beside being a useful character for taxonomy,
systematics and phylogeny studies (2, 8) of this group, might also have practical implications. Some
fungicides (e.g., organophosphorus, carboxylic acid amides) act as inhibitors of phospholipids and cell wall components biosynthesis and deposition. Therefore, it is conceivable that variations in plasma membrane and cell wall composition may result in different efficacy of these fungicides in controlling of different ochratoxigenic species.

Bioassay on artificially inoculated grape berries. Bunches of red wine-grape variety Negroamaro were collected from vineyards located in the Salento area (Southern Apulia, Italy) 7-10 days before regular harvest date. In the laboratory, berries were excised by cutting pedicels 0.5 cm above their point of insertion on the berries and thoroughly washed in tap water to remove dust and residues of pesticides. Berries were surface sterilized with 2% sodium hypochlorite for 15 min, repeatedly rinsed in sterile distilled water and let dry on blotting paper under a fume hood for 2 h. Well formed and undamaged berries were selected for use in the bioassay. Inoculum of the OTA producing strain A. carbonarius ITEM 4167 was prepared by flooding 7 to 10-day old cultures on PDA with sterile distilled water and the suspension was adjusted with sterile distilled water to $5 \times 10^4$ conidia/ml using a hemocytometer. Berries were wounded with a sterile needle (2 mm deep) in two symmetrical abaxial points and sprayed with sterile distilled water (negative control) or the conidial suspension of A. carbonarius ITEM 4167 using an EcoSpray sprayer (Labo Chemie, Aix-en-Provence, France). Enough suspension was sprayed to cover the surface of the berry without dripping. Grapes were let dry under a sterile hood and then A. carbonarius-inoculated grapes were sprayed with a solution of FP in sterile distilled water at the concentration of either 100 or 50 µg/ml. Grapes sprayed only with the A. carbonarius suspension were used as a positive control. Grapes were then transferred into moist chambers (10 berries per chamber), consisting of plastic food containers (159 × 114 × 54 mm) with moist filter paper to ensure high humidity, which were individually placed in polypropylene bags and sealed. Chambers were incubated at 26 ± 1°C for 10 days. Experiments were arranged in a fully randomized block design with at least three replicates.
per treatment. Samples from these experiments were used for assessment of the treatments efficacy in inhibiting *A. carbonarius* growth and for OTA analyses.

**Inhibition of *A. carbonarius* by FP on artificially inoculated grape berries.** Growth of the OTA producing strain *A. carbonarius* ITEM 4167 in artificially inoculated grapes was evaluated by determination of colony forming units (CFU). Grapes from each replicate (20 - 30 g) were transferred into centrifuge tubes and weighted. After adding an equal weight of sterile distilled water, grapes were homogenized in a blender (Sterilmixer II, International PBI, Milan, Italy) at high speed for 1 min, transferred into the tubes again and shaken on an orbitary shaker at 180 rpm for 1 h. Subsequently, 3 sub-samples of 1 g were drawn from each tube and used to prepare serial 1/10 dilutions in sterile distilled water. From each dilution, three 100 µl samples were plated on an Aspergillus-selective medium (24) containing (per liter): glucose 10 g, peptone 5 g, K$_2$HPO$_4$ 1 g, agar 15 g, rose bengal 25 mg, dicloran 2 mg, chloramphenicol 100 mg. Plates were incubated at 26 ± 1° C and CFU were counted in two adjacent dilutions with less than 100 CFU per plate after 2 and 5 days. Counts were corrected by the dilution factor and averaged to give CFU/g of grape pulp. Three independent trials were carried out, in which FP was applied at both 100 and 50 µg/ml rates. Interestingly, in these experiments *A. carbonarius* was isolated from the surface-sterilized berries of negative controls. This suggests an endophytic behaviour of the pathogen, which may be of importance for optimizing an effective field control strategy. The variability among different experiments in the development *A. carbonarius* infections and in the level of natural contamination of the negative control, prompted us to evaluate the results of these experiments separately (table 2). In spite of the above variability, a consistent trend was found among different experiments. FP applied at either 100 or 50 µg/ml rates resulted in a significant ($P < 0.05$) reduction of *A. carbonarius* infections. The treatment of berries with a solution of FP at 100 µg/ml resulted in a reduction of *A. carbonarius* biomass from 2 to 6 orders of magnitude. The
reduction of *A. carbonarius* growth achieved with FP at 50 μg/ml was smaller but not statistically
different (*P* < 0.05) from FP 100 μg/ml in 2 out of 3 experiments.

**Effect of FP on OTA content.** Grape samples (20 - 30 g) were homogenized by blending at
high speed for 3 min with a Sorvall Omnimixer (Sorvall Inc., Newtown, CT). The homogenized
slurry (20 g) was added to 50 ml of a solution containing 1% polyethylene glycol (PEG 8000) and
5% sodium bicarbonate, cleaned-up through an OchraTest™ immunoaffinity column (VICAM,
Watertown, MA) and analyzed for OTA content by HPLC/fluorescence detector as extensively
described by Cozzi *et al.* (14). Average recoveries of OTA from grapes spiked at levels from 1.0 to
10 ng/g ranged from 80% to 85%, with relative standard deviations (RSDs) < 2.5% (triplicate
experiments). The detection limit was 0.02 ng/g, based on a signal to noise ratio of 3:1.

The data of OTA content in artificially inoculated grapes treated with FP are shown in table
2. In all of the three experiments, berries treated with either 100 or 50 μg/ml of FP showed a
significant (*P* < 0.05) reduction of OTA content compared to positive control. The treatment with
100 μg/ml of FP prevented the accumulation of OTA in the berries to levels comparable to the
negative control (0.01 to 1% of the positive control). The reduction of OTA content obtained with
FP 50 μg/ml was apparently smaller (0.3 to 20% of the positive control), although not statistically
different from FP 100 μg/ml.

**Statistical analyses.** All the statistical analyses were performed with the InStat program
version 3.0 (GraphPad Software, San Diego, CA).

**Conclusions.** FP proved to possess a strong inhibitory activity towards three ochratoxigenic
*Aspergillus* species belonging to the section *Niger* that are the major source of OTA in grapes and
grape-derived foods and beverages.

We tested the effectiveness of FP on artificially inoculated grapes in conditions that were
highly conducive to mold development, viz. skin injuries, high level of inoculum, absence of
competitors and high relative humidity. FP applied at a rate of 100 μg/ml almost completely
controlled *A. carbonarius*. In grapes treated with half dosage (50 µg/ml) of FP, the average level of control was lower but not statistically different from the full dosage. Therefore the application rate of 50 µg/ml of FP appears to be enough to achieve satisfactory control of *A. carbonarius* under the most adverse conditions. Dramatic reductions of the OTA content were obtained with application of either 100 or 50 µg/ml of FP (average amount of OTA in 3 independent experiments: 0.6 and 5.1 ng/g of grape, respectively), compared to positive control (112.5 ng/g of grape).

In conclusion, our results show that FP is highly effective in inhibiting the growth of black aspergilli, particularly *A. carbonarius*, and prevent OTA occurrence in infected grape berries. These findings warrant further studies to assess if the use of FP is a feasible strategy for prevention of OTA occurrence in grapes and grape-derived products under field conditions.

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REFERENCES


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<td>(a) Culture collections: ITEM = Institute of Sciences of Food Production, Bari, Italy; IMI = CABI Bioscience Genetic Resource Collection, Egham, UK.</td>
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<td>(b) Range of MIC found in 3 independent experiments. Geometric means were adopted since in twofold dilutions the arithmetic mean is not an accurate measure of central tendency (40). MIC values were transformed into base 2 logs to approximate a normal distribution and subject to one-way ANOVA. Means within columns followed by different letters are significantly different ($P &lt; 0.05$) by Tukey’s multiple comparison test.</td>
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TABLE 2. Effect of FP on A. carbonarius growth and OTA production in artificially inoculated grapes.

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<td>1.5 × 10² ab</td>
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<td>6.3 × 10⁵ c</td>
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<sup>(a)</sup> Values are the means of at least 3 replicates. Data of CFU and OTA content were subjected to one-way ANOVA. Mean values within columns followed by different letters are significantly different (P < 0.05) by Tukey’s multiple comparison test.
FIGURE CAPTIONS

Figure 1. Structure of fusapyrone.

Figure 2. Effects of sub-lethal concentrations (half MIC) of fusapyrone on the growth of germinated conidia of *A. carbonarius*, *A. niger* and *A. tubingensis*. Germinated conidia of *A. niger* and *A. tubingensis* showed severe alterations of morphology, that consisted of thickening and irregular growth of hyphae that formed pronounced bulges. Abnormal swelling of cells was observed in the *A. tubingensis* isolates. Top: controls at 48 h. Middle: half MIC, 48 h exposure. Bottom: half MIC, 7 days exposure. Bar = 100 µm.
Figure 1
Figure 2