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Effects of Thymol, Carvacrol and Some Weak Organic Acids on Growth and Ochratoxin A Production by the Food Spoilage *Aspergillus ochraceus*

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Introduction
Contamination of foods and feeds with mycotoxins represents a high risk for human and animal health. One of the toxins of growing importance in the last decade is ochratoxin A (OTA), a nephrotoxic mycotoxin with carcinogenic and teratogenic properties in rats and possibly in humans (group 2B) (1). *Aspergillus ochraceus* (Ao) is an important contaminant of various substrates, such as cereals, and produce OTA (2). In humans, dietary exposure to OTA has been associated with Balkan endemic nephropathy, a chronic kidney disease linked to tumours of the renal system (3). OTA is highly nephrotoxic and may cause both acute and chronic lesions of kidneys. Similarly, neoplasia in kidney have been reported in the Bulgarian cases of mycotoxic porcine nephropathy. OTA is a moderately stable molecule that remains unaltered during most processes of food transformation and may undergo bio-concentration in some animal tissues/organs reaching concentrations in meat products that are not acceptable for human consumption (4). Moreover, this mycotoxin has been detected frequently in cereal products (5). The consumer demand for the reduced use of chemical preservatives in food has prompted the search for alternatives to fungicides in the control of postharvest diseases. Likewise, chemicals usually applied during cereal storage are becoming less favoured (6). New strategies to reduce and prevent the spoilage of cereals during storage include the use of antifungal plant extracts or weak organic acids (7). The aim of this work was to evaluate the inhibitory effects of phenolic components and weak acids on the growth and OTA production by Ao.

Methodology
Fungal inoculum. The ochratoxin producer strains *Ao* 5137 (from Dr. Perrone, Bari, Italy) was grown on YES medium (pH 7.0) at 27°C in the dark. The spores were obtained *in vitro* after 7 d of incubation. To determine the mycelial dry weight, the cultures were filtered at the end of each incubation time. The mat was dried at 80°C for 6 h and weighted.
Antimicrobial agents (AM). Aliquots of stock solutions (1 M) of carvacrol (98%), thymol (98%), citric, sorbic or propionic acids were singularly added to YES prior to sterilization to give a specific final concentration. The pH of the substrate agar prior to inoculating the culture and after the completion of growth was measured using a solid state pH meter and was approximately equal to 5.5.
Effect on the growth. *In liquid broth.* Fractions of 50 ml YES medium with streptomycin (SM) were added to 250 ml Pyrex bottles. The AM were aseptically added to each bottle to give different final concentrations (0 100 ppm). Thereafter, they were inoculated with a spore suspension (10^3 spore/ml). Cultures were incubated statically at 27°C ± 0.5 °C for 6 to 26 d. Three bottles per AM and concentration were analyzed for mycelium and toxin production. *In agar plates*. Triplicate YES agar Petri dishes (Ø 9 cm) with AM at different concentration were centrally inoculated by pouring 2 µl of 10^3 spore/ml. Periodically inoculated plates were removed briefly to be observed and to measure colony diameter. The lowest concentration that completely inhibited fungal growth was taken as the minimum inhibitory concentration (MIC; 8).
Effects on ochratoxin production. *Estimation of OTA.* After 1 to 18 days of incubation, 1-ml portions of the medium were withdrawn from the flasks cleaned-up by an immunoaffinity column (Ochraprep).

The production of OTA was determined by an HPLC system equipped with a fluorescence detector (λ_{exc} 332 nm; λ_{em} 470 nm) and a RP-C18 column (Supersphere 4 µm, ODS2, 4 × 125 mm) (Merck & Co.). The analysis was performed under isocratic conditions. The following two solvent systems were employed: aqueous buffer with glacial acetic acid 2 % and acetonitrile in isocratic gradient elution (59:41) at a flow rate of 1 ml/min in 13 min. The limit of quantification was 0.04 µmol/l. *Determination of fungal growth.* To determine the mycelial dry weight, the cultures were filtered at the end of the incubation period. The mat was dried in an oven at 80°C for 6 h and weighted.

Results

The growth in YES broth supplemented with different concentration of thymol and inoculated with 10^3 spore/ml was inhibited at concentration ≥ 100 ppm. After 9 d the growth of Ao on agar plates was drastically reduced starting by 150 ppm, and was completely inhibited at 300 ppm. After 11 d the fungal growth started slowly even at 300 ppm reaching the maximum after 22 days. Therefore in agar plates the effect of thymol was fungistatic.

At 12 and 15 d of fungal growth in liquid medium, OTA detected in medium supplemented with thymol 75 ppm was lower ($P < 0.01$) than the control (Tab. 1). The same pattern was observed in the medium with citric acid 2,000 ppm where OTA was lower ($P < 0.01$) than that detected in culture medium at 12 and 15 days of growth. As for carvacrol 75 ppm induced a reduction of OTA at levels not detectable with the described method.

Table 1. Influence of thymol, carvacrol, and citric acid on OTA concentration (ppm) 12 and 15 days after incubation in YES broth. Data are mean ± SD, n = 10. *N.D., not detectable.

Incubation time	Control	Thymol (75 ppm)	Carvacrol (75 ppm)	Citric Acid (2,000 ppm)
	[OTA]			
12 d	100.90 ± 42.96	2.60 ± 0.60	N.D.*	12.20 ± 8.58
15 d	99.52 ± 45.13	8.07 ± 11.04	N.D.	19.55 ± 16.03

Conclusions

Carvacrol, thymol and citric acid clearly showed antimicrobial activity against Ao. Our preliminary data indicated that those antimicrobial agents, at critical concentrations, gave a significant reduction in the production OTA by Ao and might be used to prevent mycotoxin contamination in animal feed.

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