

## Research Article

# Effect of Temperature on Production of Ochratoxin A by *Aspergillus niger* in Orange Juice

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This challenging study was carried out to evaluate the temporal production of ochratoxin A (OTA) by *Aspergillus niger* ATCC 16404 and wild type *A. niger* AM at different temperatures in fresh squeezed orange juice (*Citrus sinensis* [L.] Osbeck cv Tarocco). Each strain, inoculated into the filtered orange juice, was incubated at 4°C, 20°C, and 26°C for 28 days. In the juice, at 26°C and 20°C, the concentration of *A. niger* ATCC 16404 increased by more than 2 log<sub>10</sub> up to the 21st day. At 4°C it remained constant. The microbial load of *A. niger* AM decreased at all temperatures. At 26°C, the maximum OTA accumulation found was 3.44 ng/mL on the 21st day for *A. niger* ATCC 16404 and 8.44 ng/mL on the 7th day for *A. niger* AM. The OTA synthesis seemed to be an intrinsic strain-dependent mechanism. *A. niger* ATCC 16404 produced OTA in accordance with the higher temperatures and the biomass concentrations, whereas *A. niger* AM produced the toxin at all temperatures regardless of its biomass. These results showed that fresh orange juice contaminated with *Aspergillus niger* aggregate strains may contain OTA levels.

## 1. Introduction

The market of organic citrus fruit has increased during the last few years because of consumer demand for healthy food products [1]. Most of the citrus arrives at the market in the form of processed products, such as single-strength orange juice and frozen juice concentrates [2]. Before pasteurization, fruit juices contain a microbial load representative of the organisms normally found on fruits during harvest plus postharvest contaminants. Pasteurization rids juice of pathogens and other heat-sensitive microbes. However, if the original load is too high and/or the pasteurization process is inadequate, some microorganisms might survive and subsequently cause spoilage and possibly illness if the surviving organisms are virulent [3]. Therefore, these products, especially nonpasteurized single-strength juice, are not free of microbiological spoilage problems [2, 4]. Moulds and yeasts tolerate high-osmotic and low pH conditions and grow at refrigeration temperatures [2]. The mould growth may result in several kinds of food-spoilage: off-flavours and discoloration; moreover, if the contaminating microorganisms

are pathogens, it could also cause human illness [3, 5, 6]. In many tropical countries the fresh nonpasteurized orange juice is sold at all public shops along the roadside [7]. The most important aspect of mould spoilage of foods is, however, the formation of mycotoxins. Small amounts of mycotoxins remain in mould cells, as most of them are excreted in the foods. Since most of the mycotoxins are very resistant to physical and chemical treatment, a rule of thumb exists: once the mycotoxins are in the food, they stay there during processing and storage [6, 8, 9].

Ochratoxin A (OTA) was originally described as a metabolite of *Aspergillus ochraceus* Wilhem and was found soon after from several related *Aspergillus* species [10, 11]. The origin of OTA in cool and temperate climates is generally attributed to *Penicillium verrucosum*, whereas in warm temperate and tropical zones it is commonly associated with *A. ochraceus* and the black aspergilli [12, 13]. The significance of black aspergilli as toxin-producing fungi has changed since the evidence that they can produce mycotoxins that are considered a risk for human and animal health emerged [14, 15]. OTA has been widely detected in cereals,

spices, some drinks such as coffee, and wines [11, 16–19]. European Regulation lays down the maximum levels of the said substance for cereals, cereal products, raisins, roasted coffee, wine, grape juice, spices, liquorice, and food for children. The tolerable weekly intake (TWI) of ochratoxin A is 120 ng/kg body weight [20]. OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic, and immunotoxic to several species of animals and is known to cause kidney and liver tumors in mice and rats. In humans, it has been related to Balkan endemic nephropathy, although this hypothesis has not been completely demonstrated [21]. According to the International Agency for Research on Cancer, OTA is possibly carcinogenic to humans (Group 2B) [22].

The economically most important OTA producers belong to *Aspergillus* sections *Circumdati* and *Nigri* [23, 24]. The black aspergilli are spread worldwide and occur on a great variety of substrates, including spoiled fruits, vegetables, and decaying vegetation in the fields [25]. In addition, they can contaminate agricultural products at different stages including preharvest, harvest, processing, and handling. Most of them are encountered as storage moulds on plant products [26]. Section *Nigri* (black aspergilli) is the main group responsible for OTA detected in grapes, must, and wines [27, 28]. The aim of this work was to establish OTA production by *Aspergillus niger* at different temperatures in fresh squeezed orange juice.

## 2. Materials and Methods

**2.1. Fungal Strains and Growth Conditions.** *Aspergillus niger* ATCC 16404 and *Aspergillus niger* AM aggregate strains isolated from rotted citrus fruit were inoculated on malt extract agar (MEA; Oxoid, Basingstoke, UK) at 25°C for 7 days. Conidia suspension was obtained by harvesting spores and suspending them in sterile distilled water containing 0.005% of Tween 80 (Difco; Becton Dickinson, Milan, Italy). The final concentration of the spores was standardized to  $1 \times 10^7$  spores/mL using a spectrophotometer (Perkin Elmer, Lambda 15, MA, USA).

**2.2. Preparation of Orange Juice.** Tarocco oranges (*Citrus sinensis* [L.] Osbeck) were obtained from local orchards and were used after a short storage period at 4°C (no longer than 1 week). Oranges were of typical commercial maturity. Before each experiment the fruits were disinfected by dipping in a NaOCl 0.5% (Carlo Erba, Milan, Italy) solution for 1 min and in a 70% ethanol solution for 2 min, rinsed with sterile distilled water and air-dried at room temperature.

The disinfected oranges were peeled and squeezed with a domestic juicer. The juices were centrifuged at 6000 rpm for 15 min at 15°C. The supernatant was filtered twice through a Whatman # 2 filter paper chart and subsequently again filtered through a 0.45 µm sterile filter (Millipore, Bedford, USA) into sterile bottles under aseptic conditions.

**2.3. Determination of Toxigenic Potential of Fungi by ELISA.** The preliminary study was carried out *in vitro* using a basic

orange juice medium agar (OJMA) and Yeast Extract Sucrose agar (YES; Oxoid) as medium control [29]. OJMA was composed of 10% (v/v) orange juice in sterile distilled water, five-fold agar, pH 3.5. Petri plates containing 20 mL of each medium were needle-inoculated centrally with the suspensions described above and incubated at 26°C for 7 days. Then, three agar plugs (5 mm diameter) were removed from each colony. The plugs were placed in vials to which 1 mL of a mixture of methanol/formic acid (25:1, v/v) (Carlo Erba) was added. After 60 min, the extract was filtered through a Whatman # 1 filter paper. OTA accumulation was determined in every treatment using a commercial ELISA Immunoscreen OCHRA kit (Tecna S.r.l. Trieste, Italy). Direct competitive enzyme-linked immunosorbent assays were performed as described earlier [29]. Absorbance was measured at 450 nm with a microplate reader (BioRad Model 550, Laboratories Milano, Italy). According to the manufacturer's description, the detection limit was 0.05 ng/mL. Each assay was performed in triplicate.

**2.4. Challenge Test.** The orange juice samples (50 mL) were contaminated with 5 mL of the above-described standardized spores and incubated at 26°C, 20°C, and 4°C. The viable counts were determined at different intervals of time (7, 14, 21, and 28 days). Serial dilutions of each sample were made and aliquots of 1 mL were added into tubes containing molten MEA (Oxoid), mixed and poured into plates. The viable counts were expressed as spores per mL. Each viable count was performed in triplicate.

**2.5. Determination of OTA in Orange Juice.** OTA extraction was carried out as previously described [29]. Five mL of each orange juice sample was extracted with five mL of 1 M hydrochloric acid (Carlo Erba) and 10 mL of dichloromethane (Carlo Erba) each time. The mixture was shaken for 15 min and centrifuged for 8 min at 9000 rpm at 4°C. Two phases: a lower solvent and an upper aqueous were formed. Sodium bicarbonate solution (pH 8.1) (Carlo Erba) was added to the solvent phase. After centrifugation at 9000 rpm for 8 min, the aqueous phase was collected and centrifuged again to separate it from any residual solvent. The aqueous phase was taken and diluted twice with bicarbonate solution (Carlo Erba). OTA in samples was determined in triplicate using OCHRA kit as described above.

**2.6. Statistical Treatment of Results.** For the fungi counts the obtained results were fitted to simple linear regression using Microsoft Excel 2000. The experimental data of OTA concentration was interpolated using a nonlinear regression analysis. Analyses of variance (ANOVA) for individual differences were used to compare cellular viability and OTA production over time. Correlations between fungi counts and OTA production were estimated using the Karl Pearson coefficient of correlation. The statistical variables were evaluated with Mathcad 2001i Professional (MathSoft Engineering & Education, Inc.).

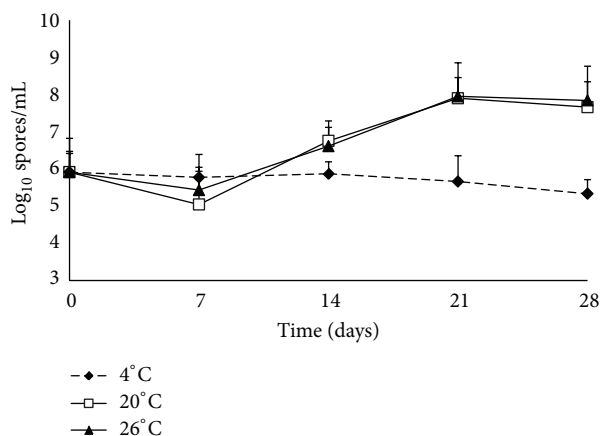


FIGURE 1: Effect of temperature on the growth of *A. niger* ATCC 16404 (*A. n.*) in orange juice. Mean values  $\pm$  standard error.

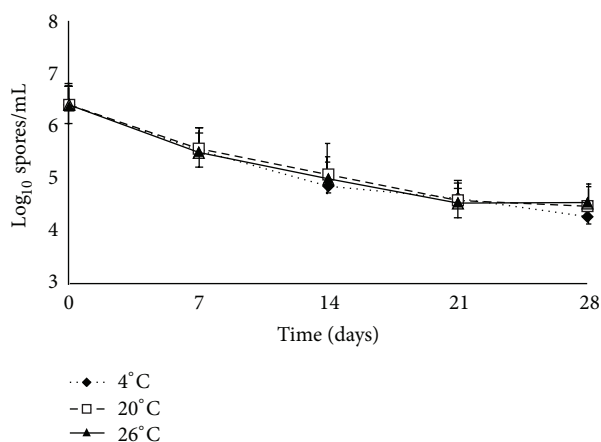


FIGURE 2: Effect of temperature on the growth of *A. niger* AM (*A. n.* AM) in orange juice. Mean values  $\pm$  standard error.

### 3. Results and Discussion

Both strains showed mycelial growth and OTA production (up to 5 ng/g) after 7 days of incubation at 26°C on OJMA. The cellular viability curves for *A. niger* ATCC 16404 and *A. niger* AM/temperature treatments on orange juice are shown in Figures 1 and 2, respectively. *A. niger* ATCC 16404 in juice incubated at 26°C and 20°C, after a latency period of about 7 days, increased in population by more than 2 log<sub>10</sub> on the 21st day, after which the number of cells remained constant. At 4°C, the strain maintained the initial concentration for up to 15 days and then decreased. The initial concentration of *A. niger* AM decreased gradually of about 2 log<sub>10</sub> until the 21st day at all temperatures. Thereafter, the populations remained constant. The cellular viability of *A. niger* ATCC 16404 was statistically significant compared with that of *A. niger* AM ( $P \geq 0.1$ ).

The mean OTA concentrations produced by *A. niger* ATCC 16404 and *A. niger* AM at each temperature and incubation time in the juice are summarized in Figures 3 and 4, respectively. The OTA concentration produced by *A. niger*

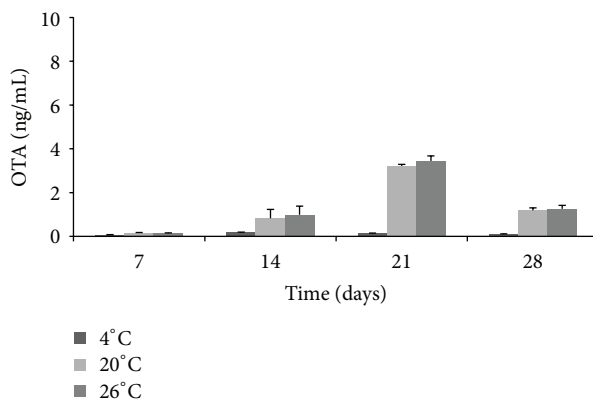


FIGURE 3: OTA concentrations produced by *A. niger* ATCC 16404 in orange juice at different temperatures. Mean values  $\pm$  standard error.

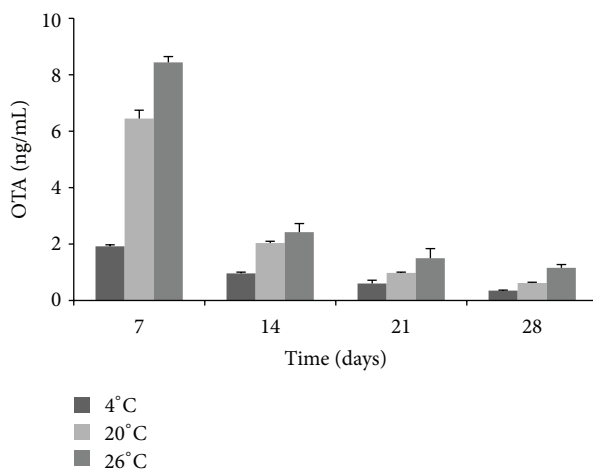


FIGURE 4: OTA concentrations produced by *A. niger* AM in orange juice at different temperatures. Mean values  $\pm$  standard error.

ATCC 16404 at 26°C and 20°C increased until the 21st day and then with time diminished. The correlation coefficient ( $R^2 = 0.99$ ) justified the significant correlation between biomass and OTA synthesis. At 4°C the concentration levels were very low during the assay. OTA production by *A. niger* AM rose until the 7th day at all temperatures; subsequently, the concentrations rapidly decreased with time. Maximum OTA accumulation was produced at 26°C. At 4°C the mould was able to produce the toxin throughout the observation period. OTA production by *A. niger* ATCC 16404, at the higher temperatures, was significantly higher compared with that of *A. niger* AM by day 21 of incubation ( $P \geq 0.1$ ). OTA produced by *A. niger* AM was synthesized in a shorter time (7 days) and in a higher concentration than that by *A. niger* ATCC 16404 at all temperatures ( $P \geq 0.1$ ).

The source of microbial contamination of fruit juices has not been fully established, but the surfaces of citrus fruits are known to harbour microbial populations which can potentially contaminate the juices. Blood orange juice features such as nutrients, low pH, and water activity seem to favour OTA production while temperature seems to be

the main factor influencing toxin production [29–33]. In this study the increase of temperature favored the growth and OTA synthesis of *A. niger* ATCC 16404. After a long latency period, at higher temperatures, *A. niger* ATCC 16404 increased in population and produced toxin in the orange juice until the 21st day. *A. niger* ATCC 16404 synthesized OTA in accordance with the higher temperatures and biomass concentrations in orange juice. Instead, the temperature did not affect the growth of *A. niger* AM, but rather the production of the toxin. *A. niger* AM produced most of the toxin at the higher temperatures within 7 days. Plant and food substrates are colonized by a diverse mycoflora that competes for nutrients and space in this context; mycotoxins could be envisaged as competitive factors, particularly in conditions of environmental stress [34–36]. In orange juice, according to our results, it appears that *A. niger* ATCC 16404 enters into an extended lag phase in which it begins to produce the toxin and reaches maximum production at the growth phase. Instead, *A. niger* AM seems to produce the toxin early on at all temperatures regardless of the biomass concentrations. It must be emphasized, for further investigation, that maximum amounts of OTA were found after a few days from the contamination by *A. niger* AM and at all temperatures. This behavior seems to be an intrinsic mechanism that is strain dependent. A possible explanation could be mycostasis. This has been envisaged as a mechanism by which propagules are protected from spontaneous germination in the absence of a substrate to be colonized [37]. After reaching peak production, the concentration of toxin gradually decreased over time probably due to the detoxification of ochratoxin A caused by *A. niger* [38]. Some authors have suggested that the strains can remove and assimilate the phenylalanine moiety from the OTA molecule because other nitrogen sources in the culture media are insufficient [39, 40]. A carboxypeptidase secreted by the *A. niger* strain could decompose OTA to ochratoxin  $\alpha$  and phenylalanine. Experiments with *Aspergillus* species showed a nearly complete conversion to ochratoxin  $\alpha$  within 5 days and after 7 days ochratoxin  $\alpha$  was further degraded [38, 41]. Despite the economic importance of citrus juices, there are few reports investigating OTA production associated with *Aspergillus niger*-aggregate group [29, 42, 43]. These results showed how fresh orange juice contaminated with *Aspergillus niger* aggregate strains may contain OTA levels. Further studies on other environmental strains of *A. niger* should be carried out to better understand the production of OTA in orange juice.

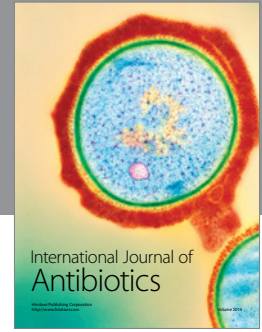
## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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