*Journal of Food Protection, Vol. 77, No. 11, 2014, Pages 1947–1952*
doi:10.4315/0362-028X.JFP-13-495


# Effect of Temperature, Water Activity, and pH on Growth and Production of Ochratoxin A by *Aspergillus niger* and *Aspergillus carbonarius* from Brazilian Grapes

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MS 13-495: Received 18 November 2013/Accepted 24 June 2014

## ABSTRACT

The growth of ochratoxigenic fungus and the presence of ochratoxin A (OTA) in grapes and their derivatives can be caused by a wide range of physical, chemical, and biological factors. The determination of interactions between these factors and fungal species from different climatic regions is important in designing models for minimizing the risk of OTA in wine and grape juice. This study evaluated the influence of temperature, water activity ($a_w$), and pH on the development and production of OTA in a semisynthetic grape culture medium by *Aspergillus carbonarius* and *Aspergillus niger* strains. To analyze the growth conditions and production of OTA, an experimental design was conducted using response surface methodology as a tool to assess the effects of these abiotic variables on fungal behavior. *A. carbonarius* showed the highest growth at temperatures from 20 to 33°C, $a_w$ between 0.95 and 0.98, and pH levels between 5 and 6.5. Similarly, for *A. niger,* temperatures between 24 and 37°C, $a_w$ greater than 0.95, and pH levels between 4 and 6.5 were optimal. The greatest toxin concentrations for *A. carbonarius* and *A. niger* (10 μg/g and 7.0 μg/g, respectively) were found at 15°C, $a_w$ 0.99, and pH 5.35. The lowest pH was found to contribute to greater OTA production. These results show that the evaluated fungi are able to grow and produce OTA in a wide range of temperature, $a_w$, and pH. However, the optimal conditions for toxin production are generally different from those optimal for fungal growth. The knowledge of optimal conditions for fungal growth and production of OTA, and of the stages of cultivation in which these conditions are optimal, allows a more precise assessment of the potential risk to health from consumption of products derived from grapes.

In Brazil, the cultivation of wine grapes is increasingly important, with several regions of the country, with different climates, involved in this activity. The sub-medium region of the São Francisco Valley, northeastern Brazil, stands out as a pioneer in the production of grapes and wine under tropical conditions and has completely different characteristics from traditional winemaking regions, located in temperate zones *(23, 27–29).* In studies of the occurrence of *Aspergillus* section *Nigri* in wine grapes *(21)* and the incidence of ochratoxin A (OTA) in wines produced in this region *(26),* the OTA produced by fungi was isolated; however, the tropical wines made in this region had values below the limit set by national law. In Brazil and in the European Community, the maximum limits allowed for mycotoxin were recently established at 2 μg/liter for OTA in wine and derivatives *(7, 10).*

OTA is a nephrotoxic mycotoxin with carcinogenic, immunosuppressive, and teratogenic properties *(14).* In humans, the ingestion of high amounts of OTA is linked to Balkan endemic nephropathy, a disease found in the rural areas of Bulgaria, Romania, Serbia, Croatia, and Bosnia *(17). Aspergillus carbonarius* is one of the main species responsible for OTA contamination in grapes, with higher production of OTA than other members of *Aspergillus* section *Nigri,* especially *A. niger (8).* Water activity ($a_w$) and temperature are the two most important factors that influence the growth of and OTA production by these mycotoxigenic species. These factors affect fungal growth, sporulation, and mycotoxin production *(16).* It is important to study the distribution of these fungi and the influence of environmental variables on their growth and OTA production *(12),* not only to add to knowledge about the behavior of a mycotoxigenic fungi isolated from a tropical region, but also to help develop predictive models to avoid or reduce production losses and risks to human health.

## MATERIALS AND METHODS

**Strain production.** In 2011 and 2012, 60 grape samples (*Vitis vinifera*) were collected from five vineyards located in the sub-medium region of the São Francisco Valley, in northeast Brazil (09°02′10″S, 40°11′06″W). The grapes, intended for the produc-

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TABLE 1. *Experimental design with real and coded variables and experimental results of the evaluation of growth and production of OTA by* Aspergillus carbonarius *and* A. niger[a]

| Expt | Temp (°C) | $a_w$ | pH | Ac (mm) | An (mm) | Ac (μg/g) | An (μg/g) |
|---|---|---|---|---|---|---|---|
| 1 | +1 (40.0) | −1 (0.93) | −1 (4.50) | 8.81 | 73.21 | 0.00 | 0.00 |
| 2 | +1 (40.0) | −1 (0.93) | +1 (6.20) | 9.02 | 87.73 | 0.005 | 0.00 |
| 3 | +1 (40.0) | +1 (0.99) | −1 (4.50) | 7.13 | 99.15 | 0.01 | 0.05 |
| 4 | +1 (40.0) | +1 (0.99) | +1 (6.20) | 8.64 | 90.01 | 0.005 | 0.01 |
| 5 | −1 (15.0) | −1 (0.93) | −1 (4.50) | 51.52 | 18.23 | 1.17 | 0.005 |
| 6 | −1 (15.0) | −1 (0.93) | +1 (6.20) | 34.26 | 31.81 | 0.23 | 0.17 |
| 7 | −1 (15.0) | +1 (0.99) | −1 (4.50) | 53.22 | 58.56 | 2.10 | 9.96 |
| 8 | −1 (15.0) | +1 (0.99) | +1 (6.20) | 63.09 | 67.36 | 11.08 | 5.17 |
| 9 | −1.68 (6.5) | 0 (0.96) | 0 (5.35) | 0.00 | 0.00 | 0.00 | 0.00 |
| 10 | +1.68 (48.5) | 0 (0.96) | 0 (5.35) | 0.00 | 0.00 | 0.00 | 0.00 |
| 11 | 0 (27.5) | −1.68 (0.91) | 0 (5.35) | 35.99 | 33.45 | 0.02 | 0.00 |
| 12 | 0 (27.5) | +1 (0.99) | 0 (5.35) | 25.87 | 90.12 | 3.12 | 0.84 |
| 13 | 0 (27.5) | 0 (0.96) | 0 (5.35) | 93.46 | 90.15 | 0.29 | 0.69 |
| 14 | 0 (27.5) | 0 (0.96) | 0 (5.35) | 93.21 | 88.90 | 0.39 | 1.47 |
| 15 | 0 (27.5) | 0 (0.96) | 0 (5.35) | 93.14 | 89.98 | 0.41 | 0.10 |
| 16 | 0 (27.5) | 0 (0.96) | −1.68 (3.92) | 93.69 | 90.00 | 0.05 | 0.82 |
| 17 | 0 (27.5) | 0 (0.96) | +1.68 (6.78) | 90.19 | 90.15 | 0.11 | 0.68 |

[a] Coded variables, −1.68, −1.0, 0, +1, +1.68; real variables in parentheses. OTA, ochratoxin A; $a_w$, water activity; Ac (mm), growth of *Aspergillus carbonarius*; An (mm), growth of *A. niger*; Ac (μg/g), OTA production by *Aspergillus carbonarius*; An (μg/g), OTA production by *A. niger*.

tion of fine red wine, were assessed for the presence of toxigenic fungi belonging to *Aspergillus* section *Nigri (20).* Strains belonging to the Culture Collection of the Department of Food Sciences (CDCA; Federal University of Lavras, Minas Gerais, Brazil), *A. carbonarius* (CDCA110) and *A. niger* (CDCA101), produce OTA and were identified and isolated from samples from one of the vineyards. The strains were transferred to petri dishes containing Czapek Dox Agar (Sigma-Aldrich, St. Louis, MO) and were incubated at 25°C for 7 days. The spore suspension was prepared using 30 ml of sterile distilled water containing 0.05% Tween 80 (Sigma-Aldrich) and then was filtered through sterile gauze (Nexcare, 3M, São Paulo, Brazil). A 10-μl aliquot of the suspension was transferred to a Neubauer chamber (Sigma, São Paulo, Brazil) to determine the final concentration of spores. The spore concentration ($10^6$ spores per ml) was standardized for all treatments.

**Preparation of semisynthetic grape culture medium with different pH and $a_w$ levels.** Studies were performed in vitro using a semisynthetic grape culture medium. Healthy Syrah variety grapes under ideal conditions for wine production were collected in a vineyard from the sub-medium region of the São Francisco Valley and were crushed. The grape medium was prepared by adding 175 ml of grape juice to 825 ml of distilled water and 20 g of agar (Merck, São Paulo, Brazil). For the treatments, the pH of the medium was adjusted to 3.62, 4.2, 5.35, 6.2, and 6.78 using 2 N NaOH. pH values were verified using a pH meter (Digimed, Digicrom Analítica Ltda, São Paulo, Brazil). The $a_w$ of the grape medium was 0.99, and it was adjusted to 0.96, 0.93, and 0.91 by adding different amounts of glycerol, according to Belli et al. *(2).* The $a_w$ of the growth medium was verified using an AquaLab CX-2 (Decagon Devices, Inc., Pullman, WA). After the preparation, 20 ml of the grape medium was distributed into petri dishes and was inoculated with 0.1 ml of spore suspension ($10^6$ spores per ml). The plates were incubated at 6.5, 15, 27.5, 40, and 48.5°C according to the experimental design used.

**Experimental design.** The experimental design and response surface methodology were used to investigate the influence of temperature, $a_w$, and pH, and the interaction of these variables, and to determine the range of values in which the fungi showed the greatest growth and production of OTA. The Central Composite Rotational Design (CCRD) was used for three independent variables in a $2^3$ factorial design, with three replicates at the center point. The coded variables were established with five levels (−1.68, −1, 0, 1, 1.68); actual corresponding values are shown in Table 1. Temperature ($X1$), $a_w$ ($X2$), and pH ($X3$) were the independent variables. Fungal growth (in millimeters) and OTA

TABLE 2. *Predicted models for the growth and production of OTA by* A. carbonarius *and* A. niger *strains*[a]

| Fungi | Predictive model | $R^2$ | $F$ | $P$ |
|---|---|---|---|---|
| *A. carbonarius* (mm) | $-1.52 + 17.01X1 - 135.91X2 + 3.18X3 - 0.14X1X2 - 4.02X1X3 + 237.34X2X3 - 0.24X1^2 - 8.14X2^2 - 1.70X3^2$ | 82.9 | 22.03 | 5.06e−13 |
| *A. carbonarius* (μg/g) | $+1.27 + 4.17X1 - 44.27X2 - 2.57X3 - 0.09X1X2 - 3.91X1X3 + 48.77X2X3 + 1.56X1^2 + 0.07X2^2 + 1.28X3^2$ | 71.7 | 6.75 | 8.63e−05 |
| *A. niger* (mm) | $-7.68 + 27.13X1 + 82.1X2 + 1.44X3 - 0.2X1X2 - 15.89X1X3 - 139.41X2X3 - 0.18X1^2 + 5.57X2^2 - 6.64X3^2$ | 85.8 | 4.69 | 0.0268 |
| *A. niger* (μg/g) | $-6.03 + 27.04X1 + 77.34X2 + 1.10X3 - 0.2X1X2 - 15.89X1X3 - 139.41X2X3 - 0.18X1^2 + 6.02X2^2 - 4.84X3^2$ | 77.1 | 8.97 | 8.49e−05 |

[a] Model growth expressed in millimeters; OTA production in micrograms per gram; $X1$, temperature; $X2$, water activity; $X3$, pH.

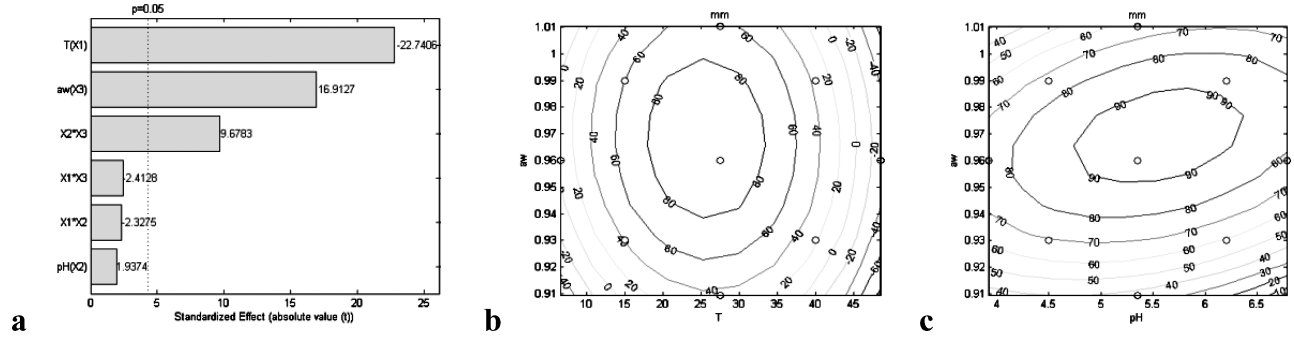


FIGURE 1. *Pareto diagram (a) showing the effects of temperature (X1), $a_w$ (X2), pH (X3), and their interactions on the growth of* Aspergillus carbonarius *and contour curves (b, c) for fungal growth considering interactions between the variables.*

production (micrograms per gram) were the response or dependent variables. The experimental design consisted of 17 assays, and the statistical analysis was performed using Chemoface software version 1.5 *(20)*.

**Assessment of strain growth and OTA extraction from cultures.** Petri dishes were examined on the 5th, 7th, and 10th days of incubation, and the colony diameter was measured in perpendicular directions using a digital caliper. OTA was extracted according to the modified method of Bragulat et al. *(6)*. Three plugs of culture were removed from the center, middle, and edge of each colony on the 10th day of the incubation period. The plugs were weighed in test tubes, and then 1 ml of methanol was added. The tubes were homogenized vigorously for 5 s and were kept at 25°C for 60 min. The extracts were filtered through polytetrafluoroethylene membranes (0.22 μm; Millipore Corp., Billerica, MA) and then were analyzed using a high-performance liquid chromatography Shimadzu coupled with two high-pressure pumps (model SPD-M20A), degasser DGU $20A_3$, interface CBM-20A, auto injector SIL-10AF, and RF-10 $A_{XL}$ fluorescence detector (Shimadzu, Kyoto, Japan). The Zorbax Eclipse XDB-C18 column (4.6 by 250 mm, 5 μm; Agilent Technologies, Palo Alto, CA) was used, and it was connected to a precolumn Zorbax Eclipse XDB-C18 4-pack (4.6 by 12.5 mm, 5 μm; Agilent). The chromatographic conditions for wavelength were 332 nm for excitation and 476 nm for emission. The flow used throughout the analysis was equal to 0.8 ml $min^{-1}$, and the injected volume of the samples and standard was 20 μl. The elution was performed using an isocratic system of 35:35:29:1 (methanol–acetonitrile–water–acetic acid). The average retention time for OTA determination was 11 $\pm$ 0.1 min. The amount of OTA in the samples was determined using an analytical curve obtained by linear regression ($y = 1.11756 \times 10^7 x - 2{,}592.1485$, where $y$ is the peak area and $x$ is the OTA concentration). The calculation defined the peak area versus the concentration of the respective standard solution, obtained by setting the coefficient of determination ($R^2$) at 0.9999. The detection limit (DL) and quantification limit (QL) were estimated through parameters obtained by the analytical curve and were calculated according to the following: DL = 3 SD/$m$ and QL = 10 SD/$m$ (where SD is the standard deviation and $m$ is the angular coefficient of the linear regression) *(13)*. The values obtained for DL and QL were 0.0004 and 0.0016 μg/g, respectively. All samples were analyzed in duplicate, and the standard OTA solutions were assessed in triplicate.

**Recovery assays.** Recovery assays were performed to ensure the analytical quality of the results. The semisynthetic culture medium was fortified with concentrations equal to 1.0, 3.0, and 6.0 μg/g in triplicate. The samples were extracted with methanol and were analyzed according to the method of Bragulat et al. *(6)*. The results of the recovery assays using 1.0, 3.0, and 6.0 μg/g were 82, 87, and 91%, respectively. These recoveries proved the remarkable reproducibility of the method and complied with Codex Alimentarius requirements for analytical methods (70 to 110% recovery) *(9)*.

## RESULTS AND DISCUSSION

Table 1 shows the real and coded variables and the experimental results of fungal growth (millimeters) and concentration of OTA (micrograms per gram) under different cultivation conditions. The results are presented for each test performed.

Experimental results from each test were used to adjust the data for first-order equations, which relate the behavior of the fungus to the studied parameters. These equations were obtained from quadratic regressions of the experimental data, using Chemoface software version 1.5. The equations obtained for the growth and production of OTA

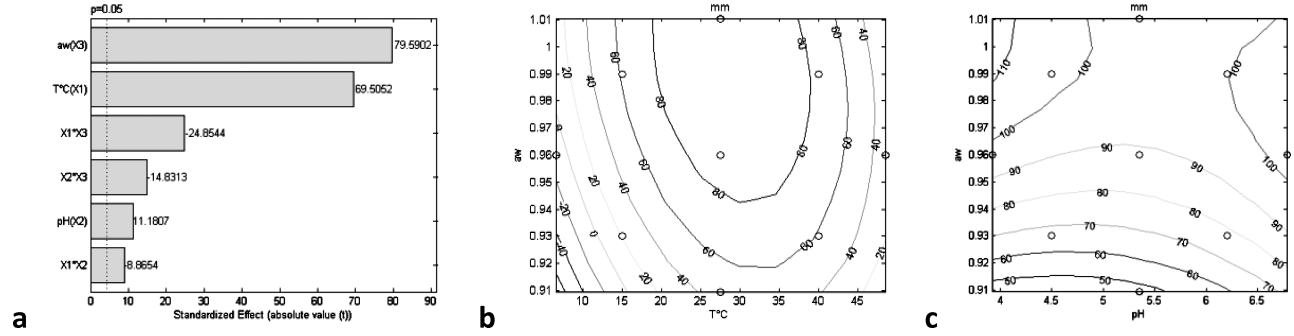


FIGURE 2. *Pareto diagram (a) showing the effects of temperature (X1), $a_w$ (X2), pH (X3), and their interactions on the growth of* Aspergillus niger *and contour curves (b, c) for fungal growth considering interactions between the variables.*

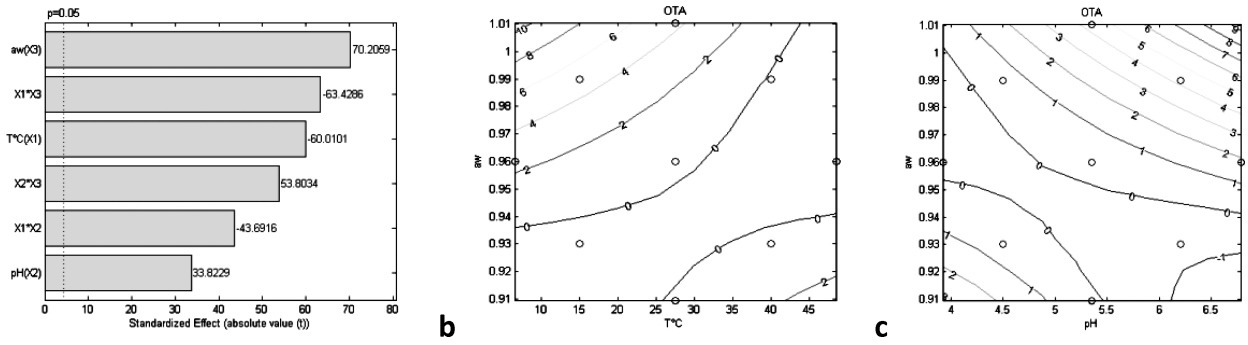


FIGURE 3. *Pareto diagram (a) showing the effect of temperature (X1), $a_w$ (X2), pH (X3), and their interactions on the production of OTA by* Aspergillus carbonarius *and contour curves (b, c) for fungal OTA production due to interactions between the variables.*

are presented in Table 2, with $R^2$. $R^2$ quantifies the quality of the adjustment, as it provides a measure of the proportion of the variation explained by the regression equation in relation to the total variation of responses.

**Growth and OTA production by *A. carbonarius* and *A. niger*.** A Pareto diagram (Fig. 1a) was used to evaluate the significance of the variables analyzed and the type of effect (synergistic or antagonistic) on the growth and OTA production of *A. carbonarius*. According to Figure 1a, only temperature and $a_w$, and the interaction between $a_w$ and pH, were significant ($P < 0.05$) for the growth of *A. carbonarius*. Temperature had the greatest influence on the growth of *A. carbonarius*. The main effect of temperature was antagonistic: the higher the temperature, the lower the fungal growth. A synergistic effect was seen with $a_w$, i.e., the higher the $a_w$, the greater the fungal growth. The pH level had no significant effect on the growth of *A. carbonarius*; however, there was a synergistic interaction between pH and $a_w$. Contour curves give a better representation of the optimal conditions for cultivation (Fig. 1b and 1c); they show that *A. carbonarius* had the highest growth (90 mm) at 20 to 33°C, $a_w$ 0.95 to 0.98, and pH 5 to 6.5. No fungal growth was observed below 10°C or above 44°C.

Regarding the growth of *A. niger,* the Pareto diagram shows that all of the studied variables and their interactions were significant ($P < 0.05$) (Fig. 2a). $a_w$ had the greatest influence on the growth of *A. niger,* followed by temperature. Both factors had a synergistic effect, i.e., the higher the $a_w$ and temperature, the greater the fungal growth. According to the contour curves (Fig. 2b and 2c), the growth of *A. niger* was optimal at 24 to 37°C, $a_w$ above 0.95, and pH 4 to 6.5. Under these culture conditions, *A. niger* showed growth of 90 mm after 10 days of incubation. The results showed that, at optimal temperature and $a_w$, the fungus grew regardless of the studied pH range. To obtain a lower growth of *A. niger* in semisynthetic grape medium, the temperature should be reduced to less than 24°C and the $a_w$ to less than 0.95.

To our knowledge, this is the first study to test culture conditions for the *A. carbonarius* and *A. niger* strains obtained from wine grapes from northeastern Brazil. Studies conducted in synthetic grape medium with *A. carbonarius* strains from different wine regions of Europe have also shown that temperature and $a_w$ influence fungal growth. However, these studies did not evaluate the influence of pH. The *A. carbonarius* strains showed optimal growth at 30 to 35°C and $a_w$ 0.95 to 0.98 *(7, 16, 18, 21, 22)*. These results are in opposition to the optimal growth conditions found in the present study, in which the greatest growth occurred between 20 and 35°C, higher temperatures than were reported in previous studies. The tolerance of *A. carbonarius* to low $a_w$ was also confirmed in the present study.

Leong et al. *(15)* assessed the growth of *A. niger* in synthetic grape medium and showed that the fungus had optimal growth at 30 to 35°C and $a_w$ 0.93 to 0.98. Similar results were found in the present study for $a_w$ (0.94 to 0.99); however, the temperature range in which the fungus showed the greatest growth was 25 to 40°C. These differences may be due to the culture medium used for the fungal growth. Moreover, the semiarid tropical climate of the São Francisco Valley, with an average annual temperature of 26°C and a maximal temperature of 33°C, are favorable conditions for the growth of *A. niger* and *A. carbonarius*.

Regarding OTA production by *A. carbonarius,* the Pareto diagram (Fig. 3a) shows that all the assessed variables and their interactions were significant ($P <$

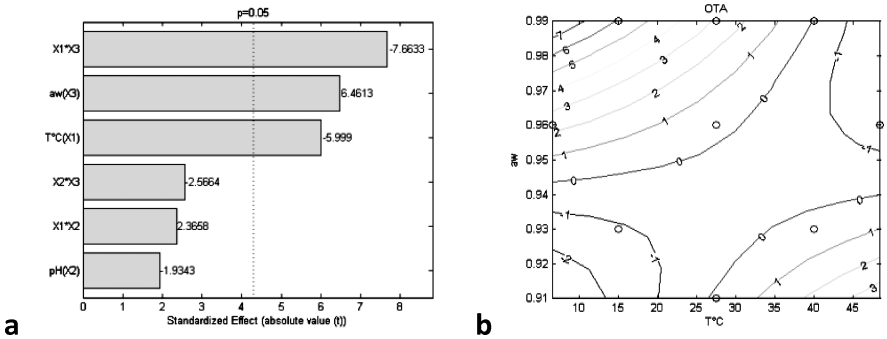


FIGURE 4. *Pareto diagram (a) showing the effect of temperature (X1), $a_w$ (X2), pH (X3), and their interactions on the production of OTA by* Aspergillus niger *and contour curves (b) for OTA production due to interactions between the variables.*

0.05). $a_w$ had the greatest influence on OTA production, followed by temperature and pH. $a_w$ and pH had synergistic effects, whereas temperature had an antagonistic effect. According to the contour curves (Fig. 3b and 3c) for *A. carbonarius,* 15°C resulted in the greatest concentration of toxin (8 to 10 μg/g), with pH above 6.0 and $a_w$ of 0.99. Reduced toxin levels (1 to 3 μg/g) were obtained at 25°C, pH above 5.0, and $a_w$ below 0.95. Although there was a trend showing an increased production of toxin at temperatures below 10°C, it is known that *A. carbonarius* does not grow at this temperature.

For *A. niger,* the Pareto diagram (Fig. 4a) shows that $a_w$ and temperature and the interaction between these two variables were significant ($P > 0.05$). $a_w$ had a synergistic effect on the synthesis of the toxin by *A. niger,* whereas temperature had an antagonistic effect on its synthesis. The effect of the interaction between these variables was antagonistic, because the greatest production of OTA was observed at the highest $a_w$ and lowest temperature. Based on the contour curves (Fig. 4b), the highest synthesis of toxin (6 to 7 μg/g) occurred at 15°C and at $a_w$ levels between 0.98 and 0.99. *A. niger* did not produce toxin at 20 to 25°C.

Temperature and $a_w$ influenced the production of OTA by *A. carbonarius,* and the culture conditions under which the maximum production of toxin occurred differed from those reported in other studies. A study of Greek strains found maximum OTA production at 20°C and at $a_w$ 0.96, and the OTA production values were 3.14 and 2.67 μg/g for the two assessed strains *(23, 25).* On the other hand, for strains from Italy, Spain, and Portugal, the maximum OTA production by *A. carbonarius* occurred at 20°C and $a_w$ levels between 0.95 and 0.98 *(3, 19).* Australian strains showed maximum OTA production (21 μg/g) at 15°C and $a_w$ 0.965. Although the OTA production by *A. carbonarius* isolated from the wine regions of Brazil was lower than that obtained for the Australian strains, the highest OTA concentration (10 μg/g) was also obtained at 15°C and $a_w$ 0.99. It was observed that the fungus could produce toxin at 25°C and $a_w$ 0.99, indicating that the Brazilian strain is able to produce toxin at various temperatures but only at high $a_w$ levels.

For *A. niger,* the highest concentrations of OTA production (7 μg/g) were obtained at 15°C and $a_w$ 0.99, similar to the results reported by Belli et al. and Leong et al. *(5, 15).* Esteban et al. *(11)* observed a maximum of OTA production at higher temperatures (20 to 25°C) and at $a_w$ values from 0.95 to 0.98.

Analysis of the two species isolated from the wine region of Brazil showed that, compared with *A. carbonarius, A. niger* had a higher growth rate at 40°C and at all the assessed values of $a_w$. Nevertheless, at 15°C and at all the tested values of $a_w$, *A. carbonarius* had a higher growth rate than *A. niger*. At optimal conditions of temperature and $a_w$, *A. carbonarius* produced greater amounts of OTA (10 μg/g) than did *A. niger* (7 μg/g). Greater toxin production by *A. carbonarius* was also found in other studies *(2, 4, 15, 19).* The adaptive differences to the climatic conditions of each wine region can be explained by the differences in the behavior of the strains tested at various sites *(1, 23, 24).*

In conclusion, the present study showed that *A. carbonarius* and *A. niger* obtained from Brazilian grapes produced the maximum concentration of toxin at 15°C, $a_w$ 0.99, and pH 5.35 when grown in vitro in grape medium. These conditions in the field before harvest could favor fungal production of OTA. Data from the current study allow more accurate assessment of the potential risk to human health from consumption of products derived from grapes. The predictive model can be used to estimate growth and OTA concentration of *A. carbonarius* and *A. niger* in winery regions of Brazil.

## ACKNOWLEDGMENTS

The authors thank CNPq and Seim-Arid EMBRAPA for financial support and the wineries for allowing us to collect samples on their properties.

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