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Effects of water activity and temperature on the growth and ochratoxin A production of three *Aspergillus* species isolated from stored wheat in Algeria

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Abstract

Wheat stored under uncontrolled conditions is susceptible to contamination by various ochratoxigenic *Aspergillus* species. Among the key environmental factors influencing the proliferation of these fungi are water activity (a_w) and temperature. In this study, three ochratoxigenic *Aspergillus* isolates (Z1, Z11, and Z14) were isolated from stored wheat samples. The combined effects of different a_w levels (0.85, 0.90, 0.95, and 0.99) and temperatures (25, 30, 35, 40, and 45°C) on lag phases and growth rates were systematically examined. The ITS nucleotide sequence analysis results identified isolate Z1 as *Aspergillus westerdijkiae*, while Z11 and Z14 were identified as *A. alliaceus* and *A. carbonarius*, respectively. The toxigenic potential of these fungal strains, assessed on the CYA medium, showed a high capacity for Ochratoxin A (OTA) production, with 3303.23 ng/g for *A. westerdijkiae*, 950.23 ng/g for *A. alliaceus* and 801.28 ng/g for *A. carbonarius* produced at 25°C. The shortest lag times prior to growth (< 24 h) were observed at 0.95 a_w/25°C for *A. westerdijkiae* (2.19 h), at 0.99 a_w/35°C for *A. alliaceus* (0.75 h) and at 0.99 a_w/30°C for *A. carbonarius* (1.03 h). The maximum growth rates were recorded as follows: *A. westerdijkiae* at 9.3 mm/day (25°C/0.99 a_w), *A. carbonarius* at 11.1 mm/day, and *A. alliaceus* at 16.5 mm/day (both at 30°C/99 a_w). In almost all compared pairs, the effects of varying a_w and temperatures on lag time before and after development rates were significantly different. These findings provide valuable insights for designing effective wheat storage strategies and ultimately improving food safety in Algeria.

Keywords – *Aspergillus alliaceus* – *Aspergillus carbonarius* – *Aspergillus westerdijkiae* – ochratoxin A – stored wheat – temperature – water activity

Introduction

Ochratoxin A (OTA) is a toxic compound produced by various fungal strains belonging to *Aspergillus* and *Penicillium*. It is known for its immunotoxic, nephrotoxic, and potentially

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carcinogenic effects in humans (IARC 1993, Xu et al. 2022, Wang et al. 2023). The presence of OTA in stored wheat and wheat-based products is generally attributed to the growth of *Aspergillus alliaceus, A. carbonarius, A. ochraceus* and *A. westerdijkiae* (Czerwiecki et al. 2002, Navale et al. 2021). These fungal species primarily originated from warm and tropical regions across the globe (Riba et al. 2008, Patriarca & Fernández Pinto 2017), where poor cereal storage practices often facilitate mold development and OTA contamination (Mahdjoubi et al. 2020). Given the critical role of wheat grain storage in food security, marketing, and distribution, it is essential to maintain the highest standards of quality and safety. Fungal growth in stored grains not only serves as a clear indicator of compromised quality but also negatively affects sensory attributes, nutritional value, and food safety (Qiu et al. 2024).

Managing the storage environment by controlling ecophysiological factors is a fundamental and effective strategy for preventing fungal infection in stored wheat (Mannaa & Kim 2017). Among these factors, water activity (a_w) and temperature are the most influential in determining the growth of grain fungi and the production of mycotoxins (Medina et al. 2017, Zingales et al. 2022). Water activity, which represents the amount of free water available for microbial activity, plays a crucial role in spore germination and mycelial development (Nanguy et al. 2010). Stored wheat can be highly susceptible to OTA contamination, especially when stored at an a_w higher than 0.70 (Kebede et al. 2020).

Fungal species, including *Aspergillus*, exhibit varying growth behaviors depending on the level of a_w. Unlike bacteria, some fungal species can thrive at relatively low a_w levels (Pitt & Hocking 2009). The optimal range for mold development typically falls between 0.80 and 0.99 a_w, while growth rates decline significantly below this threshold (Bellí et al. 2004).

Temperature is another crucial factor influencing fungal growth, with each species having a specific temperature range for optimal development. *Aspergillus* species generally thrive in warm environments, with ideal growth temperatures ranging from 25°C to 35°C. Deviations from these optimal conditions can significantly reduce growth rates or even inhibit fungal proliferation entirely (Magan & Aldred 2007).

The interplay between a_w and temperature is complex, as both factors can either synergistically or antagonistically influence fungal growth. For example, at lower a_w, the growth rate of *Aspergillus* species is more significantly affected by changes in temperature compared to higher a_w conditions (Mohapatra et al. 2017). This interaction is particularly important in storage environments, where fluctuations in both a_w and temperature can compromise the stability and safety of stored wheat (Medina et al. 2015). Additionally, the activation of metabolic regulatory genes and the production of mycotoxins by fungi can be influenced by variations in both a_w and temperature (Garcia-Cela et al. 2021).

The humid climate of Algeria, a North African country bordering the Mediterranean area, promotes mold growth and mycotoxin production (Tantaoui-Elarak et al. 2018). Studies have reported the presence of ochratoxigenic species of *Aspergillus* and OTA in stored wheat grains and their derivatives in Algeria (Riba et al. 2008, Zebiri et al. 2018, Medjdoub et al. 2023, Belasli et al. 2023). According to Zebiri et al. (2018), 69.23% of wheat grains were contaminated with OTA, with concentrations ranging from 210 to 27.310 ng/g. OTA was also detected in semolina and flour during processing, with levels ranging from 160 to 34.750 ng/g (Zebiri et al. 2018). These findings highlight a potentially high risk of OTA exposure for the Algerian population, especially since the country currently lacks regulations on the permissible levels of OTA in raw materials or food products. A recent study confirmed this exposure, detecting OTA in 83 (86.4%) of the 96 urine samples collected from Algerian workers, with levels ranging from 0.3 to 3.5 μg/L (Mendes et al. 2023).

In Algeria, limited information is available regarding the effects of temperature and a_w on the propagation of ochratoxigenic *Aspergillus* species isolated from stored wheat. As far as we know, this is the first study focusing on the impact of a_w and temperature on the ecophysiology of these species. The objectives of this study are (1) to isolate and characterize *Aspergillus* species from stored wheat, (2) to assess their potential for OTA production, and (3) to investigate the effect of

varying a_w levels and temperatures on the lag phase and development of three representative strains: *A. alliaceus*, *A. carbonarius* and *A. westerdijkiae* on a Czapek Yeast Autolysate Agar (CYA) medium.

Materials & Methods

Fungal isolates

Based on the previous study by Zebiri et al. (2018) investigating the presence and distribution of mycotoxin-producing fungi in the country's wheat supply, 200 samples were analyzed, from which numerous fungal strains were isolated. Among them, three *Aspergillus* strains were selected for their high production of OTA. The three isolates of *Aspergillus*, designated as Z1, Z11 and Z14, were obtained from durum wheat grains stored for five months. The samples were obtained from three provinces in Algeria, where durum wheat is cultivated: Sétif (northeastern Algeria), Blida (located near the Mitidja plain) and Médéa (located in the Tell Atlas region).

The direct culture technique, as outlined by Pitt & Hocking (2009), was employed to isolate these fungi from wheat samples. A hundred grams of wheat seeds underwent a superficial disinfection process by immersion in a 0.5% sodium hypochlorite solution for 1 min. After disinfection, the seeds were washed at least thrice with sterile distilled water to remove any residual disinfectant. Subsequently, ten kernels were plated on the surface of the Dichloran Rose Bengal Chloramphenicol Agar medium (DRBC, King et al. 1979). The Petri dishes were then incubated at a temperature of 25°C for five days. Following this incubation period, the plates were examined for the presence and characteristics of fungal colonies, including colony color, conidial shape, vesicle arrangement, the presence or absence of sclerotia, and their size.

Molecular characterization

The isolates were identified by sequencing the ITS1-5.8S-ITS2 regions of rDNA. The genomic DNA of fungal strains was obtained using the procedure described by Liu et al. (2000). A Nanodrop (Thermo Fisher Scientific, USA) was used to measure the quantity and quality of the DNA samples. For DNA amplification in the ITS region, primer sets ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used (White et al. 1990). The sizes of the amplicons were assessed using a 1 kb DNA ladder (Promega Corporation) after agarose gel electrophoresis. The same primer sets were used for sequencing as for amplification at Genewiz (Beckman Coulter Genomics, UK).

A BLAST search was conducted on the sequences using a pairwise sequence alignment tool available at the Westerdijk Fungal Biodiversity Institute (http://www.westerdijkinstitute.nl/). The obtained ITS sequences of the three strains were compared to 13 reference sequences retrieved from GenBank and aligned with many of the currently identified strains in *Flavi*, *Circumdati*, and *Nigri* sections. In this phylogenetic study, *Aspergillus terreus* var. *subfloccosus* CBS 117.37 (accession number: NR 149331.1) was used as the outgroup. Alignments were accomplished with MUSCLE (Edgar 2004) in MEGA 7 (Kumar et al. 2016). The sequences were deposited at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) with the GeneBank accession numbers OR685294 (Z1), OR525539 (Z11) and OR525540 (Z14). The phylogenetic tree was constructed using the maximum likelihood method with 1000 bootstrap replicates following the Tamura-Nei model (Tamura & Nei, 1993).

Ochratoxin A quantification

For the screening of OTA production, our three strains were spot-inoculated at the center of plates containing Czapek Yeast Autolysate Agar (CYA) medium and incubated at 25°C for seven days (Pitt & Hocking 1997). Subsequently, three agar plugs were extracted from each plate, weighed, and 1 mL of methanol was introduced (Bragulat et al. 2001). The obtained solutions were centrifuged at 12000 rpm for 10 min after 1 h. The resulting supernatant was filtered through a 0.45 µm Polytetrafluoroethylene (PTFE) hydrophilic filter. Each filtrate (10 µL) was then spotted on a

Thin Layer Chromatography (TLC) plate and developed in toluene-ethyl acetate-formic acid (5:4:1, v/v/v) as the mobile phase and finally, observed under UV light at 365 nm. Positive/negative scoring was based on blue-green fluorescence under 365 nm UV light after five days of incubation. The Limit of Detection (LOD) of this technique is 0.5 ng/g.

A reversed-phase High-Performance Liquid Chromatography (HPLC) system equipped with a fluorescence detector (HPLC-FLD) was used to determine OTA levels. The system used was Thermo Scientific™ Dionex™ UltiMate™ 3000 (Dionex, France). The excitation wavelength (λex) was established at 332 nm, whereas the emission wavelength (λem) was established at 466 nm. Each sample was injected using an auto-injector at a volume of 100 µL. The analytical column used was Prontosil C18, with dimensions of 150-4.6 mm, and it was preceded by a C18 guard column with dimensions of 10-4.3 mm. The temperature of the column was 30°C. The mobile phase consisted of a mixture of acetonitrile, water, and acetic acid in a ratio of 50:49.8:0.2, flowing at a rate of 0.8 mL/min. OTA in the samples was identified by comparing it with a standard OTA, which was injected under the same conditions and sourced from *A. ochraceus* (Sigma-Aldrich, Steinheim, Germany). Data processing was conducted using Chromeleon software. The concentration of OTA was determined by measuring absorbance at $\lambda = 330$ nm, with identification based on a retention time of approximately 7 min. Quantification was performed by analyzing the peak area against a standard curve, and the detection limit was established at 0.020 ng/g.

Growth rate assessment

All the assays were conducted on the CYA medium. The water employed in medium preparation was adjusted with glycerol to achieve the specified a_w levels of 0.85, 0.90, 0.95, and 0.99 a_w, as described in Marin et al. (1995).

The three *Aspergillus* strains were initially subcultured on CYA and incubated at 25°C for seven days. Using a sterile spatula, the spores from each strain were carefully removed from the colony surfaces and suspended in 10 mL of sterile distilled water containing 0.5% Tween 80 (Ramos DeSimone et al. 1999). The spore concentration was determined using a Thoma cell hemocytometer (10^6 spores/mL). Subsequently, Petri dishes containing CYA medium with varying a_w were inoculated with spore suspension (5 µL) from each strain. Following this, they were cultured at various temperatures (25, 30, 35, 40, and 45°C). Three replicates were conducted for each treatment (a_w × T° × isolate). Samples with equivalent a_w levels were then enclosed in airtight plastic boxes. The experiment lasted for twenty-one (21) days.

The growth of the three *Aspergillus* strains was monitored by measuring the diameter of the fungal growth during incubation. The daily growth measurements were noted until the fungal growth completely covered the Petri dishes (Aldred et al. 1999). For each Petri dish, two measurements of the colony diameters were taken at two perpendicular angles. The colony radii were plotted against time, and linear regression was used to determine the lag phase (λ) and the highest growth rate (µ max) (Verheecke-Vaessen et al. 2019).

Statistical analysis

The non-parametric Wilcoxon test was chosen for each pair of data to evaluate the effect of a_w and temperature on fungal growth and lag time. The statistical analyses were performed using JMP 14 software (JMP®, Version 14.0).

Results

Identification of fungal strains

The isolates Z1, Z11, and Z14 were obtained from wheat samples cultured on DRBC medium. They were initially identified based on their morphological characteristics, each presenting distinct attributes. For example, the colony of Z1 was powdery or granular, initially white, then yellow or ochre-yellow. Furthermore, isolate Z11 exhibited a white color and smooth, biseriate spores characterized by large dark sclerotia. The isolate Z14 is a black filamentous fungus

arranged biseriately with very rough and black conidia. Comparison of the sequences of the three strains with those of the closest type strains of *Aspergillus* gave percentages ranging from 99 to 100%. The phylogenetic tree generated from the ITS sequences of 13 representative fungal strains from *Aspergillus* sections *Flavi*, *Circumdati* and *Nigri* is shown in Fig. 1. According to the findings of the ITS nucleotide sequence analysis, strain Z11 was recognized as *A. alliaceus* and strains Z1 and Z14 were identified as *A. westerdijkiae* and *A. carbonarius* (biseraite cluster), respectively.

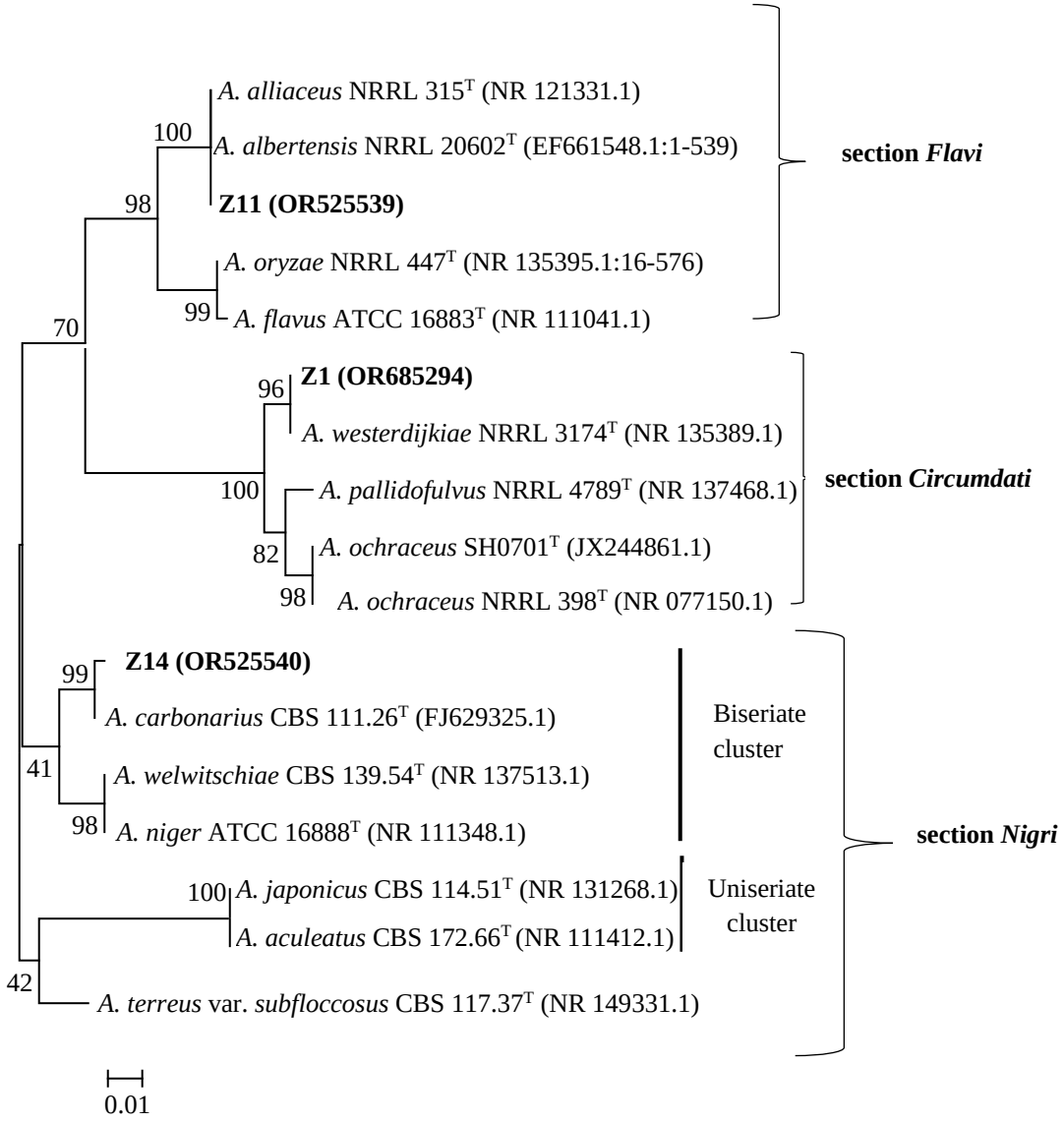

Fig. 1 – Maximum likelihood phylogenetic tree for the ITS region of *Aspergillu*s strains based on the Tamura-Nei model. Bar 0.01 nucleotide substitutions per site.

Ochratoxin A production

The toxin-producing potential of the fungal strains, assessed on CYA medium after incubation for seven days at a temperature of 25°C (Fig. 2). All isolates demonstrated significant ochratoxin A (OTA) production, with *A. westerdijkiae* yielded a high concentration of OTA (3303.23 ng/g), followed by *A. alliaceus* (950.23 ng/g) and *A. carbonarius* (801.28 ng/g).

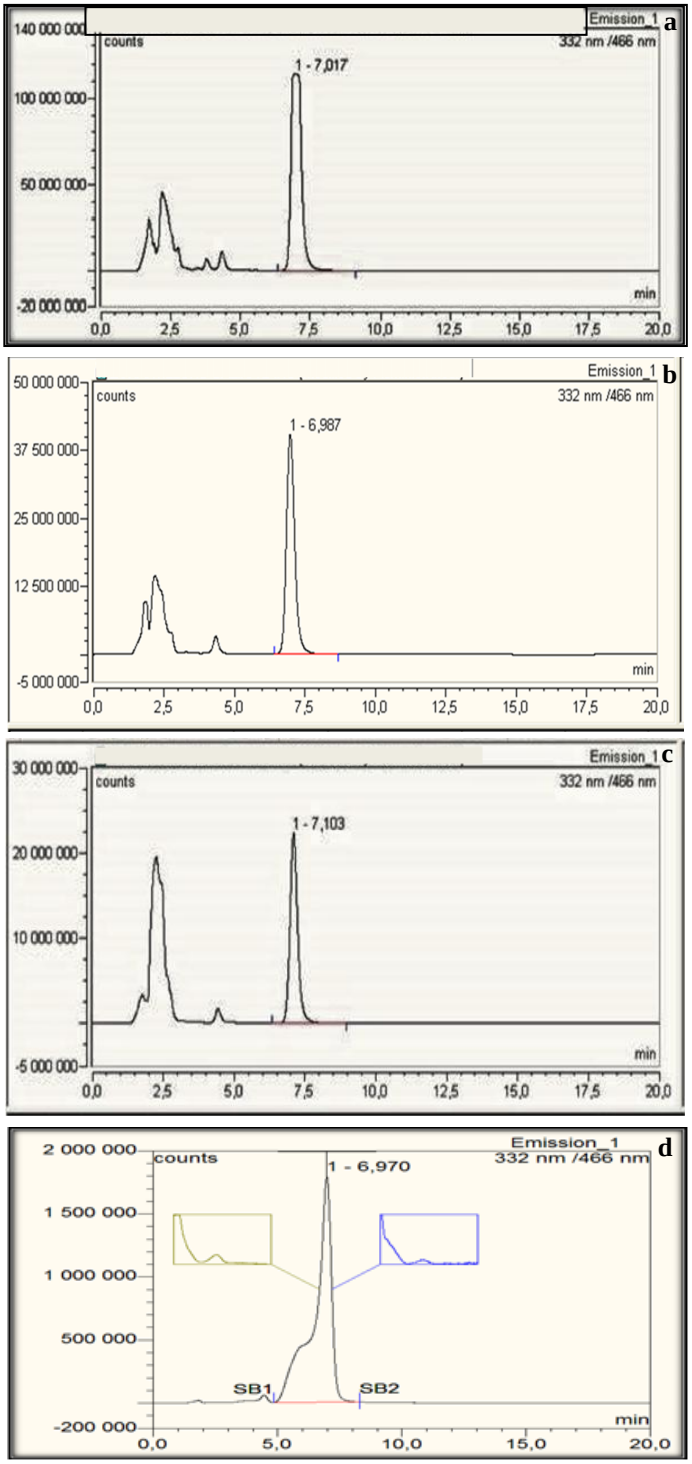

Fig. 2 – HPLC chromatograms of ochratoxin A (OTA) produced by *A. alliaceus.* (a) *A. carbonarius.* (b) and *A. westerdijkiae.* (c) cultured on CYA medium compared to the standard OTA. (d) Chromatograms were recorded at λ = 332 nm.

Effects of a_w and temperature on lag phase

The effects of a_w and temperature on the lag phases prior to growth (λ) of the studied strains are presented in Fig. 3. The findings of *A. alliaceus* show that the lag times were contrasting; for instance, between a_w pairs of 0.85-0.90 at the different temperatures, the results were non-significant. On the other hand, at 40°C, *A. alliaceus* lag times were significantly different between 0.95-0.99 a_w. In the case of *A. carbonarius*, at all the tested a_w, significant results were noticed for the lag phase at the different temperatures except at 25°C, between 0.85-0.99 a_w and at 35°C,

between 0.95-0.99 a_w. For *A. westerdijkiae*, at 25°C, the lag times were significantly different between 0.85-0.90 a_w and 0.85-0.95 a_w, while all tested conditions were significant at the different temperatures.

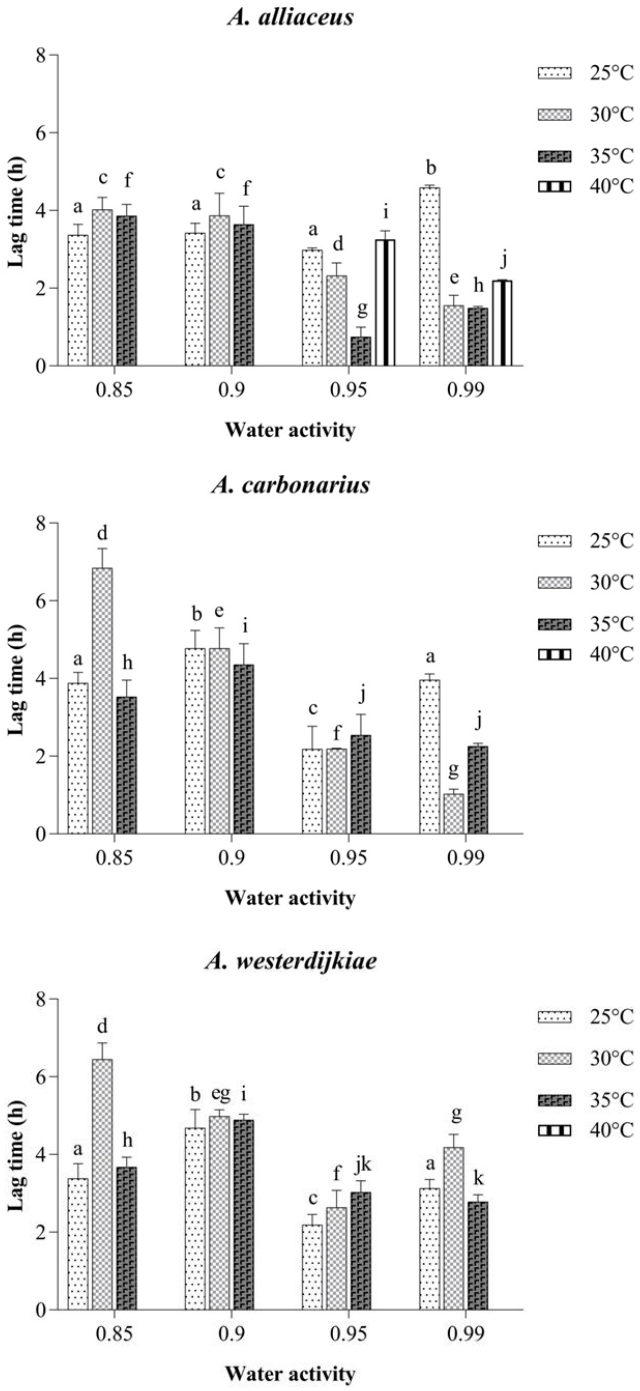

Fig. 3 – Effects of water activity at different temperatures on the lag time of *A. alliaceus*, *A. carbonarius* and *A. westerdijkiae* strains in the CYA medium. Bars indicate the standard errors of the means. Different letters indicate statistical differences in water potentials at each temperature, as determined by the Wilcoxon test ($p < 0.05$).

The lag phase is typically a period during which the cell adapts to its new surroundings before entering the exponential growth phase. Our results showed that the shortest average lag

times were under the conditions of 0.95 a_w/25°C for *A. westerdijkiae* (1.19 h), 0.95 a_w/35°C for *A. alliaceus* (0.75 h) and at 0.99 a_w/30°C for *A. carbonarius* (1.03 h).

The results of the joint effect of a_w and temperature on the latency phases of the three studied species are shown in Table 1. We noted that strain differences did not significantly impact the lag time prior to growth (0.0748 > p). However, variation of temperature (p = 0.0001) and a_w (p = 0.0001) significantly impacted the lag time prior to growth.

Table 1 Results of the Wilcoxon test with a ChiSquare test of the effects of temperature and a_w on lag time of the studied *A. westerdijkiae*, *A. alliaceus* and *A. carbonarius* strains compared two by two.

Source	FD	Prob > ChiSq
Species	2	0.0748
Temperature	4	< 0.0001*
Water activity	3	< 0.0001*

* Denotes significant values, FD = functional dependency.

Table 2 shows that the lag time is significantly different between *A. westerdijkiae* and *A. alliaceus* (p = 0.0275), while the other two combinations do not show significant differences. Furthermore, the statistical results of the effect of a_w are not significantly different between 0.85-0.90 and 0.95-0.99 (p > 0.005); otherwise, the effect is significantly different in all the compared conditions. The effect of temperature was significant on the lag phases before growth for all conditions except 30–25°C, 35–25°C and 35–30°C for all tested strains.

Table 2 Overview of pairwise statistical analyses employing Wilcoxon with a ChiSquare test of the influence of different variables on lag time data of *A. alliaceus*, *A. carbonarius* and *A. westerdijkiae* strains.

Factor	Parameter	P-value
Species comparison	*A. westerdijkiae-A. carbonarius*	0.0275*
	A. carbonarius-A. alliaceus	0.1081
	A. westerdijkiae-A. alliaceus	0.6165
Water activity	0.90-0.85	0.5153
	0.99-0.95	0.6697
	0.99-0.85	0.0105*
	0.99-0.90	0.0041*
	0.95-0.90	0.0003*
	0.95-0.85	0.0001*
Temperature (°C)	45-25	< 0.0001*
	45-30	< 0.0001*
	45-35	< 0.0001*
	40-35	< 0.0001*
	40-30	< 0.0001*
	40-25	< 0.0001*
	45-40	< 0.0001*
	30-35	0.5966
	35-25	0.2011
	35-30	0.1510

* Statistically significant P-values.

Influence of a_w and temperature on mycelial growth

The joint effects of temperature and a_w on the mycelial growth of the examined strains are revealed in Fig. 4. The obtained maximum growth rate for *A. westerdijkiae* was 9.3 mm/day at 25°C and 0.99 a_w, and the observed minimum growth rate was 3.1 mm/day at 30°C and 0.85 a_w. At 25°C and 30°C, the growth rates of *A. westerdijkiae* were not significantly different between all the tested a_w values. In contrast, at 35°C, the growth rate was significantly different at the following a_w (0.85, 0.9 and 0.95).

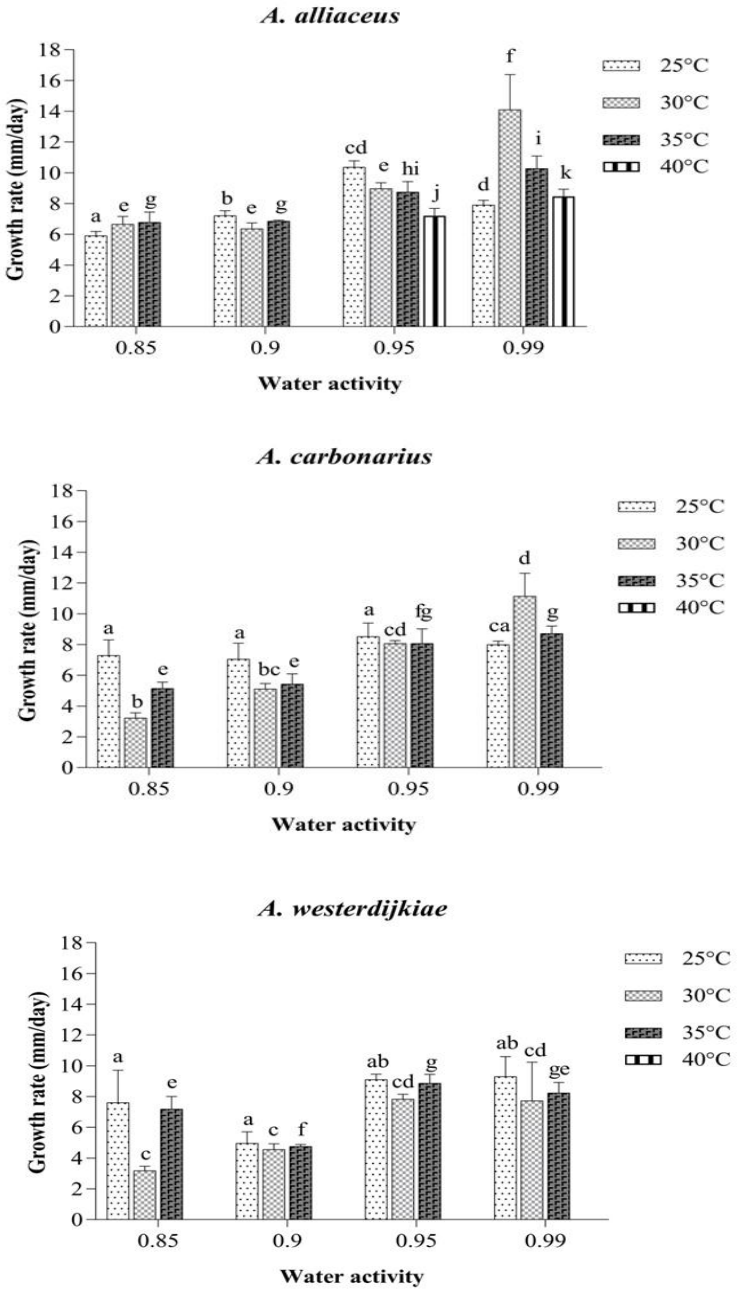

Fig. 4 – Effects of water activity and temperature on the growth rates of *A. alliaceus*, *A. carbonarius* and *A. westerdijkiae* strains. Different letters indicate statistical differences in water potentials at each temperature, as determined by the Wilcoxon test ($p < 0.05$).

The maximum growth rate obtained for *A. alliaceus* during this study was 16.5 mm/day at 30°C and 0.99 a_w, and the lowest growth rate was 5.91 mm/day at 25°C and 0.85 a_w. Additionally,

we observed a significant difference at 25°C for the tested a_w whereas non-significant results were obtained at 30°C for a_w values of 0.85, 0.90 and 0.95. For all the a_w tested, a non-significant result was noticed at 25°C for the growth rate of *A. carbonarius*, while contrasting results were found for the other temperatures. The growth of *A. westerdijkiae* collected from durum wheat grains was favoured by the lowest temperature of 25°C and by the highest a_w (0.99). For *A. carbonarius*, the observed minimum growth rate was 3.2 mm/day at 30°C/0.85 a_w, and the maximum growth rate (11.1 mm/day) was observed at 30°C and 0.99 a_w.

Through our study, the first in Algeria, we have found that the growth of our strains is enhanced and stimulated by high temperature and elevated a_w. This was confirmed by comparing the effects of the tested factors on the fungal growth of the three studied strains, as shown in Table 3. Temperature and a_w are significant factors impacting fungal growth. Regarding the impacts of a_w and temperature on the rate of growth, our results showed that at 30°C and 0.99 a_w, the growth rate was significantly the highest, and with significant differences between 25°C, 40°C and 45°C at the same a_w level.

Table 3 Results of the Wilcoxon test with a ChiSquare test of the effects of temperature and a_w on growth rates of the studied *A. westerdijkiae*, *A. alliaceus* and *A. carbonarius* strains compared two by two.

Source	**FD**	**Prob > ChiSq**
Species	2	0.0810
Temperature	4	< 0.001*
Water activity	3	< 0.001*

*Denotes significant values, FD = functional dependency.

Temperature (p = 0.001) and a_w (p = 0.001) are significant factors that affect the growth of fungi. The influence of strain variation on growth was almost significantly different. The overview of the findings is shown in Table 4. The effect of temperature on the growth rates of the three studied species was not significantly different between 35–30°C, 35–25°C and 30–25°C; otherwise, the effect was significantly different in all compared temperature pairs. The effect of a_w on the growth rates of the three strains was not significantly different between 0.9 and 0.85 and between 0.99 and 0.95. Otherwise, the effect was significantly different in all of the compared pairs.

Table 4 Overview of pairwise statistical analyses employing Wilcoxon with a ChiSquare test of the influence of different variables on the rate of growth data of *A. alliaceus, A. carbonarius* and *A. westerdijkiae* strains.

Parameter	Factor studied	P-value
A. westerdijkiae-A. carbonarius	Species comparison	0.9416
A. carbonarius-A. alliaceus		0.0568
A. westerdijkiae-A. alliaceus		0.0508
0.95-0.9	a_w	0.0002*
0.99-0.9		0.0002*
0.99-0.85		0.0006*
0.95-0.85		0.0007*
0.99-0.95		0.4132
0.9-0.85		0.8089
35-30	Temperature	0.3889
35-25		0.4371
45-40		0.0115*

Table 4 Continued

Parameter	Factor studied	P-value
30-25		0.1747
40-30		< 0.0001*
40-35		< 0.0001*
40-25		< 0.0001*
45-25		< 0.0001*
45-30		< 0.0001*
45-35		< 0.0001*

* Statistically significant P-values.

Discussion

Aspergillus species were isolated from wheat in temperate environments (Duarte et al. 2010, Kebede et al. 2020). These fungal species invade cereals in the field during growth, collection, and preservation and produce OTA if environmental conditions are favorable (Mannaa & Kim 2017). The previous study by Riba et al. (2008) displayed the occurrence of these species in Algerian wheat. Additionally, Tunisian wheat and barley were reported to be affected by *A. flavus* and *A. niger* aggregate (Jedidi et al. 2017).

The three fungal isolates (Z11, Z14 and Z1) obtained from Algerian wheat samples were conclusively identified as *Aspergillus alliaceus* (section *Flavi*), *A. carbonarius* (section *Nigri*), and *westerdijkiae* (section *Circumdati*), respectively. Morphological characterization aligned with established taxonomic criteria for these sections, with Z1 exhibiting powdery colonies transitioning from white to ochre-yellow, with lavender-purple sclerotia (1 mm diameter), hallmark traits of section *Circumdati* (Gil-Serna et al. 2020). In addition, Z11 displayed white, smooth colonies with large dark sclerotia, consistent with the *A. alliaceus*-clade in section *Flavi* (Frisvad et al. 2019). Finally, Z14 presented rough, black conidia and biseriate structures, typical of section *Nigri* (Abarca et al. 2004). Molecular validation through ITS sequencing confirmed these identifications, with 99–100% similarity to type strains. Phylogenetic analysis further resolved their placement within the *Aspergillus* sections, corroborating the utility of ITS sequencing for distinguishing closely related species (Abarca et al. 2004, Frisvad et al. 2019, Gil-Serna et al. 2020).

A. westerdijkiae produced a high concentration of OTA (3303.23 ng/g). Similar findings were reported by Frisvad (2004), Samson et al. (2006), and Gil-Serna et al. (2020), indicating that most strains of this species exhibited significant OTA production capacity. In addition, Gil-Serna et al. (2015) reported that the primary species responsible for contaminating feed and food in warm climates are *A. steynii*, *A. westerdijkiae* and *A. ochraceus*. This aligns with the high production capacity of *A. ochraceus* and *A. westerdijkiae* identified in this study.

The strain from the *flavi* section, *A. alliaceus*, produced 950.23 ng/g. Incidents of this species in food samples and its ochratoxigenic potential have been described in only a few reports (Hajjaji et al. 2006, Demirel & Sariozlo 2014, Chuaysrinule et al. 2020). In Algeria, six isolates of *A. alliaceus* were identified in flour samples (16%), and each isolate produced OTA at 230 to 11.50 ng/g (Riba et al. 2008).

The third strain, *A. carbonarius*, produced 801.28 ng/g. *A. carbonarius* was first described as an OTA producer in 1996 (Téren et al. 1996), with a majority of strains (60-100%) subsequently recognized as OTA producers (Tjamos et al. 2004, Esteban et al. 2005). The intensity of OTA production varied between the strains (Bragulat et al. 2001). Medjdoub et al. (2023) extracted this species from couscous prepared from wheat derivatives but did not test their OTA production capability.

The shortest average lag periods were observed under the following conditions: 0.95 a_w/25°C for *A. westerdijkiae* (1.19 h), 0.95 a_w/35°C for *A. alliaceus* (0.75 h), and 0.99 a_w/30°C for *A. carbonarius* (1.03 h). Romero et al. (2007) reported that the shortest lag phase (6.24 h) of *A. carbonarius* obtained from Argentina dried vine fruits and was found at 0.92 a_w and 35°C.

Lahouar et al. (2017) reported that at temperatures of 25°C and 37°C, and high a_w values (0.97 and 0.99), very short lag phases were observed for two toxinogenic isolates of *A. tubingensis*. In contrast, Samapundo et al. (2007) reported that raising temperatures of 30 to 37°C resulted in a reduction in colony growth rates and an extension of the lag phase for isolates of *A. parasiticus* and *A. flavus*. However, Astoreca et al. (2007) observed very short latency phases (< 24 h) for the growth of *A. niger* at 25–30°C when a_w ranged from 0.94 to 0.99 a_w. No growth occurred at 45°C for the studied strains and also at 40°C for *A. westerdijkiae* and *A. carbonarius* across all tested a_w levels, with a lag time of more than 21 days. According to Medina & Magan (2010), lag time is extended at marginal conditions of a_w and temperature. It was also noted that in the presence of high a_w (0.99) at 35°C for the three strains and at 40°C for *A. alliaceus*, the lag phases were short. This poses a very significant risk, especially considering that these fungi are high producers of OTA, as we have previously demonstrated. It is also possible for these strains to produce toxins in stored wheat consumed by humans under unmonitored conditions (Paterson & Lima 2010). These conditions can also occur due to poor storage practices, which enhance the growth of strains and their potential for OTA production (Yu & Pedroso 2023).

The impact of a_w and temperature on the development and sporulation of *A. westerdijkiae* was investigated by Abdel-Hadi & Magan (2009), who reported that maximum growth occurred at 30°C and 0.95 a_w. Similar profiles of temperature and a_w growth in CYA medium were obtained by Gil-Serna et al. (2015). The growth was positively influenced by high temperatures and a_w values, suggesting that *A. westerdijkiae* may be found in warm climates or storage environments (Wawrzyniak et al. 2013).

The a_w was significantly affecting *A. westerdijkiae* growth. Therefore, by controlling a_w during storage of durum wheat grains, it is possible to limit the growth of this fungus and consequently reduce OTA production (Casu et al. 2024).

A non-significant result was seen at 25°C for the growth rate of *A. carbonarius*, but divergent findings were recorded at other temperatures. These results are lower than those reported by Romero et al. (2007), where the highest growth rate of 17.46 mm/day was observed at 30°C and 0.95 a_w, no growth was detected at a_w below 0.85. These results are consistent with those reported by Bellí et al. (2004), who determined that the highest growth rate was 9.11 mm/day at 30°C and 0.98 a_w for an *A. carbonarius* isolated from wine grapes, and by Bellí et al. (2005), who reported a maximum growth rate of 10.1 mm/day at 30°C and 0.99 a_w for an *A. carbonarius* strain isolated from Spanish grapes. Leong et al. (2006) reported that the fastest growth of *A. carbonarius* strains obtained from Australian grapes occurred at 30°C and 0.96 a_w, with a maximum recorded growth rate of 7 mm/day. Abdel-Hadi (2017) confirmed the non-growth at 25°C and 0.85 a_w for *A. carbonarius* on Malt Extract Agar (MEA).

The ideal growth conditions for *A. alliaceus* were 30°C and 0.98 a_w (Hajjaji et al. 2006). In this study, the growth was observed at 40°C, but only at high water activity levels (0.95 and 0.99 a_w), with corresponding growth rates of 7.2 mm/day and 8.4 mm/day, respectively. These findings align with those of Frisvad et al. (2019) and Bouti et al. (2020), who reported that some strains of *A. alliaceus* can grow on CYA at 42°C. This indicates a significant risk, as prolonged incubation at elevated temperatures and high a_w levels can lead to OTA production by heat-tolerant strains, posing a potential threat to public health and food safety.

Conclusion

This study is the first to investigate the effect of water activity (a_w) and temperature on the ecophysiology of three ochratoxin A (OTA)-producing species—*Aspergillus westerdijkiae*, *A. alliaceus*, and *A. carbonarius*—isolated from stored wheat in Algeria. The findings show that each species has distinct optimal conditions for achieving the shortest lag times and highest growth rates. Notably, high temperatures and elevated a_w levels significantly enhance the growth of these strains, leading to shorter lag phases. This is particularly concerning given that these fungi are prominent OTA producers. Prolonged exposure to such favorable conditions can increase OTA

production, underscoring the critical need for controlled storage environments to prevent fungal contamination and improve food safety in Algeria.

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Data availability

No data were utilized for the research outlined in this article.

Declaration of competing interest

The authors state that they have no recognized competing financial interests or personal relationships that might have influenced the work presented in this paper.

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