

The effect of the interactions of water activity, and temperature on OTA, OTB, and OT α produced by *Penicillium verrucosum* in a mini silo of natural and inoculated wheat using CO₂ production as fungal activity sentinel

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ABSTRACT

Ochratoxin A (OTA) is a nephrotoxin that contaminates grains in storage. Moisture and temperature sensors give delayed responses due to their slow kinetic movement within the silo. This study examines if CO₂ production could predict OTA contamination and identify storage conditions exceeding the maximum limit (5 µg/kg). The impact of water activity levels (0.70–0.90 a_w), temperatures (15 and 20 °C), and storage duration on (a) *Penicillium verrucosum* population, (b) CO₂ respiration rates (RR), and (c) ochratoxins concentrations in stored wheat was investigated. 96 samples were analysed for ochratoxins with LCMS-MS. RR was >7 times higher at wetter conditions than at drier a_w levels. A positive correlation between CO₂, OTA, OTB, and OT α was observed at the wettest conditions. OTA exceeded the limit at >0.80 a_w (16% moisture content) with RR > 0.01 mg CO₂ kg⁻¹ h⁻¹. The knowledge of the RR of stored grain would alert grain farmers/managers to improve grain storage management.

1. Introduction

Penicillium verrucosum is a competitive fungal species dominating cereal grains pre- and post-harvest under conducive environmental conditions (Magan et al., 2003). Its occurrence in grains indicates ochratoxin A contamination and is prevalent in temperate regions with high humidity such as Northern Europe (Cabañes et al., 2010; Lund & Frisvad, 2003; Ramakrishna et al., 1996). OTA is also produced by *Aspergillus ochraceus*, *Aspergillus carbonarius*, and sometimes *A. niger* mostly under warmer climatic conditions (Alborch et al., 2011; Birzele et al., 2000; Lindblad et al., 2004; Ramos et al., 1998).

OTA has been reported to occur in a wide variety of foods ranging from cereal grains, cereal products, cheese and meat products, dried fish, nuts, beans, pepper, grapes, wine, and dried fruits among others (EFSA, 2006; El Khoury & Atoui, 2010; Walker & Christian Larsen, 2005). OTA is a potent nephrotoxin, carcinogen, and immunosuppressant, posing significant health risks to humans and animals upon ingestion (Berger et al., 2003; Duarte et al., 2010). Chronic exposure to

OTA through contaminated wheat products has been associated with various adverse health effects, including renal diseases, immune system disorders, and urinary tract tumors (Bui-Klimke & Wu, 2015). The International Agency for Research on Cancer (IARC) classifies OTA as a possible human carcinogen (Group 2B) based on sufficient evidence of its carcinogenicity in animals and limited evidence in humans (Ostry, Malir, Toman, & Grosse, 2017).

OTA, within the family of structurally related ochratoxins, stands out as the most potent toxin. Its toxicity surpasses that of its dechlorinated counterpart, Ochratoxin B (OTB), yet remains similar to the toxic effects observed with its ethyl esterification derivative, Ochratoxin C (OTC) (Zhang et al., 2017; Zhihong et al., 2015). Both OTB and OTC have been implicated in various adverse health effects, including immunotoxicity, hepatotoxicity, kidney toxicity, renal toxicity, genotoxicity, and cytotoxicity (Müller et al., 2003; Heussner et al., 2006; EFSA, 2020). Although OTB has been detected in a range of food items such as cereals, coffee, and wine, its presence generally occurs at lower levels compared to ochratoxin A.

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Ochratoxin alpha (OT α), a less potent variant of OTA, emerges through chemical alterations in OTA's structure during metabolism or food processing (Berthiller et al., 2013; EFSA CONTAM Panel et al., 2020; Rychlik et al., 2014). Abrunhosa et al. (2010) elucidated the enzymatic hydrolysis of OTA's peptide bond, catalyzed by numerous peptidases and lipases, leading to the formation of OT α . Additionally, Ringot et al. (2006) documented OT α 's generation from OTA metabolism by rumen microorganisms following ingestion. Consequently, OT α has been identified as a reliable biomarker for OTA in various biological matrices, including raw milk, plasma, and urine in both humans and animals (Boudra & Morgavi, 2006; Klapac et al., 2012; Muñoz et al., 2010). A maximum limit of 5 $\mu\text{g/kg}$ has been imposed by the European Commission for OTA in cereals and cereal products. The process of determining a maximum limit for OTC, OTB, and OT α is still in progress. Consequently, it is imperative to focus on detecting and minimising these compounds in food and animal feed.

The growth of *Penicillium verrucosum* and OTA production are influenced mostly by the moisture content of grains and temperature within the storage environment and storage practices (Magan et al., 2003). OTA reduces the quality and safety of stored grains resulting in significant economic losses for farmers and traders and poses significant health risks when consumed (Cinar & Onbaşı, 2019; Ganesan et al., 2022). Cairns-Fuller et al. (2005) examined the growth rate and OTA production by *P. verrucosum* under different conditions of a_w , temperature, and gas compositions using both wheat grains and a 2% wheat-based medium. Optimum growth was established in vitro on the wheat-based media at 0.98 a_w at 25 °C and in vivo in wheat grains at 0.95 a_w at 25 °C. The report also shows minimal growth at 0.85 a_w with no production of OTA after 56 days of incubation.

Other studies reported that higher water availability $>0.95 a_w$ and temperatures >28 °C could impact the metabolism of *P. verrucosum* for low growth rate and OTA formation in cereal grains and cereal products (Arroyo et al., 2005; Czaban, Wroblewska, Stochmal, & Janda, 2006; Lindblad et al., 2004; Ramakrishna et al., 1996). This suggests that OTA synthesis can occur within a certain range of water activity levels smaller than the levels for the growth of *P. verrucosum* which can still thrive at low water activity levels. The detection of OTA produced by *Aspergillus* species in different foods in vitro and in situ has been largely investigated (Chiotta et al., 2015; Gil-Serna et al., 2015; Gizachew et al., 2019). Only a few studies have considered OTA produced by *Penicillium verrucosum* species. Also, studies on the effects of the interactions of environmental conditions on the growth of *P. verrucosum* and OTA, OTB, and OT α production in stored wheat are limited.

In addition to temperature and water availability that influence grain deterioration in storage, it is important to consider the gaseous state of the storage environment. Post-harvest grains continue to respire during storage with the results of the metabolic process as CO₂, heat, and water (Kaleta & Górnicki, 2013). In addition, the presence of living organisms such as fungi and insects may increase the CO₂ levels within the storage environment (Maier et al., 2010). Therefore, monitoring the CO₂ concentrations released in grain silos could indicate the onset of spoilage and this can be measured using CO₂ sensors. Due to the rapid result alertness from the CO₂ sensors, it is preferable to moisture and temperature sensors to monitor grain quality in storage (Neethirajan et al., 2009; Raudienė et al., 2017; Zhang et al., 2014).

Several studies have used CO₂ measurement to monitor grain quality in storage. Mylona et al. (2012) used the cumulative CO₂ production data measured with Gas Chromatography to quantify the effect of different storage conditions on dry matter loss relating it to deoxynivalenol, zearalenone, and fumonisins in stored wheat and maize. Their findings indicated that CO₂ production increased as storage conditions increased, showing distinct production patterns for the three toxins. A similar method and results were reported by Garcia-Cela et al. (2018). However, these studies did not measure the real-time respiration rates in the stored grains, and the relationship between the CO₂ produced, toxins produced, and the grain mycobiota in storage was not established.

Therefore, to accurately evaluate grain storage conditions using CO₂ level measurements, it's crucial to understand the CO₂ production rate of wheat and its dynamics at different moisture levels and temperatures.

This study aimed to examine if real-time CO₂ production in stored wheat silos could predict the early risk of OTA contamination and identify storage conditions exceeding maximum limit (5 $\mu\text{g/kg}$). The objectives of this study are to investigate the impact of storage conditions (0.70–0.90 a_w , 15 and 20 °C) on (a) *Penicillium verrucosum* populations (b) CO₂ respiration rates (RR) (c) ochratoxins concentrations produced in naturally contaminated and *Penicillium verrucosum* inoculated wheat and (d) to examine the relationship between the CO₂ and ochratoxins concentrations in the contaminated wheat.

2. Materials and methodology

2.1. Wheat grains and moisture adsorption curve analysis

Wheat grains from Northern Ireland (2021 harvest) were used in the study. They were stored 6 months before the storage experiment. The initial water activity of grains was analysed using (AquaLab water activity meter 4 TE Decagon devices, Inc., Pullman, USA). For the moisture content analysis, grains were oven-dried at 105 °C overnight and were calculated on a dry weight basis.

10 g sub-samples of wheat were placed in ten 25 ml glass Universal bottles. Known amounts of water (0.1–3.5 ml) were added to the grains. The bottles were sealed and mixed thoroughly and kept at 4 °C for 24 h with regular shaking. The samples were equilibrated at room temperature for 1 h and the water activity of grains was measured (AquaLab water activity meter 4 TE Decagon devices, Inc., Pullman, USA). Moisture contents (MC) of the samples were calculated following oven drying at 105 °C overnight. Three replicates were analysed. The amount of water added was plotted against a_w values to achieve the targeted water activity levels for grain modification. The relationship between the MC (dry weight basis) and a_w values was plotted and noted.

2.2. Fungal strain and spores harvesting

Penicillium verrucosum (OTA11) known to produce ochratoxins was used in this study. The strain was stored at –20 °C in glycerol: water (70:30, v/v) in the Applied Mycology Group culture collection, Cranfield University. *Penicillium verrucosum* was grown on Malt Extract Agar (MEA) with an anti-bacterial agent (chloramphenicol) incubated at 25 °C for 7 days.

Spores were harvested aseptically from the 7-day-old *Penicillium* plates scraped with a sterile spatula with sterile Tween 80 water (0.05% v/v). 10 ml of the suspension was transferred into a sterile tube and shaken. Spores targeted concentrations (1.5×10^5 spores/ml) were counted and calculated with the Thoma cells formula using an Olympus microscope (U-ULH model, Olympus Optical, Japan). The spore suspension was further diluted with Tween 80 water to achieve the targeted volume of 20 ml for the inoculation experiment.

2.3. Mini-silo CO₂ sensing storage experiment

3 kg of wheat grains were weighed into a 12 l box. The grains were rehydrated to targeted water activities (0.70, 0.80, 0.90, 0.95 a_w) with a known amount of water and 20 ml less for spore inoculum for inoculated grains. The grains were vigorously mixed and stored at 4 °C overnight. The following day, the grains were equilibrated at the targeted temperature (15 and 20 °C) rooms for 3 h and the water activities of the equilibrated grains were measured. Grains were transferred into a 5 Litre coffee flask with a centrally placed ATEX-compliant sensor probe. Cotton wool was placed in the headspace of the flask to minimise gaseous movement in and out of the flask. There were two flasks (replicates) for each water activity level. The ten flasks were stored at each temperature condition. The probe comprises CO₂, temperature, and

relative humidity sensors connected to a computer system where their respective real-time monitoring readings from the stored grains were collected. The grains were stored for 20 days. 60 g of contaminated samples were collected on day 10 and day 20 from each flask with a sterile probe into sterile jars having a good representation of the bulk sample. The water activity of the grains was monitored before and after sampling. 40 g of the sub-sample grains were dried in the oven at 50–55 °C overnight, ground, and stored at –20 °C before further analysis. The CO₂ sensors were calibrated before each experiment for each treatment studied. After sampling on day 10, the grain inside the flask is 60 g less, the new weight is considered for calculating the respiration rates for day 20. The respiration rate was calculated using the diffusion formula in Eq. (1) adapted from Raudienė et al. (2017).

$$\text{Respiration rates} = \frac{\Delta\text{CO}_2 \times M_{\text{CO}_2} \times V_h}{V_m \times m \times \Delta t} \quad (1)$$

where ΔCO_2 is the change in CO₂ volumetric concentration in ppm, M_{CO_2} is the molar mass of CO₂ gas = 44.01 (g mol^{–1}), V_h is the volume of headspace in the jar (L), V_m is the molar volume of a gas (L mol^{–1}), m is the mass of the wheat sample (kg) and Δt is the duration (h) of ΔCO_2 .

The molar volume was determined by analyzing the storage temperatures.

$$V_m = \frac{R \times T}{P}$$

where R is the gas constant (L mol^{–1} K^{–1}), T is temperature (K), P is pressure (atm).

2.4. Fungal population and isolation

2.4.1. Fungi Enumeration (Serial dilution method)

1 g of whole kernel contaminated grain was weighed and soaked in sterile 9 ml peptone water in a 25 ml universal bottle for 3 h. 1 ml was transferred into the 10^{–2} dilution bottle and was serially diluted to the 10^{–4} bottle. Sterile tips were used between each dilution and the universal bottle's content was shaken using a vortex. 100 µl of the dilution aliquots were reversely plated onto Dichloran Glycerol 18 agar (DG18+: Oxoid CM0729) and Malt Extract Agar (MEA+: Oxoid CM59) media in 3 replicates and spread out with a sterile metal spreader. The plates were incubated at 25 °C for 7 days. Fungal genera were identified visually with a stereoscope. Colonies were counted with a colony counter and expressed as Log₁₀ CFUs/g calculated on a dry weight basis.

2.4.2. Fungi Isolation (direct plating method)

25 grains were directly inoculated on DG18+ and MEA + media to identify dominant fungal genera in the contaminated grains. 5 grains were placed equidistant on each medium plate of five replicates and incubated at 25 °C for 7 days. Fungal genera were identified visually with stereoscope and morphological characteristics. The frequency of isolation was calculated using Eq. 1 below.

$$\% \text{Frequency of isolation} = \frac{\text{Total contaminated grains}}{\text{Total grains in plates (25 grains)}} \times 100 \quad (2)$$

2.5. Mycotoxin analysis

The initial mycotoxin concentrations of the wheat grains were analysed. After the storage experiment, 96 contaminated wheat samples (including replicates) were analysed for mycotoxins. 5 g of ground contaminated wheat grains were mixed with 20 ml of the extraction solvent (acetonitrile (ACN): water (H₂O): acetic acid (HOAC) 79: 20:1, v/v/v). Samples were extracted for 90 min using a GFL Orbital Shaker 3017 (Berlin, Germany). After extraction, 500 µl of the extracted aliquot was diluted with 500 µl dilution solvent (ACN: H₂O: HOAC 20:79:1, v/v/v),

yielding a 1:1 dilution in a vial. The mix was vortexed and 5 µl of the diluted extracts were injected into the Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) system. The chromatographic method and mass spectrometric parameters were adapted from Sulyok et al. (2020). The accuracy of the method is verified on a continuous basis by participation in a proficiency testing scheme organised by BIPEA (Gennevilliers, France). The rate of satisfactory z-scores of $-2 < z < 2$ is >96% for the 2200 results submitted so far. The limit of detection (LOD) and quantification (LOQ) of OTA and OTα are 0.08 µg/kg and 0.28 µg/kg respectively while for OTB, LOD was 0.12 µg/kg and LOQ was 0.39 µg/kg.

2.6. Statistical analyses

The data sets were analysed with Statistica 14.0.1 and R 4.4.1 software. The normal distribution of data was examined with the normal plots of the residuals. When data failed the normality test, data were transformed to square root or logarithm values to achieve normality. Transformed data were normally distributed; therefore, factorial ANOVA was used to analyze the effect interactions between the storage conditions on respiration rate and ochratoxin concentrations. The Tukey Honest Significant Difference (HSD) post hoc test evaluated differences among means. Statistical analyses performed were considered significant when p values were < 0.05. The concentrations of ochratoxins below the limit of detection and quantitation were assigned with values of LOD/2 and LOQ/2, respectively, for statistical analysis (EFSA, 2018). A linear regression model was done in R software on transformed data to examine the relationship between CO₂ and OTA concentrations in both wheat treatments.

3. Results

3.1. Visual images of contaminated grains

The visual representation of *Penicillium* contamination in wheat grains is illustrated in Fig. 1.

3.2. Fungal populations in contaminated grains

Penicillium populations in both contaminated wheat were significantly ($p < 0.05$) impacted by the interactions between a_w x temperature, a_w x treatment, a_w x temperature x treatment, and a_w x days x treatment as shown in Fig. 2. However, temperature and days as a single or combined factor do not significantly affect the *Penicillium* populations.

In the naturally contaminated wheat at 15 °C *Penicillium* populations were high at 0.95 a_w but significantly decreased with temperature rise. Meanwhile, at 20 °C, *Penicillium* populations were highest at 0.90 a_w . In the inoculated wheat, *Penicillium* populations were highest at 0.90 a_w at



Fig. 1. Images showing contaminated wheat on day 20 with *Penicillium* growth at (a) 0.90 a_w and (b) 0.95 a_w . Grains at drier levels, 0.70 and 0.80 a_w , showed no visible mould growth.

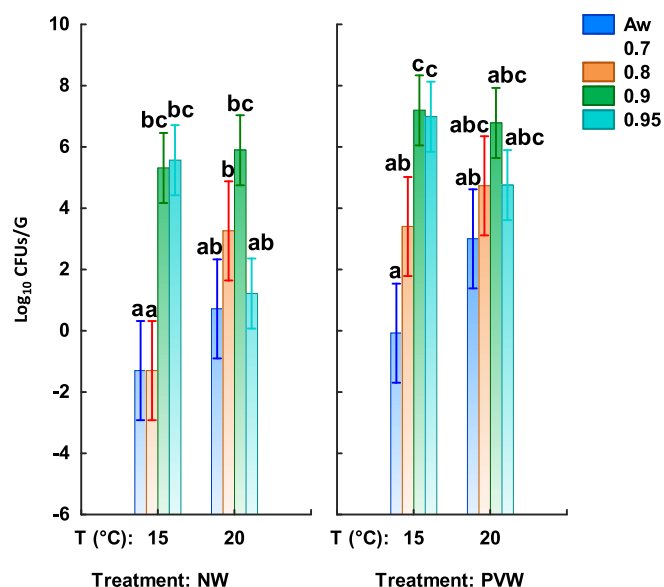


Fig. 2. The influence of water activity, temperature, and treatment interactions on *Penicillium* populations in naturally contaminated and inoculated wheat on Malt Extract Agar media. Different letters indicate significant differences in populations using the Tukey HSD test. CFUs/G- colony forming units per gram. A_w - water activity. T- Temperature. NW- naturally contaminated wheat. PVW- *P. verrucosum* inoculated wheat.

both temperatures. A similar trend in population pattern was observed in both wheat at 20 °C. The mean values of the colony-forming units and the frequency of isolation of *Penicillium* in both wheat treatments and media are in Table 1a and 1b.

3.3. CO₂ production in contaminated wheat

3.3.1. Respiration rates in wheat

The respiration rates (RR) were steady for the first 4 days in the naturally contaminated wheat at 0.90 a_w while in the inoculated wheat, an increase in RR was observed after day 1 at both temperatures (Figs. 3a and b). As the temperature and water activity level of grains increased, RR increased. RR at 0.90 a_w significantly differed between both treatments throughout the storage experiment. RR at 0.95 a_w was significantly different in both treatments for the first 8 days at 15 °C and the first 3 days at 20 °C. The mean values of respiration rates (mg CO₂ kg⁻¹ h⁻¹) in contaminated wheat at all storage conditions are shown in Table 2 while its graphical representation is shown in Fig. A.1 in the supplementary data.

Table 1a

Mean values ($n = 6 \pm$ S.E) of *Penicillium verrucosum* populations (Log₁₀ CFUs/G) in contaminated grains at all storage conditions.

Grains	T (°C)	Days	0.70 a_w		0.80 a_w		0.90 a_w		0.95 a_w	
			MEA+	DG18+	MEA+	DG18+	MEA+	DG18+	MEA+	DG18+
NW	15	10	0	0	0	2.6	4.3 ± 4.1	3.8 ± 3.6	6.9 ± 6.5	6.9 ± 6.4
		20	0	0	0	0	6.4 ± 4.9	6.2 ± 5.7	4.5 ± 4.4	4.5 ± 4.4
	20	10	0	2.6 ± 2.5	3.3 ± 3.2	5.2 ± 5	5.4 ± 4.9	5.5 ± 5.4	2.3 ± 2.6	0
		20	2.8 ± 2.3	3.1 ± 3.1	5.4 ± 5.1	5.0 ± 4.9	6.4 ± 4.9	6.3 ± 5.8	4.6 ± 4.6	4.6 ± 4.6
PVW	15	10	3.3 ± 3.2	0	4.6 ± 3.3	4.9 ± 4.2	7.0	7.0 ± 5.3	6.9 ± 6.2	7.0 ± 6.4
		20	0	0	5.5 ± 5.4	5.3 ± 5.2	7.4 ± 6.8	7.3 ± 6.0	7.4 ± 7.3	7.2 ± 7.1
	20	10	3.1 ± 2.6	2.9	4.5 ± 4.4	4.6 ± 4.4	6.9 ± 6.3	6.0 ± 5.3	6.1 ± 6.0	6.2 ± 6.1
		20	3.0 ± 2.3	2.3 ± 2.3	5.3 ± 4.7	5.2 ± 4.1	7.0 ± 6.9	7.1 ± 6.9	7.1 ± 6.9	7.2 ± 7.1

S.E: Standard Error. NW: Naturally contaminated wheat. PVW: *Penicillium verrucosum* wheat. T: Temperature. a_w : water activity. DG18+: Dichloran Glycerol 18 agar + Chloramphenicol. MEA+: Malt Extract Agar + Chloramphenicol.

3.3.2. The influence of storage conditions on respiration rates in contaminated wheat

Respiration rates significantly increased with the rise in water activity and differed substantially between wheat treatments. Respiration rates were more than seven times higher at wetter conditions than at drier a_w levels. The interactions between a_w x temperature, a_w x treatment, a_w x temperature x treatment, and a_w x days x treatment significantly affected the respiration rates in both wheat treatments (Fig. 4).

3.4. The influence of storage conditions on Ochratoxins production in contaminated wheat

The mean values of all ochratoxin concentrations in the contaminated wheat at all storage conditions are in Table 3.

3.4.1. Ochratoxin A

The concentrations of OTA in the wheat were significantly affected by water activity and storage days. Various interactions significantly influenced OTA concentrations except for the interactions between days x treatment, temperature x days x treatment, and a_w x temperature x days x treatment. In the naturally contaminated wheat, OTA concentrations were highest at 0.95 a_w at 15 °C with mean values of 1592 µg/kg but significantly decreased as temperature increased to 20 °C. However, the highest OTA concentrations in the inoculated wheat were observed at 0.90 a_w at 20 °C. OTA concentrations were generally low at drier conditions of 0.70 and 0.80 a_w at both temperatures in both wheat treatments as shown in Fig. 4.

3.4.2. Ochratoxin B

Water activity, storage days, and treatment as individual factors impacted on OTB concentrations. The interaction a_w x temperature, a_w x days, a_w x treatment, temperature x treatment, a_w x temperature x treatment, and a_w x days x treatment influence OTB production in the contaminated wheat. OTB concentrations were highest (60 µg/kg) at 0.95 a_w at 15 °C on day 20 in the naturally contaminated wheat with a significant reduction (15 times) as temperatures increased to 20 °C at the same a_w level and storage day. The highest concentrations of OTB were observed in the inoculated wheat at 0.90 a_w for both temperatures. The mean concentration of OTB was 16 µg/kg at 15 °C, which increased seven times when the temperature changed to 20 °C at the same water activity level (Fig. 4).

3.4.3. Ochratoxin alpha

All storage conditions (a_w , days, temperature, and treatment) as single factors significantly impacted the concentrations of Ochratoxin alpha in the contaminated wheat. The interactions between the storage conditions also influence OTα concentrations except for the interactions between temperature x days, temperature x treatment, days x treatment, and a_w x temperature x treatment. The Tukey HSD post hoc test showed that the highest concentration of OTα observed at 0.95 a_w at 15 °C on day 20 (61 µg/kg) was significantly different from OTα concentrations at

Table 1b
Frequency of isolation (%) of *Penicillium verrucosum* in contaminated grains at all storage conditions.

Grains	T (°C)	Days	0.70 a _w		0.80 a _w		0.90 a _w		0.95 a _w	
			MEA+	DG18+	MEA+	DG18+	MEA+	DG18+	MEA+	DG18+
NW	15	10	8	6	10	6	98	100	88	100
		20	2	8	10	18	100	100	0	34
	20	10	6	18	18	24	100	100	22	0
		20	22	22	98	62	100	100	34	48
PVW	15	10	100	82	100	100	100	100	100	100
		20	100	100	100	100	100	100	78	100
	20	10	96	98	100	100	96	98	0	50
		20	100	63	100	100	58	62	26	46

NW: Naturally contaminated wheat. PVW: *Penicillium verrucosum* wheat. T: Temperature. a_w: water activity. DG18+: Dichloran Glycerol 18 agar + Chloramphenicol. MEA+: Malt Extract Agar + Chloramphenicol.

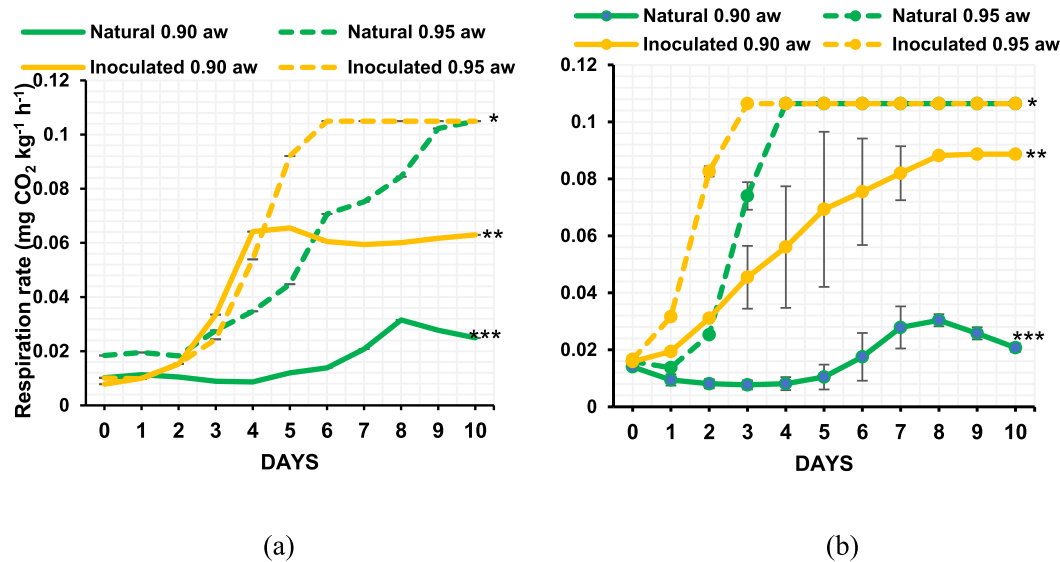


Fig. 3. The respiration rates in contaminated wheat for the first 10 days at 0.90–0.95 a_w for both treatments at (a) 15 °C and (b) 20 °C. a_w- water activity. Natural- naturally contaminated wheat. Inoculated- *P. verrucosum* inoculated wheat.

Table 2
Mean values (n = 2) of respiration rates (mg CO₂ kg⁻¹ h⁻¹) in contaminated wheat.

Grains	T (°C)	Days	0.70 a _w	0.80 a _w	0.90 a _w	0.95 a _w
Naturally contaminated wheat	15	0	0.00087	0.00074	0.00548	0.01458
		10	0.00163	0.00124	0.01354	0.10669
		20	0.00158	0.00129	0.01356	0.10669
	20	0	0.00224	0.00494	0.01402	0.01587
		10	0.00123	0.00830	0.02077	0.10641
		20	0.00184	0.00863	0.01611	0.11137
<i>Penicillium verrucosum</i> inoculated wheat	15	0	0.00197	0.00387	0.00781	0.00176
		10	0.00798	0.02237	0.06299	0.10842
		20	0.00889	0.01390	0.08409	0.10838
	20	0	0.00307	0.00534	0.01587	0.01666
		10	0.00238	0.01325	0.08868	0.10641
		20	0.00478	0.01749	0.09141	0.11108

T- Temperature. a_w- water activity.

other storage in the naturally contaminated wheat.

In the inoculated wheat, only temperature significantly impacted the concentrations of OTα. OTα concentrations were mostly below LOD with the highest concentration (2 µg/kg) at 0.90 and 0.95 a_w at 20 °C.

3.5. Relationship between respiration rates and OTA in wheat

The relationship between CO₂ respiration rates and OTA in the

contaminated wheat was examined with a linear regression model with adjusted R² = 0.6108 which indicates a moderate prediction. Among all the storage parameters used in the model, CO₂ and Treatment PVW are strong predictors (with p values of <0.05) of ochratoxin concentrations at different water activity levels (Fig. 5). The model was cross-validated by plotting the predicted OTA values against the known ones (Fig. A.2 in supplementary data). The linearity of the plot was not satisfactory. Other regression models such as a generalized linear model and a logistic regression model were performed but were not satisfactory. We attribute this to the non-measurable values (< LOD) of OTA concentrations at drier a_w levels (0.70–0.80 a_w). However, high OTA concentrations were observed at higher a_w (0.90–0.95 a_w) with higher respiration rates having a positive and significant correlation coefficient (r) of 0.76. It is therefore important to stress the influence of other storage conditions on the relationship between CO₂ production and OTA concentrations in the wheat. The correlations between CO₂, *Penicillium* populations, and all ochratoxin concentrations are shown in Table A.1 in the supplementary data.

4. Discussion

Moulds and grains respire, producing CO₂, which has been used as an early indicator of mould growth and mycotoxin contamination in stored grains (Garcia-Cela et al., 2018; Mylona et al., 2012). However, the use of CO₂ production to predict the early risk of ochratoxin contamination in wheat under different storage environments is not fully documented.

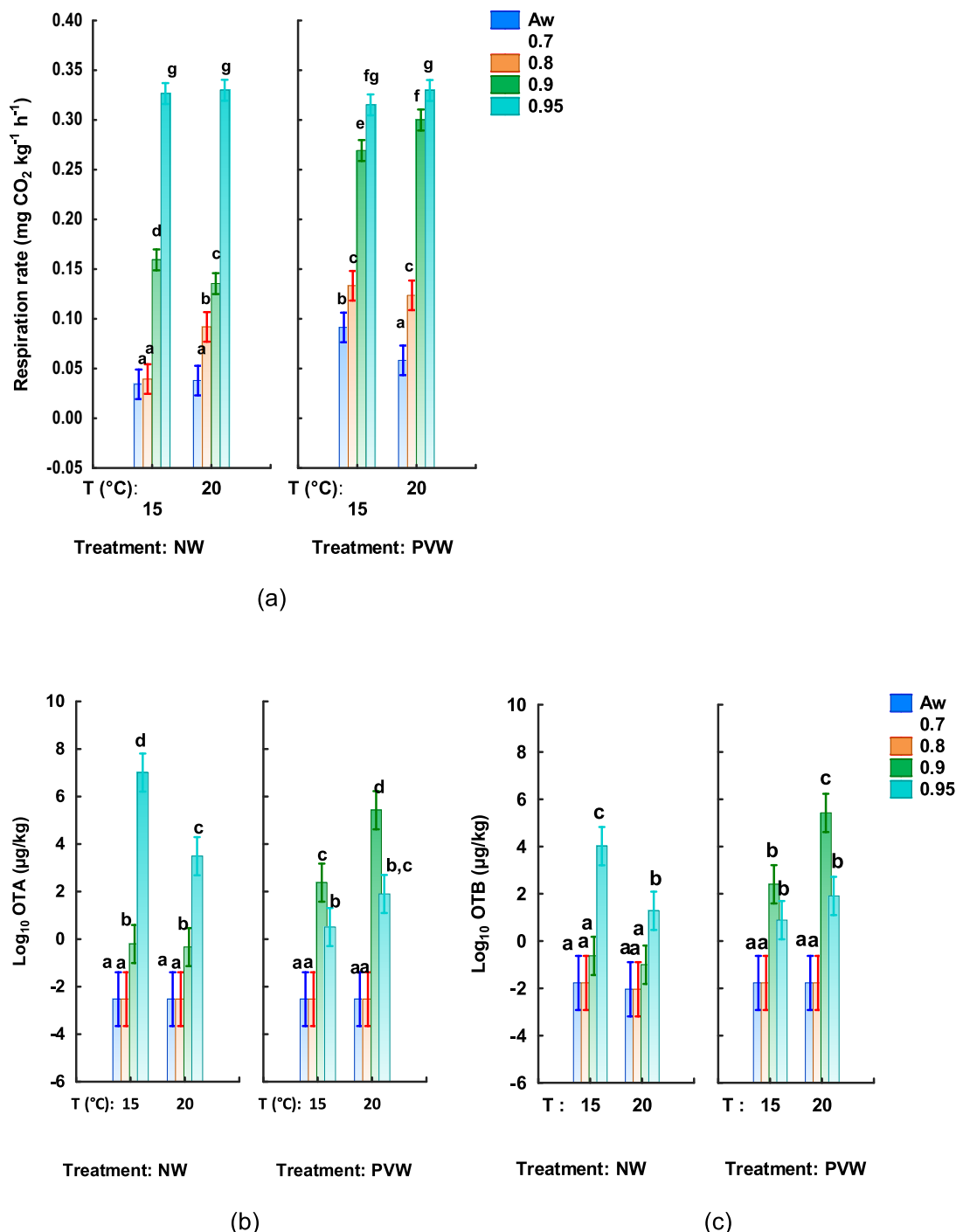


Fig. 4. The influence of water activity, temperature, and treatment interactions on respiration rates, ochratoxins concentrations in contaminated wheat. Different letters show significant differences in the mean of respiration rates and ochratoxins concentrations at each storage condition using the Tukey HSD test. A_w - water activity. T- Temperature. NW- naturally contaminated wheat. PVW- *P. verrucosum* inoculated wheat.

This study employed the use of CO₂ sensors to measure the real-time respiration produced in wheat, examining the prediction of OTA production in wheat stored at 0.70, 0.80, 0.90, 0.95 a_w at 15, and 20 °C to determine the storage conditions where OTA concentrations exceed the legislative limit. The specified water activity levels correspond to the wheat moisture content of 14%, 16%, 22%, and 25% (wet weight basis) (Chulze et al., 2020). These storage conditions were selected as they are commonly used for storing cereals in temperate zones and promote fungal growth in stored grains.

Water activity and temperature are critical factors that affect mould

growth and mycotoxin contamination in foods. Their impacts on the growth of different microbes especially fungi in various grains have been widely studied (Mannaa & Kim, 2017; Oluwakayode et al., 2024; Peter Mshelia et al., 2020; Wawrzyniak et al., 2018). As a xerophilic fungus, *Penicillium* can thrive below <0.85 a_w , although in temperate climates, it can grow as high as 0.95 a_w . Nevertheless, hydrophilic fungi could outgrow it above 0.95 a_w (Kaneekar & Kaneekar, 2022; Magan & Lacey, 1984; Mannaa & Kim, 2017).

This study shows a significant interaction between water activity and temperature on *Penicillium* populations in naturally contaminated

Table 3Mean values ($n = 4$) of Ochratoxins concentrations in contaminated wheat.

Treatment	T (°C)	a_w	OTA ($\mu\text{g/kg}$)		OTB ($\mu\text{g/kg}$)		OT α ($\mu\text{g/kg}$)	
			DAY 10	DAY 20	DAY 10	DAY 20	DAY 10	DAY 20
Naturally contaminated wheat	15	0.7	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		0.8	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		0.9	< LOD	17	< LOD	5	< LOD	< LOD
		0.95	873	1593*	60	61	< LOD	61
	20	0.7	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		0.8	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		0.9	< LOQ	6	< LOD	1	0.4	< LOD
		0.95	47	25	4	4	8	7
<i>Penicillium verrucosum</i> inoculated wheat	15	0.7	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		0.8	0.4	< LOD	< LOQ	< LOD	< LOD	< LOD
		0.9	7	331*	0.8	16*	< LOD	< LOD
		0.95	< LOD	54*	< LOD	3	< LOD	< LOD
	20	0.7	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		0.8	< LOD	< LOD	< LOD	1.3	< LOD	< LOD
		0.9	181	1091*	36	126*	0.6	2
		0.95	10	7	< LOQ	0.4	< LOD	2

OTA- Ochratoxin A. a_w - water activity. T- Temperature. * represents significant differences (Tukey HSD test) in ochratoxins concentrations between days for each treatment in each row.

wheat. *Penicillium* populations increased with high water activity and peaked at 15 °C at 0.90–0.95 a_w with no statistical differences between both a_w levels, indicating a 62% increase compared to 0.70 a_w . In a previous study by Cairns-Fuller et al. (2005), a similar water activity level of 0.95 a_w was reported as the optimal a_w level for *Penicillium* growth in wheat grains, but at 25 °C. Another study reported the growth rate of ochratoxigenic *P. verrucosum* isolates on barley meal extract agar to be highest at 0.95 a_w at 30 °C (Pardo et al., 2006). Other xerophilic fungal species; *Aspergillus fresenii* and *Aspergillus sulphureus* were reported to grow optimally at 0.94 a_w and 30 °C on Nyjer seeds. (Gizachew et al., 2019). However, growth rates in these studies were monitored by mycelia measurement and not colony counts as used in our research.

To obtain a distinct dominance of *Penicillium* species at the start of the incubation period for adequate growth and OTA production, the wheat grains were inoculated with *P. verrucosum* spores, to have higher CFUs than other fungi on the grain. It was found that the *Penicillium* populations in the inoculated grains were not significantly higher compared to the naturally contaminated grains except at a few conditions (0.80 a_w at 15 °C and 0.95 at 20 °C). Populations increased by 58% at a wet condition of 0.90 a_w compared to populations at the drier condition of 0.70 a_w at 15 °C.

Ochratoxin A, an important storage mycotoxin has gained research attention over the years with more focus on OTA production by *Aspergillus* species, the main OTA producer in grapes and wine. Passamani et al. (2014) evaluated the levels of Ochratoxin A produced by *A. carbonarius* and *A. niger* obtained from Brazilian grapes grown in a grape medium in vitro. The study found that the highest concentrations of OTA were observed at 15 °C, 0.99 a_w , and pH 5.35. Another study shows that the highest concentrations of Ochratoxin A produced by two *Aspergillus* strains, *A. carbonarius*, and *A. niger* in maize kernels were observed at 15 °C despite the higher temperatures used in the study (Alborch et al., 2011). These studies indicate that OTA is optimally produced at a temperature of 15 °C and > 0.94 a_w . However, other researchers have reported that OTA can also be produced optimally at higher temperatures, ranging from 30 to 37 °C and > 0.90 a_w . A study by Pardo et al. (2004) reported the highest OTA concentrations at 0.99 a_w at 30 °C produced by three isolates of *A. ochraceus* in irradiated barley. Lahouar et al. (2017) found that *A. tubingensis* produced the highest OTA concentrations in sorghum seeds at 37 °C and 0.97 a_w .

Although storage/ incubation days did not significantly impact the *Penicillium* populations, it had a significant impact on the concentrations of OTA and its interactions with a_w and temperature. Some studies have reported that incubation times affect OTA production, although this is influenced by water availability or temperature changes and substrate

type. Chiotta et al. (2015) reported OTA production by *A. carbonarius* at 20 °C and 0.950–0.965 a_w , within 14 days of incubation in synthetic grape juice medium. Meanwhile, *A. ochraceus* produced the highest amount of OTA on Corinth's raisins on the 24th day of incubation (Kapetanakou et al., 2009) and both *A. niger* and *A. carbonarius* produced OTA from the 5th day of incubation in maize kernels (Alborch et al., 2011). The variation in OTA production time may be due to indigenous microflora found in various substrates which can either detoxify OTA or reduce its production. This study only analysed OTA levels on day 10 and day 20 and not on a series of days. The concentrations on day 10 were mostly below the limit of detection (LOD) and were significantly different from those on day 20. Due to variations in LOD values and fungal species, it is difficult to make direct comparisons between OTA concentrations in our study to those in other studies. Generally, at wet conditions (0.90–0.95 a_w), OTA concentrations were significantly higher than OTB and OT α concentrations. At, 15 °C, OTA concentrations were twenty-six times higher than its dechlorinated (OTB) and less potent variant (OT α) in the naturally contaminated wheat.

The byproducts of the respiration of grain and its natural microbiota are carbon dioxide, water vapor, and heat. Lower RR were observed in the wheat at drier conditions while higher water activity levels had higher RR. This was supported by the findings of Huang et al. (2013) and Dillahunty et al. (2000) on the effect of a_w on respiration rates in corn and rice respectively. The CO₂ sensors, used to measure carbon dioxide concentrations, have a maximum measuring capacity of 50,010 ppm. Once the limit was reached, the readings remained constant throughout storage. Hence, in both contaminated wheat, samples at the wettest water activity (0.95 a_w) had the same respiration rates on day 10 and day 20. During the initial phase of the experiment, the respiratory rate (RR) remained low and stable for a few days before increasing. However, the RR increased as the temperature and water activity level increased. This finding contradicts the observation of Raudienė et al. (2017), who reported high RR at the beginning of their experiment because the grains used were not equilibrated. Therefore, after grain rehydration, the RR was high due to water on the grain surface. However, as water infiltrated the grains, the respiration process decelerated and was influenced by alterations in temperature and water availability.

Surprisingly, *Penicillium* populations were negatively correlated with RR and Ochratoxin concentrations in the naturally contaminated wheat (Table A.1 in supplementary data). The highest RR was observed at 0.95 a_w at 20 °C where *Penicillium* populations and OTA concentrations were low. However, the increase in RR due to fungal activity indicates the start of grain deterioration which can be examined visually or with a stereoscope as shown in Fig. 1. The presence of other fungal genera

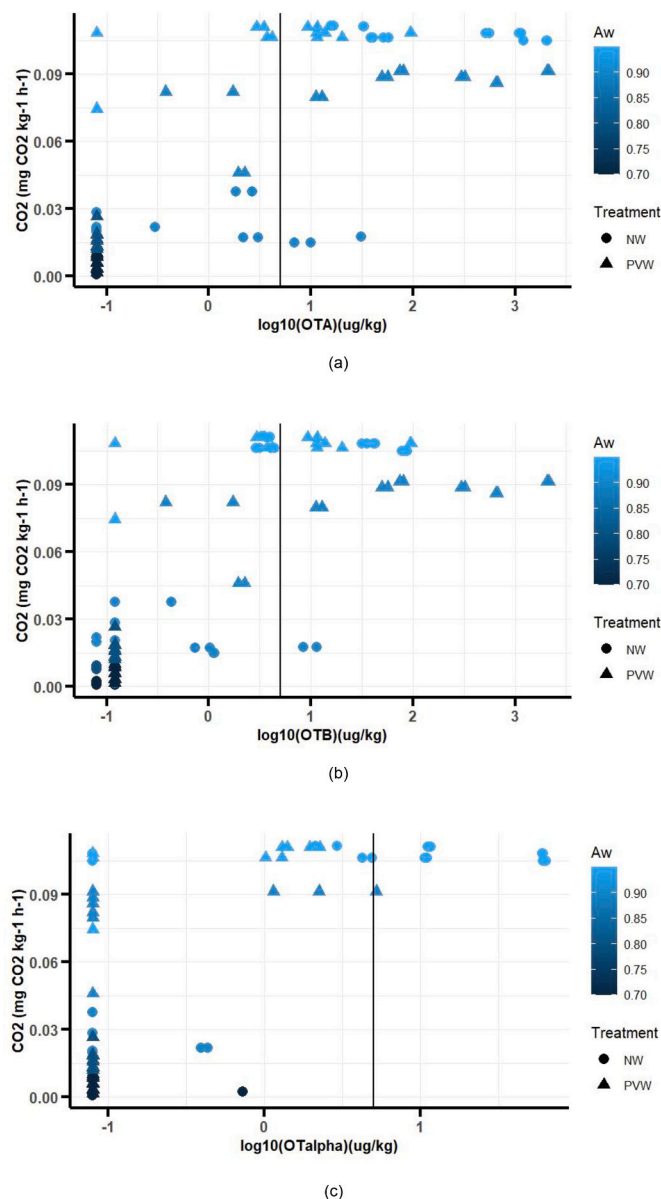


Fig. 5. The relationship between the respiration rates and ochratoxin concentrations with water activity in both wheat treatments. Vertical lines indicate maximum levels (5 $\mu\text{g/kg}$) of ochratoxin A concentrations.

found in the contaminated wheat grains; *Aspergillus*, *Fusarium*, *Aspergillus* section *Aspergillus* (previously called *Eurotium*), *Cladosporium*, *Mucor*, *Rhizopus*, and *Alternaria* may contribute to the increased RR. Other fungal genera were also impacted by changes in water and temperature in the stored wheat.

Temperature did not significantly impact the RR, but a significant difference exists between RR in both wheat treatments. Although the relationship between the CO₂ RR and ochratoxin concentrations at drier a_w levels was not fully established due to low measurable values ($< \text{LOD}$) of OTA, our observation is that, at a higher a_w level, RR was high and ochratoxin concentrations were high. Therefore, the knowledge of optimal and marginal levels of ecological factors including the CO₂ production within a grain silo could significantly improve the storage management of grains.

5. Conclusion

Grain quality can quickly deteriorate during storage due to

environmental factors such as moisture and temperature. If storage is not properly managed, mycotoxins can spread from a hotspot area to the entire grain mass in the silo. To monitor this, CO₂ sensors integrated with relative humidity (RH) and temperature sensors were installed in the wheat silos and coupled to a decision support system (DSS). This linked the real-time RR data to biological boundary models for moisture content and temperature conditions which were either favourable or unfavourable for the growth of *Penicillium verrucosum* and ochratoxin production in the wheat. This study found that CO₂ RR in stored wheat was significantly affected by changes in grain moisture and temperature. It also established the relationship between CO₂ RR and ochratoxin concentrations at the wettest conditions. Installing such real-time systems will allow the identification of the areas within silos that may represent hot spots and improve the pro-activeness of grain managers to take remedial action, to reduce food waste.

CRediT authorship contribution statement

Abimbola Oluwakayode: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Michael Sulyok:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Rudolf Krska:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Funding acquisition, Conceptualization. **Angel Medina:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2024.140590>.

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The effect of the interactions of water activity, and temperature on OTA, OTB, and OT produced by *Penicillium verrucosum* in a mini silo of natural and inoculated wheat using CO₂ production as fungal activity sentinel

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