Fungal Biology, Volume 125, Issue 2, February 2021, pp. 115-122
DOI: 10.1016/j.funbio.2019.11.001

Interacting climate change factors (CO_2 and temperature cycles): effects on growth, secondary metabolite gene expression and phenotypic ochratoxin A production by *Aspergillus carbonarius* strains on a grape-based matrix.

Carla Cervini[a,b] , Carol Verkeecke-Vaessen[c], Massimo Ferrara[a], Esther García-Cela[c], Donato Magistà[a], Angel Medina[c], Antonia Gallo[d], Naresh Magan[c], Giancarlo Perrone[a]

[a]*Institute of Sciences of Food Production (ISPA) National Research Council (CNR), Bari, Italy*

[b]*Department of Economics, University of Foggia, Foggia, Italy*

[c]*Applied Mycology Group, Environment and AgriFood Theme, Cranfield University, Cranfield, UK*

[d]*Institute of Sciences of Food Production (ISPA) National Research Council (CNR), Lecce, Italy*

Corresponding Author:

Email address*:* carla.cervini@ispa.cnr.it

ABSTRACT

Little is known on the impact that climate change (CC) may have on *Aspergillus carbonarius* and Ochratoxin A (OTA) contamination of grapes, especially in the Mediterranean region - a hot spot for the impact of CC scenarios with temperature expected to increase by +2-5°C and CO_2 to double or triple (400 vs 800/1200 ppm). This study examined the effect of (i) current and increased temperature in the alternating 11.5h dark/12.5h light cycle (15-28°C vs 18-34°C), representative of the North Apulia area, South Italy and (ii) existing and predicted CO_2 concentrations (400 vs 1000 ppm), on growth, expression of biosynthetic genes (*AcOTApks*, *AcOTAnrps*, *AcOTAhal, AcOTAp450*, *AcOTAbZIP*) and regulatory genes of Velvet complex (*laeA*/*veA*/*velB*, "velvet complex") involved in OTA biosynthesis and OTA phenotypic production by three strains of *A. carbonarius*. The experiments made on a grape-based matrix showed that elevated CO_2 resulted in a general stimulation of growth and OTA production. These results were supported by the up-regulation of both structural and regulatory genes involved in the OTA biosynthesis in elevated CO_2 condition. Our work has

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The final published version (version of record) is available online at DOI:10.1016/j.funbio.2019.11.001. Please refer to any applicable publisher terms of use.

shown for the first time that elevated CO_2 concentration in the Mediterranean region may result in an increased risk of OTA contamination in the wine production chain.

KEYWORDS: black aspergilli, alternating temperatures, carbon dioxide, mycotoxins, ecophysiology, biosynthetic genes.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus* and *Penicillium* species (Frisvad et al., 2004a,b) responsible for contaminating many foodstuffs including cereals (Scudamore et al., 2004), coffee (Taniwaki et al., 2003), cocoa (Bonvehí, 2004), grapes and derivatives (Abrunhosa et al., 2001; Varga and Kozakiewicz, 2006; Zimmerli and Dick, 1996). Many studies have shown that the *Aspergillus* section *Nigri*, commonly known as "Black Aspergilli", are responsible for OTA contamination of grapes (García-Cela et al., 2016; Palumbo et al., 2011; Battilani et al., 2003). In particular, *A. carbonarius* is considered the major species responsible, with other closely related biseriate species, *A. niger* and *A. welwitschiae* of less importance (Perrone et al., 2008; Cabañes and Bragulat, 2018). OTA is a potent nephrotoxin, classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC, 1993). Based on toxicological and exposure data, the European Commission, has established maximum levels for the presence of OTA in wine, fruit wine, grape juice, grape nectar and grape must for human consumption (2 $\mu g \cdot kg^{-1}$) and dried vine fruits (10 $\mu g \cdot kg^{-1}$) (EC, 2006/1881). OTA contamination of grapes is strongly related to plant phenology, geographical area, meteorological conditions/microclimate and varies from season to season (Visconti et al., 2008; Somma et al., 2012). Indeed, climate represents the key-factor in the agro-ecosystem that influences fungal colonization and mycotoxin production in crops (Magan et al., 2003). Based on the 5th Report of the Intergovernmental Panel on Climate Change (CC) (IPCC, 2014), anthropogenic Green House Gases (GHG) emissions have increased since the pre-industrial era, driven by economic and population growth. Their effects, have been one of the dominant causes of the observed global warming since the mid-20th century. Based on recent prediction model, atmospheric concentration of CO_2 is expected to double or triple (from 400 to 800-1200 ppm) and temperature to increase (+2-5°C), in the next 25-50 years.

Such CC scenarios are projected to have a profound effect on the productivity and quality of crop and livestock production world-wide exacerbating food insecurity especially in areas vulnerable to hunger and undernutrition (Wheeler et al., 2013). In addition, food safety could be compromised, with mycotoxins being one of the most important hazards to be most likely affected by CC (Miraglia et al., 2009; Medina et al., 2017). In this regard, numerous studies based on the development of

predictive models (Van Der Fels-Klerx et al., 2012, Van Der Fels-Klerx et al., 2016; Battilani et al., 2012; Battilani et al., 2016) have tried to estimate the changes in risks of mycotoxin contamination of staple crops due to possible CC scenarios. These studies mainly focused on an increase in temperature of +2 or +5°C in Europe and the related changes in the contamination of wheat by deoxynivalenol (DON) (Van Der Fels-Klerx et al., 2012) and of maize by aflatoxin B_1 (AFB_1) (Battilani et al., 2012). However, such studies did not include an evaluation of CO_2 increasing level and the interactions between temperature and elevated CO_2. A study by Medina et al. (2015) showed that interactions between increased temperature (+4°C), elevated CO_2 (350 vs 650 or 1000 ppm CO_2) and drought stress had little effect on growth but significant effects on AFB_1 production in *A. flavus*, as confirmed by gene expression analysis of some structural and regulatory genes involved in toxin biosynthesis. A similar study has been carried out on *A. carbonarius* in relation to changes in temperature and elevated CO_2 on growth and OTA production by Akbar et al. (2016). They found that on coffee-based media and stored coffee beans there was more impact of CC on OTA production in *Aspergillus westerdijkiae* than in *A. carbonarius*. However, no detailed studies have been carried out in relation to grape matrices and different day/night temperature cycles.

An examination of the relative risk of OTA contamination of grapes by the European Commission (2002) reported that there was an increasing gradient of the incidence and OTA levels from Northern (50.3% and 0.18 $\mu g \cdot kg^{-1}$) to Southern (72.3% and 0.64 $\mu g \cdot kg^{-1}$) European countries. Similarly, wines produced in Southern Italy, where climatic conditions favor the growth of OTA-producing fungi in grapes, has generally resulted in higher OTA contamination levels (Visconti et al., 2008) than those produced in Central and Northern Italy. Moreover, in Apulia region (Southern Italy) higher OTA contamination levels have been reported in red wines produced in Southern area (e.g. Primitivo, Negramaro) historically more affected by the presence of OTA than those produced in the Northern area (e.g. Nero di Troia, Aglianico) (Perrone et al., 2007).

In our opinion, the understanding of whether changes in CC scenarios could increase the risk of OTA contamination is of crucial interest in this important wine producing region of Italy. Indeed, the objectives of this study were to examine the effect of two different temperature cycles (15-28 vs 18-34°C, 11.5h/12.5h dark/light), representative of the North Apulia, which is an area not yet affected by severe OTA contamination levels, and interaction with existing and future CO_2 exposure concentrations (400 vs 1000 ppm) on (a) growth rate, (b) gene expression of toxin biosynthesis related genes (*AcOTApks, AcOTAnrps, AcOTAhal, AcOTAp450, AcOTAbZIP)* and the regulatory "Velvet complex" (*laeA/veA/velB*), and (c) phenotypic OTA production, in different strains of *A. carbonarius*.

2. Materials and methods

2.1. Fungal strains

Three OTA-producing strains of *A. carbonarius* (ITEM 7444, ITEM 5010 and ITEM 18515) isolated from Italian wine grapes from Apulia region and held in the Agri-Food Toxigenic Fungi Culture Collection of the Institute of Sciences of Food Production, CNR, Bari (www.ispa.cnr.it/Collection), were selected.

2.2. Media preparation and inoculation

A basal Grape Juice Medium (GJM; 0.99 water activity (a_w)) was prepared by diluting (50% v/v) a commercial pasteurized grape juice (composition per 100 mL: fat, 0.02 g; proteins, 0.3 g; carbohydrates, 16.10 g) with distilled water and supplemented with 1.2% agar (Ioannidis et al., 2015). The a_w of GJM was measured using the AquaLab 4TE water activity meter. The pH of the medium was adjusted to 3.5 using 1M KOH.

Fungal inocula were prepared from fungal colonies grown on Potato Dextrose Agar (PDA) plates for 7 days at 25°C. A sterile loop was used to dislodge conidia and place them into 9 mL of sterile water containing a 0.05% Tween 80 in a 25 mL glass Universal bottle. The concentration was quantified with a haemocytometer and diluted with sterile water to obtain a conidial suspension of 10^5 conidia·mL^{-1} for each *A. carbonarius* strain. Each conidial suspension was used to centrally inoculate the GJM plates previously overlaid with sterile cellophane membranes (8.5 cm diameter). Fungal mycelium was harvested by scraping cellophane membrane surfaces after 4 and 7 days post inoculation (dpi), and stored at -20°C for OTA content analysis or -80°C for RNA extraction. Experiments were carried out with three replicates per treatment and repeated twice.

2.3. Incubation conditions and CO_2 treatment

The *in vitro* agar cultures were placed in 12-L air tight containers and incubated in a dynamic climatic chamber (SANYO Electric Co., Ltd., MLR-350H) at two different 11.5h/12.5h dark/light temperature cycles simulating nowadays (15-28°C) and CC scenario (18-34°C) of the Northern area of the Apulia region. The containers included a beaker (500 mL) of water in order to minimize moisture loss. They were flushed twice a day either with current (400 ppm) or increased (1000 ppm) CO_2 concentrations by using specialty grade mixtures gas cylinders (British Oxygen Company, Guildford, Surrey, UK), with a flow rate of 3 L·min^{-1} in order to renew 3x the air volume of the incubation chambers (Verheecke-Vaessen et al., 2019).

2.4. Growth measurements

Fungal growth was assessed daily until 7 dpi and expressed as the average measurement (mm) of two orthogonal diameters of centrally inoculated colonies. The colony diameters (mm) were plotted against time (days) and the linear regression model was used for calculating the growth rate ($mm \cdot day^{-1}$) and the lag phase prior to growth (hours). The square of the linear correlation coefficient was ≥ 0.98.

2.5. RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from frozen mycelium by using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St Louis, Missouri, U.S.A.), according to the manufacturer's instructions. RNA concentration was determined by using Genova Nano (Jenway, Stone, UK) and its integrity was checked on 1% agarose gel. First strand cDNA was synthesized using 1 µg of total RNA with the Omniscript® Reverse Transcription kit (Qiagen, Hilden, Germany), following the manufacturers' instructions.

The expression levels of the biosynthetic (*AcOTApks, AcOTAnrps, AcOTAhal, AcOTAp450, AcOTAbZIP*) and regulatory "Velvet complex" (*laeA/veA/velB*) genes of *A. carbonarius* were analyzed by using quantitative reverse transcription-PCR (qRT-PCR). The *β-tubulin* gene was used as an internal reference. Nucleotide sequences of primers used in the qPCR assays are shown in the Table S1. qPCR reactions were performed using the Viia 7 Real Time PCR (Thermo Fisher Scientific, Waltham, USA) system programmed to hold at 50°C for 2 min and at 95°C for 2 min and to complete 40 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s. Specificity of the PCR amplifications was confirmed by dissociation curve analysis and qPCR efficiency of each oligonucleotide pair was calculated from each linear regression of standard curves. Real time PCR experiments were conducted in 96-well plates by using SYBR Select 2x MasterMix (Thermo-Fisher Scientific, Waltham, USA) and different concentrations of primers pairs, as reported in Table S1, for each gene. Data analysis was assessed using QuantStudio™ RT-PCR Software (Applied Biosystems, version 1.3).

Relative gene expression was assayed in triplicate for each individual biological replicate. The relative quantification of gene expression was established using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schimittgen, 2001) and fold changes at 1000 ppm were expressed as $\log_2 (2^{-\Delta\Delta CT})$. Significant differences between fold changes at 1000 ppm vs 400 ppm (control) for each gene, were assessed by using Sidak's multiple comparison test (Table S4).

2.6. Ochratoxin A analysis

For the quantification of OTA in fungal cultures, HPLC-FLD analysis was performed. About 100 mg of freeze-dried mycelium were extracted with 2 mL of a mixture of methanol:acetonitrile:water (30:30:40 v/v/v) by shaking for 2 h at room temperature. After extraction, samples were centrifuged for 15 min at 4500 rpm and 600 µL of supernatant were diluted with 400 µL of acetonitrile:water:acetic acid (35.0:62.5:2.5 v/v/v) and vortexed for 30 s. The diluted extract was filtered (0.22 µm) and 100 µL were injected into the HPLC-FLD apparatus. The mobile phase was an isocratic mixture of acetonitrile:water (45:55 v/v) containing 1% acetic acid. The HPLC-FLD apparatus was a 1100 series liquid chromatography (LC) system comprising a binary pump, an autosampler, and a fluorescence detector (excitation wavelength, 333 nm; emission wavelength, 460 nm) from Agilent Technologies (Waldbronn, Germany). The column used was a Zorbax Eclipse Plus C18, 150 mm × 4.6 mm, 3.5 µm particles (Agilent, USA). The flow rate of the mobile phase was 1 $mL \cdot min^{-1}$, with a run of 15 minutes. The retention time for OTA was 9.5 minutes. The standard solution of OTA in acetonitrile were purchased from Romer Labs Diagnostic GmbH (Tulln, Austria). Calibration solutions of OTA were prepared in HPLC mobile phase, and calibration curves were prepared in the range of 3.9 $ng \cdot mL^{-1}$ to 1000 $ng \cdot mL^{-1}$. The volume of injection was 10 µL both for the samples and the standards. The limit of detection and quantification were 1.50 $ng \cdot g^{-1}$ and 5 $ng \cdot g^{-1}$, respectively.

2.7. Data analysis

Statistical analyses were performed using GraphPad Software (version 7.0, Prism, La Jolla California, USA). After checking the normal distribution of datasets using the Shapiro-Wilk test, the Analysis of Variance (ANOVA) was performed in order to test the effect of each single variable (CO_2, temperature) and their interaction (temperature x CO_2). Furthermore, significant differences among the means were established by using Tukey HSD *post hoc* test ($p \leq 0.05$).

3. Results

3.1. Growth measurements

Table 1 shows the effect of temperature cycles and exposure to either 400 or 1000 ppm CO_2 on the lag phases (h) prior to growth and the relative growth rates ($mm \cdot day^{-1}$) of the three *A. carbonarius* strains. Overall, there was a reduction in the lag phases when 2.5x higher CO_2 concentration was imposed. Under the combined effect of the temperature cycle of 15-28°C x 1000 ppm, the lag phases of two strains (ITEM 7444, ITEM 18515) were significantly shorter than those observed in the 15-28°C cycle x 400 ppm. The ANOVA analysis (Table S2) showed that CO_2 variation significantly impacted on lag phases prior to growth of all the three *A. carbonarius* strains,

while the temperature cycle and its interaction with CO_2 had a significant effect only on the lag phases of the strain ITEM 7444.

Data on the relative growth rates (see Table 1) showed that the three *A. carbonarius* strains behaved similarly and the exposure to increased CO_2 concentration (1000 ppm) resulted in faster growth compared to the current CO_2 level (400 ppm). In addition, when the temperature cycle was elevated to 18-34°C, growth rates were significantly higher than those observed in the 15-28°C cycle. The different ecophysiological behavior of *A. carbonarius* strains, both in terms of growth and sporulation, under the combined effect of two temperature cycles and CO_2 air concentrations, was evident on 7 days centrally inoculated plates (Figure 1). Statistical analyses showed that single factors (temperature cycle, CO_2) had a significant impact on the growth of all three strains, while their interaction (temperature cycle x CO_2) did not have a significant effect (Table S2).

3.2. Gene expression and ochratoxin A production

Figure 2 shows the effect of interacting CC factors on the expression of biosynthetic and regulatory genes in the three *A. carbonarius* strains when grown on a grape-based substrate after 4 days incubation. This time point was chosen on the basis of previous studies on the correlation between kinetics of OTA production and transcription levels of OTA biosynthetic genes in *A. carbonarius* (Gallo et al., 2014; Ferrara et al., 2016). Indeed, they reported that genes' activation occurs in the earlier days of incubation and precede the later increase of OTA production. This finding was also reported in *Penicillium nordicum*, another ochratoxin A producer (Geisen et al., 2004).

In elevated CO_2 condition (1000 ppm) and in the 15-28°C temperature cycle a general upregulation of the synthetic OTA genes (*AcOTApks*, *AcOTAnrps*, *AcOTAhal*, *AcOTAp450*, *AcOTAbZIP*) was observed. In particular, for the *A. carbonarius* strain ITEM 5010, there was an increased expression of the gene *AcOTAnrps* exhibiting almost a 5-fold higher transcription level. Under this interacting condition, the gene coding for a *bZIP* transcription factor, was also more than 2-fold overexpressed. This occurred in all three strains examined.

In addition, in the 15-28°C cycle x 1000 ppm CO_2, the regulatory velvet complex genes, *laeA*, *veA* and *velB,* resulted upregulated, with the same trend observed for the key OTA cluster biosynthetic genes. Interestingly, the *veA* gene was a 5-fold upregulated in strain ITEM 7444.

Under the higher 18-34°C temperature cycle, a more variable pattern in the transcriptional profile of all the genes occurred. Under this growth condition, the main OTA cluster genes (*AcOTApks*, *AcOTAnrps*, *AcOTAhal)* were upregulated only in strain ITEM 5010. With regard to the Velvet complex genes (*laeA*/*veA*/*velB*), their expression levels were almost unchanged in all three *A.*

carbonarius strains except for the *veA* gene in strain ITEM 7444 strain which showed a >3-fold upregulation in the 18-34°C cycle x 1000 ppm CO_2 treatment.

Figure 3 compares the temporal effect of both temperature cycle x CO_2 exposure concentrations on the OTA production by the three *A. carbonarius* strains. Although the three strains behaved similarly under the different interacting CC factors, intra-species differences in the amounts of OTA detected were observed, with the ITEM 18515 strain being a much higher toxin producer than the other two strains examined. Furthermore, the OTA concentration produced was higher after 4 dpi than after 7 dpi.

In general, higher OTA production occurred in the elevated CO_2 concentration (1000 ppm) treatment, especially in the 15-28°C temperature cycle. In this scenario after 4 dpi, each strain produced higher amounts of OTA, which was significantly different from that detected in current CO_2 (400 ppm) conditions for two out of three strains (ITEM 7444, ITEM 5010).

In current CO_2 conditions (400 ppm), no differences were found between the two temperature cycles (15-28°C vs 18-34°C) tested for all three *A. carbonarius* strains. Statistical analysis (Table S3) shows that the temperature cycles significantly influenced OTA production only in strains ITEM 5010 and ITEM 18515, while CO_2 variation and the interaction between CO_2 x temperature cycle had a significant impact on all three *A. carbonarius* strains examined.

4. Discussion

This is the first study where the effect of interacting CC factors (T x CO_2) have been investigated on growth, relative expression of a range of structural and regulatory genes and OTA production by *A. carbonarius* strains grown on a grape-based matrix. This may be particularly important for the Apulia region, which is considered an important wine producer area in Italy. In particular, we aimed to study the effect of current and projected climate conditions in the North Apulia region, where the OTA risk is now relatively low, selecting two day/night temperature cycles (15-28°C vs 18-34°C, 11.5h/12.5h dark/light), and two CO_2 concentrations (400 vs 1000 ppm).

The effect of day/night temperature associated with photoperiod on the ecophysiology of *A. carbonarius* grown on substrates simulated grape composition has been already investigated. Oueslati et al., 2010, studied the combined effect of three alternating temperatures (20-30°C, 20-37°C, 25-42°C) and 11h/13h light/darkness photoperiod on *A. carbonarius* strains isolated from Tunisian grapes and grown on a Synthetic Nutrient Medium (SNM) at 0.99 a_w. They found that mycelia growth rate was faster at 20-30°C, compared to the other conditions. Similarly, García-Cela et al., 2012, studied the effect of two day/night temperature (20-30°C and 25-37°C) and 16h/8h

light/darkness on growth and OTA of two *A. carbonarius* isolated from Spanish grapes and found that 20-30°C cycle resulted in higher risk of contamination. In our study, the different effect of two day/night temperature cycles applied with the same photoperiod, was evident both on mycelial growth rate and sporulation of colonies. In particular, 18-34°C temperature cycle determined a faster growth indicating that the projected +6°C diurnal temperature may be associated to a higher risk of *A. carbonarius* contamination.

In addition, our results, suggested that the expected increase of 2.5x CO_2 atmospheric concentration might increase the *A. carbonarius* colonization rate resulting in a possible increased risk of spoilage in grapes and grape-derived products. Such data constitutes new evidence about the ecophysiology of *A. carbonarius* and differs from previous studies where growth was generally found to be relatively unaffected by elevated CO_2 exposure when compared to mycotoxin production. In this regard, Akbar et al. (2016), suggested that lag phases prior to growth and growth rates of different strains of *Aspergillus* sections *Circumdati* (*A. westerdijkiae*, *A. steynii*, *A. ochraceus*) and *Nigri* (*A. niger*, *A. carbonarius*) grown on a coffee-based media, were generally not significantly stimulated by the effect of increased CO_2 atmospheric concentration (1000 ppm). Similarly, Medina et al. (2015) found that the growth of *A. flavus* on a conducive medium (YES) was relatively unaffected by the addition of 2x and 3x existing CO_2 levels (350 vs 650/1000 ppm). However, these studies excluded the effect of photoperiods and temperature cycling and in addition, they were conducted on other substrates, differently from us. Thus, the increased growth rate of *A. carbonarius* in the present study was probably different because of the interaction between the temperature cycles and elevated CO_2.

The present study has also examined the impact of such interacting CC factors on the transcriptional profile of key genes related to OTA biosynthesis, and the possible correlation with the phenotypic OTA production. The combination of the current temperature cycle (15-28°C) with elevated CO_2 level (1000 ppm) resulted in an overall upregulation of biosynthetic genes – *AcOTApks*, *AcOTAnrps*, *AcOTAhal*, and in particular – *AcOTAbZIP* encoding for a transcription factor, which were positively correlated with the phenotypic production of OTA under this treatment. Moreover, *laeA*, *veA*, *velB* genes were upregulated following a similar expression pattern of the other structural genes, suggesting that 15-28°C x 1000 ppm CO_2 condition may directly influence the global regulatory velvet complex and thus OTA production. These results are in accordance with Crespo-Sempere et al., 2013 that showed the need of *veA* and *laeA* genes for OTA production in *A. carbonarius*.

In general, information about the expression of mycotoxin related genes under different CC factors are still limited for toxigenic fungi. The limited studies, available until now, reported

discordant evidences about the pattern of expression of key genes related to the mycotoxin production and the phenotypic toxin amount detected. In particular, Medina et al. (2015) studied the three-way interacting effects of CC factors (a_w x T x CO_2) on *A. flavus* grown on YES and found that high temperature (37°C) and increasing CO_2 concentration (350 vs 650/1000 ppm) induced the upregulation of both structural (*aflD*) and regulatory (*aflR*) genes which positively correlated with the AFB_1 production. In contrast, Gallo et al. (2016), found that only the structural genes (*aflD*, *aflO*) showed increased expression levels in correlation with higher AFB_1 production when they examined the impact of two-way interacting factors (a_w x T) on *A. flavus* grown on an almond-based medium.

Recently, Verheecke-Vaessen et al. (2019) examined the effect of three-way interacting CC factors (a_w x T x CO_2) on growth, biosynthetic genes and phenotypic T-2 and HT-2 toxin production by *Fusarium langsethiae* on oat-based media and on stored oats. They found that the combinations of a higher temperature (25 vs 30°C) x slight water stress (0.98 a_w) x elevated CO_2 concentration (400 vs 1000 ppm) caused a significant increase in both the expression of biosynthetic toxin genes (*Tri5*, *Tri6*, *Tri16*) and phenotypic production of T-2/HT-2.

With regards to OTA production, very little is known about the impact that interacting CC factors may have on the regulation of the OTA cluster and how such drivers may influence mycotoxin production. Recently, Lappa et al. (2017), monitored the temporal expression of two OTA biosynthetic genes (*AcOTApks*, *AcOTAnrps*) and a regulatory (*laeA*) gene in *A. carbonarius* in relation to temperature and water stress. They reported that while water availability was the key factor affecting OTA production, only temperature acted as the key trigger influencing the transcript levels of the toxin biosynthesis genes.

In the present study, it has been shown, for the first time, that there is an impact of projected CC scenarios on the risk of OTA in a specific area in the Mediterranean region.

Recent studies by the European Commission (2018) pointed out that the Mediterranean basin is already being impacted by CC and extreme weather events, that are drastically changing the landscape of this region. Such CC scenarios are likely to affect the natural ecosystem of important staple crops leading to food/feed safety and security uncertainty (Donatelli et al., 2017). With respect to food safety, mycotoxins are probably one of the major hazards likely to be affected by such changes (Miraglia et al., 2009; Medina et al., 2017). Toxigenic fungi have specific ecological niches for crop colonization and toxin production which reflect their distribution around the world (Van Der Fels-Klerx et al., 2016). Indeed, in hotspot regions for CC it is expected that both pests and diseases may migrate to areas which match their ecological niches and where they are more resilient (Lobell and Gourdji, 2012; Battilani et al., 2016). In addition, there is potential that such

CC scenarios will impact on selection or evolution of new fungal genotypes producing different secondary metabolites which may require alternative mitigation strategies than those being used now (Moretti et al., 2019).

On the basis of the predicted CC scenario impacts on the Mediterranean region, the present ecophysiological conditions affecting the colonization by *A. carbonarius* and the OTA contamination in grapes and grape-derived products might change. Under the CC scenarios examined in the present study, we found that the increase of more than 2.5x CO_2 concentration in the North Apulia region, resulted in an increase of colonization rate by *A. carbonarius* and phenotypic OTA production. This was supported also by a molecular analysis of the transcriptional profile of both structural and regulatory genes involved in toxin biosynthesis. Our findings, suggest that the predicted CC may have a detrimental effect on the wine production chain in this area. This type of data could be used for the development of predictive models of the potential impact and possible increase in risks of OTA contamination of grapes and grape-based products in areas already identified as hot spots for CC impacts in the Mediterranean region.

Acknowledgements

The authors thank Monika Jodkowska (Cranfield University, UK) for the skilled technical assistance in HPLC-FLD analysis.

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Table 1: Lag phase (h) and growth rate (mm·day^{-1}) of *A. carbonarius* ITEM 7444, ITEM 5010, B7 at 15-28°C and 18-34°C, 400 ppm vs 1000 ppm. Letters indicate significant differences (Tukey HSD, $p<0.05$).

Lag phase and growth rate

Strains	Conditions	ppm	Lag Phase (h)	Growth rate (mm·day^{-1})
ITEM 7444	15-28°C	400	32.05 ± 0.1[a]	12.54 ± 0.1[b]
		1000	26.55 ± 0.2[b]	15.5 ± 0.49[c]
	18-34°C	400	26.79 ± 0.9[b]	15.86 ± 0.02[c]
		1000	24.63 ± 0.7[b]	18.9 ± 0.07[a]
ITEM 5010	15-28°C	400	26.21 ± 0.6[a]	14.99 ± 0 [b]
		1000	24.73 ± 1.24[a]	16.6 ± 0.07[c]
	18-34°C	400	26.74 ± 1.17[a]	16.38 ± 0.1[c]
		1000	24.99 ± 0.67[a]	18.4 ± 0.39[a]
B7	15-28°C	400	28.55 ± 1.1[a]	14.08 ± 0.1[b]
		1000	24.22 ± 0.30[c]	16.7 ± 0.04[c]
	18-34°C	400	27.36 ± 0.08[a,b]	17.50 ± 0.1[c]
		1000	25.84 ± 0.31[b,c]	19.0 ± 0.42[a]

(A)

(B)

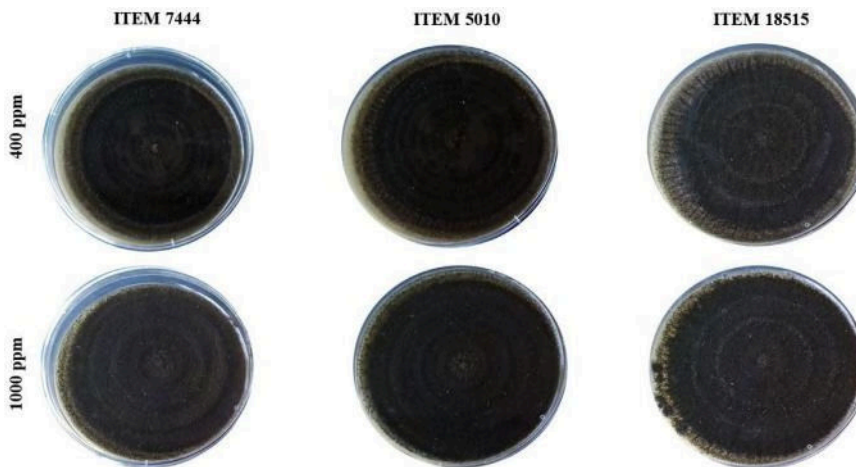

Figure 1: (A) Pictures of *A. carbonarius* ITEM 7444, ITEM 5010, ITEM 18515 colonies centrally inoculated taken after 7 days at 15-28°C 11.5h/12.5h dark/light, 400 and 1000 ppm. (**B)** Pictures of *A. carbonarius* ITEM 7444, ITEM 5010, ITEM 18515 colonies centrally inoculated taken after 7 days at 18-34°C 11.5h/12.5h dark/light, 400 and 1000 ppm.

Strain ITEM 7444

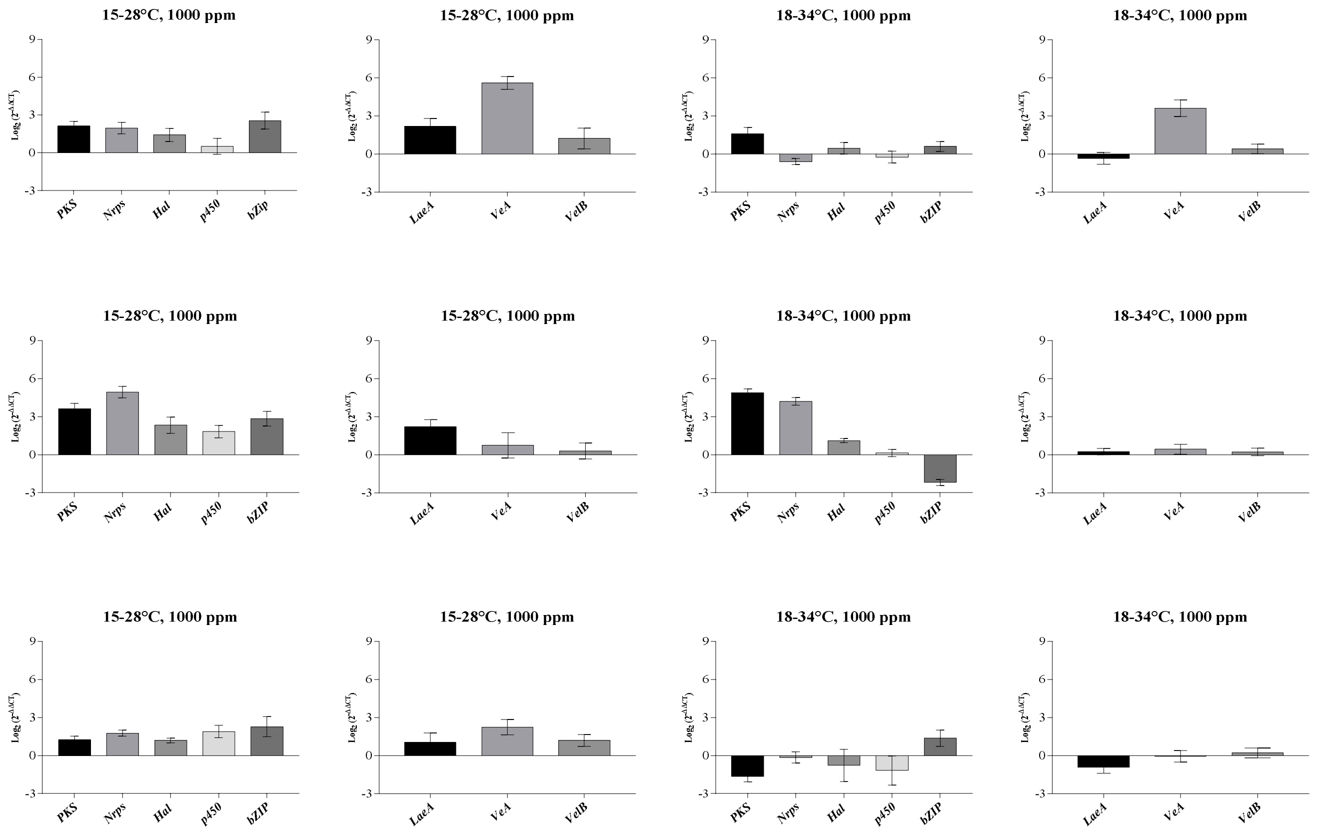

Figure 2: Relative expression of OTA biosynthetic (*AcOTApks, AcOTAnrps, AcOTAhal, AcOTAp450*), transcription factor (*AcOTAbZIP*) and regulatory (l*aeA*/*veA*/*velB*) genes of *A. carbonarius* ITEM 7444, ITEM 5010, ITEM 18515 grown on 0.99 a_w GJM at 15-28°C and 18-34°C 11.5h/12.5h dark/light at 1000 ppm vs 400 ppm (control), after 4 days.

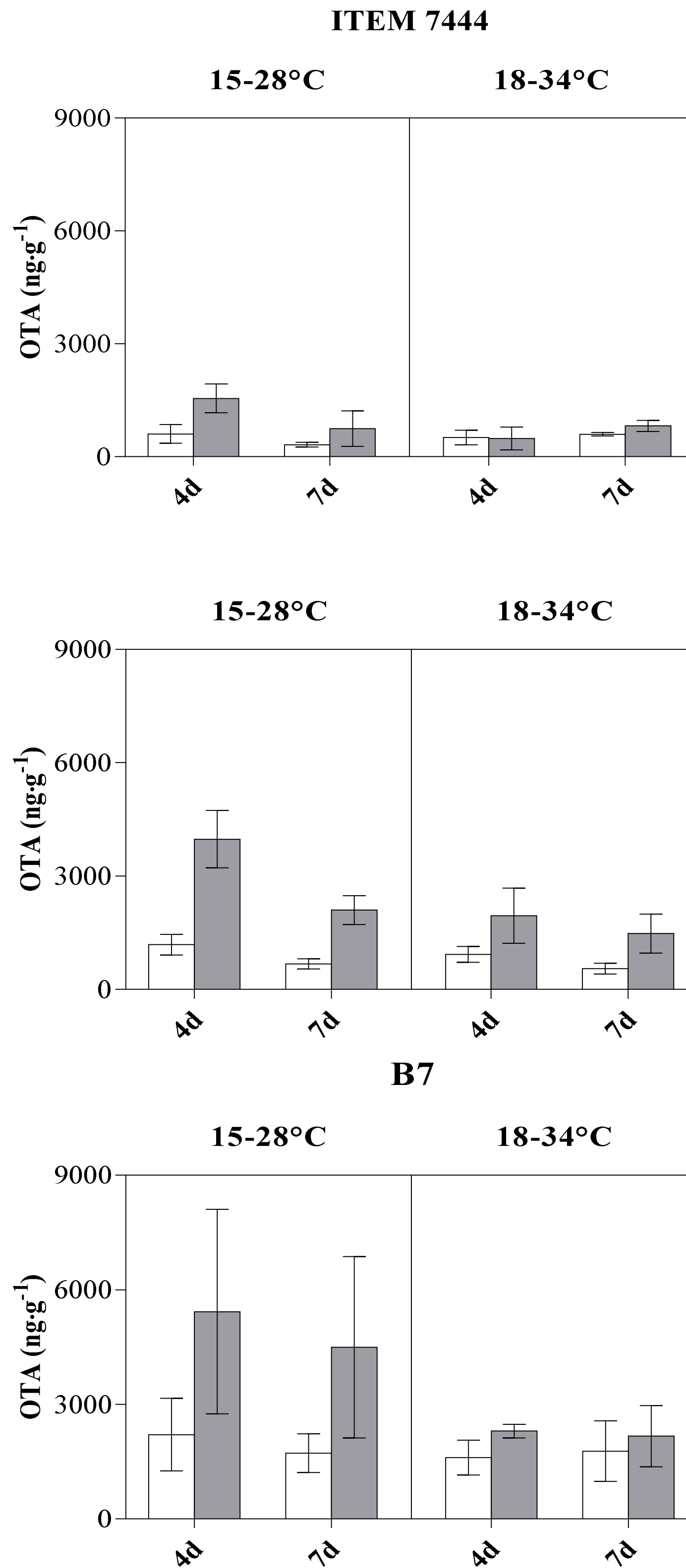

Figure 3: Ochratoxin A production (ng·g^{-1}) by *A. carbonarius* ITEM 7444, ITEM 5010, ITEM 18515 at 15-28°C and 18-34°C 11.5h/12.5h dark/light, 400 ppm (white bars) vs 1000 ppm (grey bars) at 4 and 7 dpi. Letters indicate significant differences (Tukey HSD, $p<0.05$).

SUPPLEMENTARY MATERIALS

Table S1: Primers sequences for qRT-PCR used in this study.

Primer	Concentration	Sequence (5'-3')	Reference
RT_OTApks_Ac_FOR	200 nM	CGTGTCCGATACTGTCTGTGA	Gallo et al. 2014
RT_OTApks_Ac_REV	200 nM	GCATGGAGTCCTCAAGAACC	
RT_nrps_Ac_FOR	200 nM	ACGGGTCGCTGCTCTATATC	Ferrara et al. 2016
RT_nrps_Ac_REV	200 nM	ACTCACCACATCAACCACGA	
RT_hal_Ac_FOR	200 nM	GAACGCCAGTAGAGGGACAG	Ferrara et al. 2016
RT_hal_Ac_REV	200 nM	ATGGAGGTGGTGTTGTTGTG	
RT_AcOTAp450_F	200 nM	GTGGTTATCCCGCCCAATAC	Ferrara et al. 2016
RT_AcOTAp450_R	200 nM	TGCCAGATTCATCCCGATAC	
RT_Ac_OTAbZIP_for	250 nM	AATGGAACCAGCATTGATCTC	Ferrara et al. 2016
RT_Ac_OTAbZIP_rev	250 nM	GACCCAAGCATTCGCTCTA	
RT_AClaeA-1F	150 nM	AATGGGACCGCAATGAGTC	This study
RT_AClaeA-2R	150 nM	TCCTGCTCCTGTTCGTCAC	
RT_Ac_veA_FOR	150 nM	GGTGAATGAGACCGAGCA	This study
RT_Ac_veA_REV	150 nM	GCATTGTAGGCGAAGGTGA	
RT_Ac_VelB_For	150 nM	AGTGCGTTCCGACTGACTG	This study
RT_Ac_VelB_Rev	150 nM	TGGACTGATTACCGACATTTACA	
RT3 BT Ac_F	200 nM	CAAACCGGCCAGTGTGGTA	Ferrara et al. 2016
RT3 BT Ac_R	200 nM	CGGAGGTGCCATTGTAAACA	

Table S2: Summary of ANOVA analysis for lag phase (hours) and growth rate (mm/day) of *A. carbonarius* ITEM 7444, ITEM 5010 and B7 strains in relation to CO_2 (400 vs 1000 ppm) and temperature variations (15-28°C vs 18-34°C).

Strains	**Significance**			**Response**
	CO_2	*Temperature*	*CO_2 x Temp.*	
ITEM 7444	**	**	*	Lag Phase (hrs)
	***	***	ns	Growth rate (mm/day)
ITEM 5010	*	ns	ns	Lag Phase (hrs)
	***	***	ns	Growth rate (mm/day)
B7	*	ns	ns	Lag Phase (hrs)
	***	***	ns	Growth rate (mm/day)

Levels of significance (*p*-values): 0.12=ns; 0.033=*; 0.002=**; < 0.001=***

Table S3: Summary of ANOVA analysis for ochratoxin A production of *A. carbonarius* ITEM 7444, ITEM 5010 and B7 strains in relation to CO_2 (400 vs 1000 ppm) and temperature variations (15-28°C vs 18-34°C).

Strains	**Significance**		
	CO_2	*Temperature*	*CO_2 x Temp.*
ITEM 7444	**	ns	*
ITEM 5010	***	**	*
B7	*	*	*

Levels of significance (*p*-values): 0.12=ns; 0.033=*; 0.002=**; < 0.001=***