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6 **Effects of temperature and water activity change on ecophysiology of ochratoxigenic**
7 ***Aspergillus carbonarius* in field-simulating conditions**

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18 **ABSTRACT**

19 Ochratoxin A (OTA) is the primary mycotoxin threat in wine and dried vine fruits. Its presence in
20 grape and wine is strongly related to climatic conditions and the expected climate change could
21 represent a risk of increasing fungal colonization and OTA contamination in grapes. In this regard,
22 the interacting effect of i) different conditions of water availability (0.93 and 0.99a_w) and ii) different
23 10h/14h dark/light alternating temperature conditions simulating a nowadays (18/31°C) and climate
24 change scenario (20/37°C) in high OTA risk areas of Apulia region, were studied. Lag phases prior
25 to growth, mycelial growth rate, the expression of biosynthesis, transcription factors and regulatory
26 genes of OTA cluster and OTA production were analyzed in *Aspergillus carbonarius* ITEM 5010
27 under the combined effect of different climatic factors. At 18/31 °C and under water stress conditions
28 (0.93 a_w) the growth rate was slower than at 0.99 a_w; on the contrary, at 20/37°C a higher growth rate
29 was observed at 0.93 a_w. An over-expression of OTA genes and genes belonging to the global
30 regulator Velvet complex was observed at 18/31°C and 0.99 a_w, with the specific OTA pathway
31 transcription factor *bZIP* showing the highest expression level. The up-regulated transcription profile
32 of the genes positively correlated with OTA production higher at 18/31°C than at 20/37°C and 0.99
33 a_w; while no OTA production was detected at 0.93 a_w at each of the temperature conditions tested.
34 These findings provide preliminary evidence that the possible increase of the temperature, likely to

happen in some areas of the Apulia region, may results in a reduction of both *A. carbonarius* spoilage and OTA production in grapes.

KEYWORDS: climate change; growth; lag phase; gene expression; ochratoxin A; grape

1. Introduction

Ochratoxin A (OTA) is one of the most abundant food contaminating mycotoxins produced by species belonging to *Aspergillus* and *Penicillium* genera; it is a potent nephrotoxin, classified as a possible human carcinogen (Group 2B) (IARC, 1993). To date, the European Union has established maximum OTA levels for different food products and the limit for grapes and wine was set at 2 μg/kg (Commission of the European Communities, 2006 Reg 1881). Studies performed over the last decade have provided evidence that all fungi responsible for OTA production in grapes belong to *Aspergillus* section *Nigri*, the so called "Black Aspergilli". In particular, *A. carbonarius* has been identified as the major cause of contamination in berries (Cabañes and Bragulat 2018; Perrone et al., 2008). This contamination is strongly related to various factors such as climatic conditions, geographical regions, grape varieties, damage by insects, growing season, with fluctuations in contamination rate occurring from one year to another (Somma et al., 2012; Visconti et al., 2008). A number of ecophysiological studies simulating field conditions revealed that *A. carbonarius* growth and OTA production were determined by environmental factors such as temperature, water availability and photoperiod (Bellí et al., 2006; Oueslati et al., 2010). In this respect, climate has always been one of the key-factors in the agro-ecosystem that influences fungal colonization and mycotoxin production in crops (Bellí et al., 2005a; Magan et al., 2003). Climate change is thus expected to have a profound effect both on our landscape worldwide and on sustainable food production system. It is estimated that the environment in which crops will be grown in the next 10-25 years may change markedly with atmospheric CO_2 concentration expected to double or triple, accompanied by episodes of heavy rainfall or periods of extreme drought. Because of the increase of greenhouse gases, the global temperature could rise between +2 and +5 °C (Botana et al., 2015; Medina et al., 2015). With respect to food safety, one of the most important hazards likely affected by climate change will be the contamination by mycotoxins of economically important commodities (Miraglia et al., 2009). Mycotoxigenic fungi have their own specific temperature and humidity range for crop infection, mycotoxin production and survival, which reflects their geographical distribution and determines a gradient of mycotoxin contamination worldwide. Some species might shift their geographical distribution in response to global warming, leading to changes in the pattern of mycotoxin occurrence

(Battilani et al., 2016). Recent studies have reported diverse observations on the effect of climate change on mycotoxins production. Under elevated CO_2 concentration, *Fusarium verticillioides*, a well-known pathogen of maize, showed an increased virulence, although fumonisins production was relatively unchanged compared to control conditions (Vaughan et al., 2014). The interaction of environmental factors (water activity x temperature x CO_2) was reported to have a little effect on growth of *A. flavus* on maize kernels, while a significant increase in the production of the mycotoxin Aflatoxin B_1 was detected (Medina et al., 2014). Differential effects of interacting climate change factors on growth and OTA production in most species of *Aspergillus* on coffee were observed by Akbar et al. (2016). In particular, within section *Circumdati*, *A. westerdijkiae* strains were the ones showing a significant stimulation of OTA production at 0.90 a_w under elevated CO_2 concentrations. Moreover, a preliminary study on *A. ochraceus* and *A. carbonarius* evidenced a reduction of growth and OTA production when temperature increased (García-Cela et al., 2012). There is limited research investigating the toxigenic risk in grapes and wines towards a climate change scenario. In the present work, we evaluated the interacting effect of 0.99 and 0.93 a_w, reported to be representative of more and less conducive conditions, respectively, for the colonization and OTA contamination by *A. carbonarius* (Bellí et al., 2004, 2005a) and different alternating temperature conditions, 18/31°C and 20/37°C (10h/14h dark/light), simulating a nowadays and climate change scenario, respectively, in high OTA risk areas of Apulia region during the month preceding harvest. In fact, from early veraison to harvest, the incidence of *A. carbonarius* black rot in grape berries increases with a peak at ripening, due to the change in composition of berries that become mature and soft during this period (Leong et al., 2006).

In this work, we studied the behavior of *A. carbonarius* ITEM 5010 grown on grape medium under different conditions related to a possible increase of temperatures and drought stress, by analyzing lag phase prior to growth, mycelial growth rate, expression levels of OTA biosynthetic genes and genes belonging to the regulatory Velvet complex in relation to OTA production.

2. Materials and methods

2.1 Fungal strain and growth conditions

The *A. carbonarius* strain ITEM 5010 isolated from Italian wine grapes and held in the Agro-Food Microbial Culture Collection – ITEM of the Institute of Sciences of Food Production, CNR, Bari, Italy (www.ispa.cnr.it/Collection), was used for this study.

To test the influence of temperature, water activity (a_w) and dark/light variation, a Grape Juice Medium (GJM) was prepared by mixing 50% (v/v) commercial pasteurized grape juice (composition per 100 ml: fat, 0.02 g; proteins, 0.3 g; carbohydrates, 16.10 g) and 1.2% agar in distilled water (Ioannidis et al., 2015). The a_w of GJM, measured by an AquaLab 4TE a_w meter at 25°C, was adjusted to 0.99 and 0.93 by adding the required grams of glucose. The pH of the medium was adjusted to 3.5 using 1 M KOH. Fungal inocula were prepared from cultures grown on Potato Dextrose Agar (PDA) plates for seven days at 25°C.

Different alternating conditions simulating a nowadays and climate change scenario of high OTA risk areas of Apulia region (18/31°C and 20/37°C, 10h/14h dark/light) were tested using a dynamic climate chamber (WTC Binder, Labortechnik GmbH, Tuttingen Germany).

Grape Juice Medium plates overlaid with sterile cellophane membranes were uniformly spread with 100 µl of a conidial suspension (10^5 conidia/ml) of *A. carbonarius* strain ITEM 5010. Plates cultures with the same a_w were incubated into a transparent plastic box containing water or a water/glucose solution to minimize moisture loss and maintain constant the a_w value during incubation at different conditions. Fungal mycelium was harvested at 4 days and 7 days post inoculation (dpi) and stored at -20°C for OTA content analysis. Fungal mycelium was harvested at 4 days dpi and stored at -80°C for RNA extraction.

2.2 Growth measurements

Fungal growth was assessed daily for seven days and expressed as the average measure (mm) of two orthogonal diameters of centrally inoculated colonies (3 mm drop). The colony diameters (mm) were plotted against time (days) and the linear regression model, already described by (Medina et al., 2014), was used for calculating the growth rate expressed as the slope of the line. The lag phase (hours) was calculated by equaling the linear regression formula to the diameter of the inoculated drop. The square of the linear correlation coefficient was ≥ 0.98. Experiments were carried out with three replicates per treatment.

2.3 RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from frozen mycelium ground in liquid nitrogen using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA concentration was determined by using Nanodrop and its integrity was evaluated by doing a 1% agarose gel. First strand cDNA was synthesized using about 1 µg of total RNA in a reaction mix containing oligo $(dT)_{18}$ primer, random examers and SuperScript III Reverse Transcriptase (Invitrogen, San Diego, CA) according to the manufacturer's protocol.

136 The expression levels of OTA biosynthetic genes (*AcOTApks*, *AcOTAnrps*, *AcOTAhal*, *AcOTAp450*,
137 *AcOTAbZip*) and heterotrimeric velvet complex genes (*VelB*,*VeA,LaeA*) of *A. carbonarius* were
138 analyzed by using real-time quantitative reverse transcription-PCR (qRT-PCR); *β-tubulin* was used
139 as internal reference gene. Nucleotide sequences of primers used in the qPCR assays are shown in
140 Table 1. Real time PCR were performed using the Viia 7 Real Time PCR system programmed to hold
141 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,
142 72°C for 30 s. Specificity of the PCR amplifications was confirmed by dissociation curve analysis.
143 Real time PCR experiments were conducted in 96-well plates by using SYBR Select MasterMix and
144 different concentrations of primers pairs for each gene in a final volume of 10 µl. Data analysis was
145 assessed by QuantStudio™ RT-PCR Software. The relative quantification of gene expression was
146 established using the comparative $2^{-\Delta\Delta CT}$ method. PCR efficiency of each oligonucleotide pair was
147 calculated from each linear regression of standard curves. Relative gene expression analysis was
148 performed on three biological replicates for each of which three technical replicates were assayed.
149
150 *2.4 Determination of ochratoxin A*
151 For the determination of OTA in fungal mycelium, HPLC-FLD analysis was performed on three
152 biological replicates
153 An aliquot of lyophilized mycelium (100 mg) was extracted with 2 ml of a mixture of
154 methanol:acetonitrile:water (30:30:40 v/v/v) by shaking for 2 h at room temperature. After extraction,
155 samples were centrifuged for 15 min at 3901 g and 600 µl of supernatant were diluted with 400 µl of
156 acetonitrile:water:acetic acid (35.0:62.5:2.5 v/v/v) and vortexed for 30 s. This solution was filtered
157 (0.22 µm) and 100 µl were injected into the HPLC-FLD apparatus. The mobile phase was an isocratic
158 mixture of acetonitrile:water (45:55 v/v) containing 1% acetic acid. The limit of detection (LOD) of
159 this method for OTA was 6.78 ng/g.
160 The HPLC apparatus was an Agilent 1260 Infinity system equipped with a G1312 binary pump, a
161 G1367E autosampler with a 100 µl loop, a G1316C column thermostat set at 30°C, and a fluorescence
162 detector (G1321B) (excitation wavelength, 333 nm; emission wavelength, 460 nm) from Agilent
163 Technologies (Waldbronn, Germany). The column used was a 150 x 4.6 mm i.d., 5 µm, Zorbax C18,
164 (Phenomenex, Torrance, CA, USA) with a 3 mm i.d. and a 0.45 µm pore size guard filter (Rheodyne,
165 Cotati, CA, USA). The flow rate of the mobile phase was 1 ml/min. The standard solutions of OTA
166 in acetonitrile were purchased from Romer Labs Diagnostic GmbH (Tulln, Austria). Calibration
167 solutions of OTA were prepared in HPLC mobile phase, and calibration curves were prepared in the
168 range of 0.1 to 100 ng/ml.
169

170 *2.5 Data analysis*

171 Statistical analysis was performed by using GraphPad Software (version 7.0, Prism, La Jolla
172 California, USA). After checking the normal distribution of lag phases, growth rates and OTA
173 production datasets with D'Agostino-Pearson test, the analysis of variance (ANOVA) was performed
174 in order to test the effect of each single variable and their interaction on the results obtained.
175 Furthermore, significant differences among the means were established by using Tukey HSD *post*
176 *hoc* test.

177

178

179 **3. Results**

180

181 *3.1 Lag phase, growth rate and sporulation*

182 As shown in Figure 1A, at the two alternating temperatures 18/31°C, the drought stress (0.93 a_w)
183 caused a lag phase considerably longer (40 h) than at 0.99 a_w (26 h), while at 20/37°C, the lag phase
184 exhibited no significant differences when a_w of the substrate was changed. As regards growth rate, at
185 18/31°C, it was faster at 0.99 a_w (19 mm/day) than at 0.93 a_w (10 mm/day); on the contrary, at 20/37
186 °C, the fungus showed a faster growth at 0.93 a_w (11 mm/day) than at 0.99 a_w (6 mm/day) (Fig. 1B).
187 The different ecophysiological behavior of *A. carbonarius*, both in terms of growth and sporulation,
188 was macroscopically evident comparing mycelial growth on plates at 7 days after inoculation, as
189 illustrated in Figure 2. In particular, at 20/37°C a less sporulated mycelium was observed at 0.93 a_w
190 than at 0.99 a_w, although the growth rate resulted to be faster in water stress condition, as previously
191 described. In order to confirm this new evidence, the experiment was repeated two times also on
192 different strains (data not shown).
193 ANOVA analysis (data not reported) showed that both lag phase and growth rate were significantly
194 influenced ($p<0.001$) by temperature conditions, a_w, and their interaction.

195

196 *3.2 Gene expression and OTA production*

197 The effect of the two alternating day/night temperature on the expression of both biosynthetic and
198 regulatory genes in *A. carbonarius* was analysed after 4 days of growth at 0.99 a_w, which seems to be
199 the most reliable time to detect the activation of the genes involved in OTA biosynthesis, preceding
200 the later increase of OTA production. As shown in Figure 3, a general up-regulation of structural
201 OTA biosynthesis genes (*AcOTApks, AcOTAnrps, AcOTAhal* and *AcOTAp450*) was observed at
202 18/31°C compared to 20/37°C. In particular, a minimum of about 2 fold change in the expression
203 level was detected for the genes located in the OTA cluster with a peak of about 12 fold change of

204 the gene coding for the cytochrome *p450* oxidase protein, expected to be involved in the oxidase step
205 for the formation of the precursor metabolite OTβ in the biosynthesis pathway (Ferrara et al., 2016).
206 Moreover, at 18/31°C, the gene coding for a *bZIP* transcription factor protein, likely acting as
207 regulator of OTA gene expression, exhibited a 34 fold higher transcription level (Fig. 3A). The genes
208 *laeA*, *veA*, and *velB*, belonging to the global transcriptional complex regulating fungal secondary
209 metabolism known as Velvet complex, showed a higher level of expression between 2 and 5 fold at
210 18/31°C temperature cycle as well (Fig. 3B).
211 Gene expression analysis performed after 4 days of incubation, was supported by the results of OTA
212 production.
213 At 0.99 a_w, the highest OTA production was observed in *A. carbonarius* grown at 18/31°C. In
214 particular, at these temperatures OTA content was slightly higher at 4 days (554 ng/g) than at 7 day
215 (512 ng/g) after inoculation. At 20/37°C, OTA accumulation was lower at 4 days (185 ng/g) than at
216 7 days (226 ng/g) (Fig. 4). The longer production timing at 20/37 °C is likely due to the slower growth
217 rate observed at these higher temperature conditions. Under strong water stress conditions, *A.*
218 *carbonarius* appeared unable to produce OTA, in fact no OTA was detected at 0.93 a_w , under the
219 limit of detection, in any of the temperature conditions tested. ANOVA analysis evidenced that OTA
220 production was significantly affected by the different temperature conditions. (Fig. 4)
221
222 **4. Discussion**
223 The studies of European Commission (2018) on adapting to climate change in Europe suggest that,
224 in Southern Europe, changes may equate to a temperature increase of 4–5°C coupled with longer
225 drought periods. The climate change scenario hypothesized in our study takes EU suggestions into
226 account and is derived from climatic data series related to the high OTA risk areas of Apulia region
227 as retrieved from "ilmeteo.it" website (https://www.ilmeteo.it/, 2017).
228 The combined effect of a_w and temperatures on the ecophysiology of *A. carbonarius* grown on
229 substrates that simulate grape composition has been largely investigated (Astoreca et al., 2007; Bellí
230 et al., 2005b; Chiotta et al., 2015;), while only few studies have considered day/night alternating
231 temperatures associated with the photoperiod (García-Cela et al., 2012; Oueslati et al., 2010).
232 Performing *in vitro* studies which consider interacting climate change factors, may provide
233 ecophysiological data quite similar to a real field situation. In our study, when the alternating cycle
234 at 18°C for 10 h darkness and 31°C for 14 h light, corresponding to the nowadays climate condition
235 in Apulia, was applied, we observed a higher growth rate and a shorter lag phase at high-water
236 availability (0.99 a_w), compared to water stress (0.93 a_w). Our results are consistent with previous
237 studies, reporting that the optimum for the growth of *A. carbonarius* varied from 25 to 30°C at 0.96-

238 0.99 a_w and that water stress caused a general reduction of growth rate and extension of lag time
239 (Astoreca et al., 2010). Moreover, Oueslati et al. (2010) studied the combined effect of three
240 alternating temperatures (20/30, 20/37 and 25/42°C) and 11h/13h light/darkness photoperiod of *A.*
241 *carbonarius* strains isolated from Tunisian grapes and grown on a Synthetic Nutrient Medium (SNM)
242 at 0.99 a_w. They found that mycelia growth rate was significantly enhanced at 20/30°C than at
243 20/37°C and it was even slower at 25/42°C. This evidence supports our results in which, under water
244 availability condition (0.99 a_w), *A. carbonarius* grew faster at 18/31°C than at 20/37°C.
245 In fact, at 20/37°C, simulating a climate change scenario, we observed a reduction of *A. carbonarius*
246 growth rate at 0.99 a_w, which was in turn lower than that observed at 0.93 a_w at the same temperature,
247 even though the mycelium appeared less sporulated and no differences were observed in terms of lag
248 phase. These findings diverge from previous studies on *A. carbonarius*, in which it was reported that
249 higher a_w determined a faster growth rate compared to lower values, regardless of temperature (Bellí
250 et al., 2005b). However, other works underline how significant is the impact of the combination of
251 a_w and temperature on fungal growth and mycotoxin production (Selouane et al., 2009).
252 In our study we considered the sporulation behavior of *A. carbonarius* only from the morphological
253 point of view which revealed that a water stress condition (0.93 a_w) determined a visible reduction of
254 sporulation compared to high water availability (0.99 a_w). In addition, a small but more sporulated
255 colony was observed at the increased day/night temperature (20/37°C). No work has been carried out
256 on the sporulation rate of *A. carbonarius* while few evidences exist on other mycotoxigenic fungi.
257 Such studies suggested that middle water stress conditions (0.95-0.97 a_w) combined with constant
258 high temperature (30-35°C) are generally suitable for spore production (Abdel-Hadi & Magan, 2009;
259 Gervais and Molin, 2003; Giorni et al., 2008; Parra et al., 2004).
260 Ample evidence exists to suggest that environmental factors, like temperature and light and humidity,
261 affect mycotoxin production by activating biosynthesis genes through a complex regulatory
262 mechanism that occurs at several levels from cluster-specific regulators to global transcriptional
263 complex (Keller, 2019).
264 Recent studies have made it possible to identify the main structural genes in the OTA biosynthesis
265 cluster of *A. carbonarius*. The genes *AcOTApks*, *AcOTAnrps*, *AcOTAhal*, and *AcOTAp450* encoding
266 for a polyketide synthase, a non ribosomal-peptide synthase, an halogenase and a cytochrome p450
267 monooxygenase, respectively, contribute to determine the molecular structure of OTA (Perrone and
268 Gallo, 2017). In the cluster, the *AcOTAbZIP* gene, encoding for a bZIP (basic leucine zipper)
269 transcription factor, is also present as a pathway specific regulator of OTA cluster as suggested by its
270 transcription profile highly correlated to OTA production, observed in a previous study (Ferrara et

271 al., 2016). An homologous *bZIP* gene has identified in the OTA cluster of *A. ochraceus* and its role
272 as regulator of OTA biosynthesis has been corroborated by gene inactivation (Wang et al., 2018).
273 At 18/31°C, all the OTA genes analysed in this work showed expression levels higher than at
274 20/37°C. These results were positively correlated with OTA production which was higher and
275 significantly different at 18/31°C than at 20/37°C (Fig. 4). Different studies reported that optimum
276 conditions for the production of OTA by *A. carbonarius* are high a_w (0.95-0.99) at temperature
277 ranging from 20°C to 30 °C (Astoreca et al., 2010; Bellí et al., 2005b; Mitchell et al., 2004; Selouane
278 et al., 2009) however, this variation is also depending on the origin (Lasram et al., 2010) and
279 intraspecific variability (Garcia et al., 2011) of the strains. It is generally agreed that increasing
280 temperatures and decreasing a_w cause a reduction of OTA production by *A. carbonarius* (Bellí et al.,
281 2005b; Marin et al., 2006). Indeed, no OTA production was observed under the limit of detection,
282 when we analysed *A. carbonarius* grown at 0.93 a_w at both the two temperature conditions tested and
283 after extension of incubation time at 10 and 14 days (data not shown). The lack of OTA production
284 at 0.93 a_w may be due to the glucose added in the medium to lower the a_w value; in fact, it was reported
285 that increasing glucose in the medium causes a decreasing of OTA production in *A. carbonarius* (Stoll
286 et al. 2013).
287 Our findings are also concordant with a research reporting that the increasing of night temperature
288 from 20°C to 25 °C and diurnal temperature from 30°C to 42°C induced a drastic reduction in OTA
289 accumulation (Oueslati et al., 2010). In our case, the major diurnal temperature increase (+6°C) rather
290 than the slight nocturnal one (+2°C) could have negatively affected OTA production.
291 To date, very little has been reported about the molecular mechanisms by which environmental factors
292 regulate OTA cluster activation. In a recent study on the production of OTA under the influence of
293 temperature and a_w in *A. carbonarius*, the authors have observed that while a_w was the key factor
294 affecting OTA production, only temperature acted as the key factor influencing transcript levels of
295 some biosynthesis structural genes; however, no transcriptional factor internal to the cluster was
296 investigated (Lappa et al., 2017). Generally, temperature and other abiotic factors affect mycotoxin
297 production by acting on the transcription levels of biosynthesis genes, with the increasing production
298 coupled to the rising expression of the structural genes. Instead, not conclusive or contradictory
299 results about the expression levels of specific transcription factors in the cluster have been reported
300 to date. In most of the studies on aflatoxin biosynthesis, for example, the transcript levels of the two
301 regulatory genes, *AflR* and *AflS*, did not change significantly in comparison to the levels of structural
302 genes, when conducive or repressive temperature conditions for mycotoxin production were applied
303 (Gallo et al., 2016; O'Brian et al., 2007). On the contrary, we found that the highly increased
304 expression levels of the putative transcription factor of OTA cluster (*AcOTAbZIP*) mirrored the

305 increased expression of OTA structural genes and then the increased OTA production. Not all the
306 genes including in the cluster of OTA biosynthesis and their corresponding functions have been
307 identified. From the analysis of genomic sequences of ochratoxigenic *Aspergillus* species, a likely
308 second transcription factor, a zinc finger DNA-binding protein, is present in the cluster and it could
309 also have a role in the regulation of OTA production (Wang et al., 2018). So, the regulation of OTA
310 biosynthesis is far to be completely clarified. Furthermore, the regulatory mechanism of secondary
311 metabolites such as mycotoxins, is composed of overlapping and interconnected pathways, which
312 comprise global regulators and multiprotein complexes that respond to environmental clues. One of
313 most important is the heterotrimeric Velvet complex (*VelB/VeA/LaeA*) that links sexual development
314 with secondary metabolism in fungi in response to light, through a regulated spatial
315 compartmentalization of the three proteins (Bayram et al., 2008). In details, under dark conditions,
316 *VeA* is transported from cytoplasm to the nucleus where it reacts with *LaeA* and *VelB*, regulating
317 production of secondary metabolites, and with *VelB*, controlling fungal sexual development (Palmer
318 et al., 2013). In *A. carbonarius,* Crespo-Sempere et al. (2013), found that *VeA* and *LaeA* have an
319 important role in regulating conidiation and OTA production in response to light, since the loss of
320 *VeA* and *LaeA* led to a reduction of OTA and conidial production in mutant strains. In wild-type
321 strain, they observed an increasing of OTA production under dark conditions and an activation of
322 conidiation by light; however, the transcription of the *VeA* and *LaeA* genes appeared relatively
323 constant under both light and dark conditions, suggesting that in the regulatory mechanism the
324 subcellular localization of the protein is more important than the transcriptional level of the
325 corresponding genes. Interestingly, in our study on *A. carbonarius* exposed to a light/dark cycle, the
326 expression of the three genes of the global regulator complex appeared from 2 to 5 fold higher at the
327 temperature conditions favorable to OTA production, 18/31°C, than at 20/37°C, in correlation with
328 the transcription profile of biosynthesis genes. Actually, the role of *VeA* and more generally of the
329 Velvet complex in the regulation of certain secondary metabolite clusters was suggested to be
330 temperature-dependent as observed in a recent study on *A. fumigatus* under different temperature
331 conditions (Lind et al., 2016). Therefore, the Velvet complex appears to be involved in both
332 temperature and light based regulation of secondary metabolism in *Aspergillus*, providing support to
333 the fact that regulation of mycotoxin production occurs in response to multiple environmental cues.
334 The localization of VeA in the nucleus under dark conditions seems to be crucial for the Velvet
335 complex in controlling the dark responsive secondary metabolism in fungi, while the precise
336 mechanisms by which this complex and the interaction of its components regulate temperature
337 influence on the secondary metabolism is still to be elucidated. A complex regulatory network
338 involving multiple interconnecting proteins may be responsible to the response to various

339 environmental stimuli. With the present *in vitro* study, we investigated for the first time the behavior
340 of *A. carbonarius* under the combined effect of two environmental factors in simulating field
341 conditions of an area at high risk of OTA contamination, according to the expected values in a climate
342 change scenario. On the basis of our results, we found that the possible increase of the temperature
343 from 18/31 °C to 20/37°C 10h/14h dark/light, likely to happen in some areas of the Apulia region,
344 may results in a reduction of both *A. carbonarius* spoilage and OTA production in grapes.
345 Furthermore, molecular measurements gave us useful information not only as a more sensitive
346 support to the chemical analysis, but also highlighting the complexity of the OTA biosynthesis by the
347 involvement of some regulatory genes not necessarily located in the cluster.
348
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508 **Table 1:** Primers 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	

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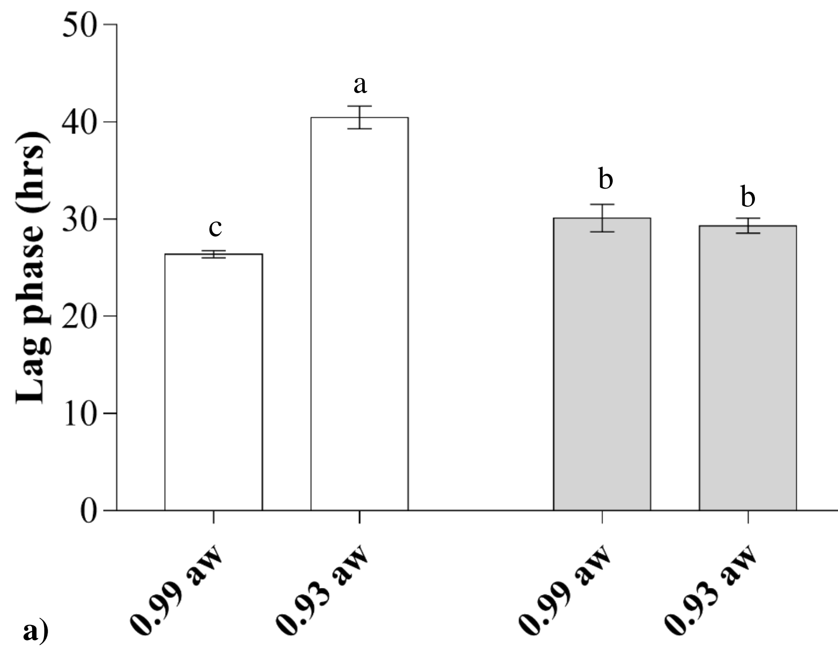
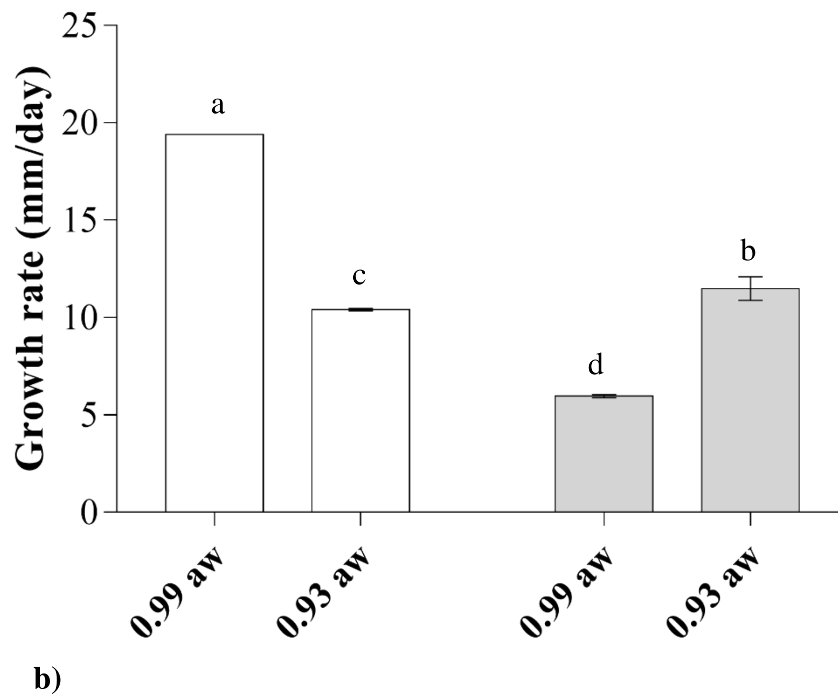
511 **Figure 1 (a,b)**





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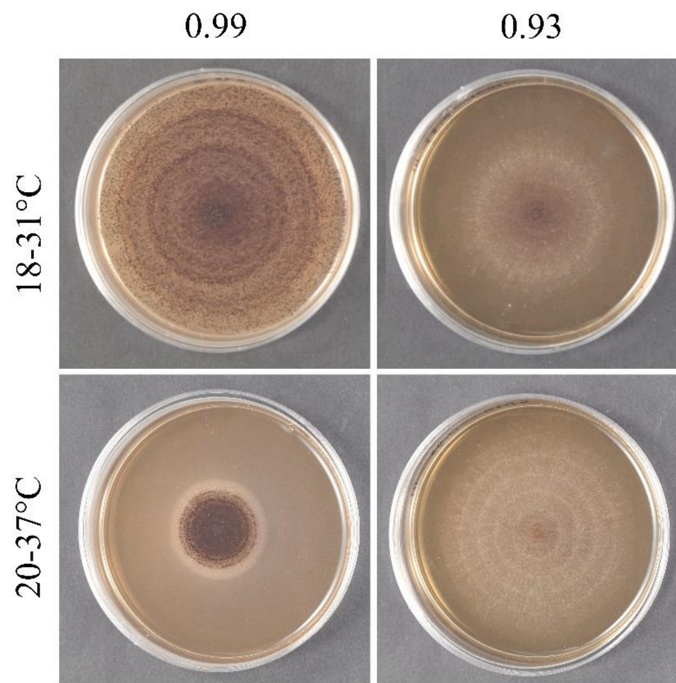
515 **Figure 2**



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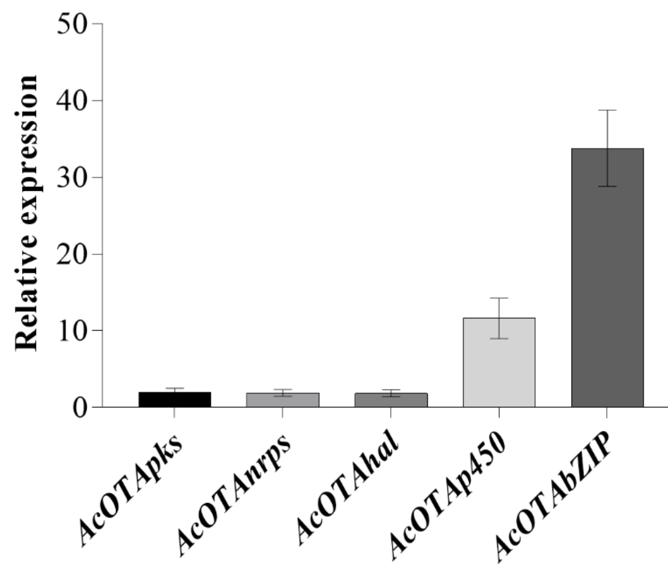
519 **Figure 3**



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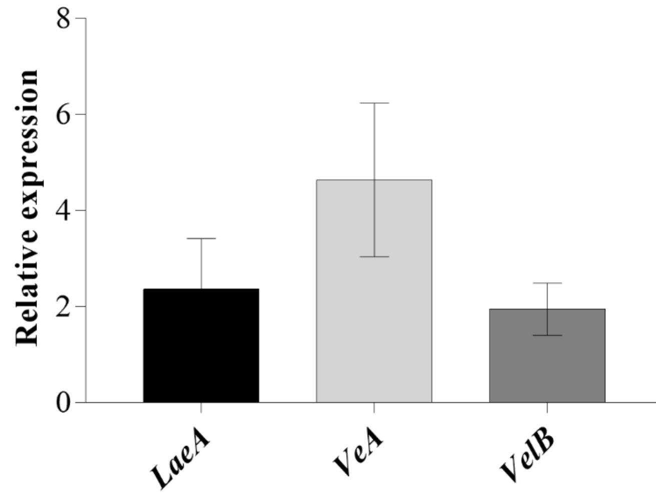
523 **Figure 4**



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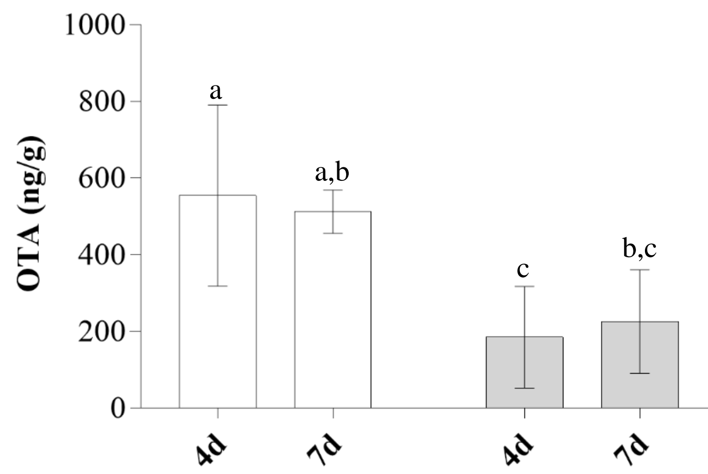
527 **Figure 5**



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