

1 **Effect of oxidant stressors and phenolic antioxidants on the**  
2 **ochratoxigenic fungus *Aspergillus carbonarius***

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24 **Abstract**

25 BACKGROUND. There are few studies dealing with the relationship between  
26 oxidative stress and ochratoxin A (OTA). In this work we have analyzed the effect  
27 of the oxidant stressor menadione and the antioxidants 3,5-di-tert-butyl-4-  
28 hydroxytoluene (BHT), catechin, resveratrol and a polyphenolic extract on growth,  
29 generation of reactive oxygen species (ROS), OTA production and gene  
30 expression of antioxidant enzymes of *Aspergillus carbonarius*.

31 RESULTS. Exposure to menadione concentrations higher than 20  $\mu$ M led to  
32 increases in ROS and OTA levels and to a decrease in growth rate. Exposure to  
33 2.5-10 mM BHT also led to higher ROS and OTA levels, although growth rate was  
34 only affected above 2.5 mM. Naturally occurring concentrations of catechin,  
35 resveratrol and polyphenolic extract barely affected growth rate, but they produced  
36 widely different effects on OTA production level depending on the antioxidant  
37 concentration used. In general, gene expression of antioxidant enzymes  
38 superoxide dismutase (SOD) and peroxiredoxin (PRX) was down regulated after  
39 exposure to oxidant and antioxidant concentrations that enhanced OTA  
40 production.

41 CONCLUSION. *A. carbonarius* responds to oxidative stress increasing OTA  
42 production. Nevertheless, the use of naturally occurring concentrations of  
43 antioxidant phenolic compounds to reduce oxidative stress is not a valid approach  
44 by itself for OTA contamination control in grapes.

45

46 **Keywords:** *Aspergillus carbonarius*; ochratoxin production; oxidative stress; grapes.

47 **INTRODUCTION**

48           *Aspergillus carbonarius* is an ochratoxin A (OTA)-producing fungus, which  
49 has been considered the main species responsible for OTA contamination of  
50 grapes and derived products <sup>1-2</sup>. The greatest concern about this contamination  
51 lies in the fact that OTA is a nephrotoxic, carcinogenic, teratogenic and  
52 immunotoxic mycotoxin <sup>3</sup>. Thus, OTA has been classified by the International  
53 Agency for Research on Cancer <sup>4</sup> in group 2B (possible human carcinogen).  
54 Fungal growth and production of OTA has been shown to be affected by  
55 environmental and nutritional factors, such as pH, temperature, water activity and  
56 carbon and nitrogen sources <sup>5</sup>. However, little is known about the effect of other  
57 factors such as oxidative stress and the influence of phenolic antioxidants on OTA  
58 production level and fungal growth of *A. carbonarius*.

59           Oxidative stress is recognized as a trigger of mycotoxin biosynthesis.  
60 Jayashree and Subramanyam <sup>6</sup> were among the first researchers who evidenced  
61 the relevance of reactive oxygen species (ROS) and oxidative stress on mycotoxin  
62 production. They found that aflatoxin production is a consequence of increased  
63 oxidative stress leading to enhanced lipid peroxidation and ROS generation.  
64 These results were subsequently confirmed when it was observed that oxidative  
65 stressors such as hydrogen and lipid hydroperoxides induce the accumulation of  
66 deoxynivalenol by *Fusarium graminearum* <sup>7</sup>, aflatoxins by *Aspergillus parasiticus*  
67 and *Aspergillus flavus* <sup>8-9</sup> and sterigmatocystin by *Aspergillus nidulans* <sup>10</sup>. By  
68 contrast, it has also been demonstrated that antioxidant molecules have an  
69 inhibiting effect on the formation of mycotoxins such as aflatoxins and fumonisins  
70 <sup>11-13</sup>. Indeed, some of these compounds have also been used safely as  
71 alternatives to fungicides to control aflatoxin and fumonisin contamination in  
72 various food and agricultural products <sup>14-16</sup>.

73 Correlation between oxidative stress and mycotoxin biosynthesis has been  
74 less demonstrated in OTA than in other mycotoxins. Palumbo *et al.*<sup>17</sup> studied the  
75 inhibition effect of phenolic antioxidants on OTA production and fungal growth of  
76 different ochratoxigenic *Aspergillus* species. Butylated hydroxyanisole (BHA) and  
77 the antimicrobial agent propyl paraben (PP) have also been tested as alternatives  
78 to fungicides to control *Aspergillus* section *Nigri* species in peanuts during storage  
79 <sup>18-19</sup>. Some of these antioxidant compounds included in these studies such as  
80 BHA, caffeic acid and resveratrol are known to inhibit lipoxygenases. Moreover, it  
81 is also known that the lipoxygenase biosynthesis pathway yields oxylipins, which  
82 trigger signaling mechanisms that activate mycotoxin production<sup>20</sup>. Actually, the  
83 involvement of a lipoxygenase in OTA production by *Aspergillus ochraceus* has  
84 been demonstrated by Reverberi *et al.*<sup>21</sup>. Based on this background information,  
85 De Rossi *et al.*<sup>22</sup> demonstrated that resveratrol is able to control OTA production  
86 level by *A. carbonarius* through the inhibition of lipoxygenase activity. This led us  
87 to hypothesize that the oxidant/antioxidant balance affects OTA biosynthesis and  
88 that oxidative stress might be another important factor involved in triggering of  
89 OTA biosynthesis.

90 The present study was undertaken to investigate how oxidative stress  
91 influences OTA production by *A. carbonarius*. To this aim, the effect of the oxidant  
92 stressor menadione and phenolic antioxidants on fungal growth, generation of  
93 ROS, OTA production level and gene expression of antioxidant enzymes of *A.*  
94 *carbonarius*, was studied.

## 95 **MATERIALS AND METHODS**

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## 97 **Fungi and culture conditions**

98

99 The OTA-producing *A. carbonarius* strain W04-40 was isolated from a  
100 Spanish vineyard by Martínez-Culebras and Ramón <sup>23</sup> and deposited in the  
101 Institute of Agrochemistry and Food Technology of the Spanish National Research  
102 Council (IATA-CSIC).

103 *A. carbonarius* strain was grown on Petri dishes containing Malt Extract  
104 Agar (MEA) medium in the dark at 28 °C for 6 days to achieve conidia production.  
105 Conidia were collected with a sterile solution of 0.005% (v/v) Tween 80 (J.T.  
106 Baker, Holland) and were adjusted to 10<sup>6</sup> conidia mL<sup>-1</sup> using a Thoma counting  
107 chamber. One hundred microliters of the conidial suspension was homogeneously  
108 spread on Petri dishes containing Czapek Yeast Extract Agar (CYA) medium and  
109 incubated in the dark at 28 °C. For analysis of OTA production level, ROS  
110 generation and gene expression, *A. carbonarius* strain W04-40 was incubated for  
111 48 h. For growth assessment, CYA plates were inoculated centrally with 5 µL of  
112 conidia suspensions (10<sup>6</sup> conidia mL<sup>-1</sup>). Two perpendicular diameters of the  
113 growing colonies were measured daily over four days until the colony reached the  
114 edge of the Petri dish. The radius of the colony was plotted against time, and a  
115 linear regression was applied in order to obtain the growth rate as the slope of the  
116 line to the X-axis. All the assays were performed in triplicate.

117 To study the effect of oxidative stress on *A. carbonarius* the oxidant agent  
118 menadione (2-methyl-1,4-naphthoquinone) (0, 10, 20, 40, 60, 80 and 100 µM) was  
119 added to CYA plates. As antioxidant agents, catechin and resveratrol (both 0, 10,  
120 25, 50, 100, 250, 500, 1000 and 1500 µM), the synthetic antioxidant 3,5-di-tert-  
121 butyl-4-hydroxytoluene (BHT) (0, 1, 2.5, 5 and 10 mM), and a grape polyphenol

122 extract obtained in the laboratory (described below) (0, 5, 10, 15, 20, 25, 30 and  
123 35 ppm) were added to CYA plates. Dimethylsulfoxide (DMSO) (0.1 % final  
124 concentration) was used as solvent for menadione and BHT while catechin,  
125 resveratrol and the polyphenol extract were dissolved in methanol (0.1 % final  
126 concentration). The same percentage of solvent was added to control cultures  
127 without oxidant or antioxidant agents. All oxidant and antioxidant compounds were  
128 purchased from Sigma-Aldrich, UK.

129

### 130 **Extraction of phenolic compounds from grapes**

131

132 The extraction of phenolic compounds from red grapes (Bobal variety) was  
133 performed just from the grape skins. Grapes were peeled and skins washed with  
134 distilled water. Then, skins were dried at 80°C for three hours. Eighty grams of  
135 dried skins were frozen and ground with liquid nitrogen, and 600 mL of methanol-  
136 water (80:20) were added for the extraction of phenolic compounds. After  
137 homogenization with a polytron PT 45/80 (Kinematica AG, Switzerland)) for 30  
138 seconds, four times, the extract was incubated at 45°C for 10 min. Then, the  
139 homogenate was centrifuged at 5000  $\times g$  for 30 min. The supernatant was  
140 recovered and the residue was re-extracted with another 600 mL of methanol-  
141 water (80:20). Methanol was evaporated in a rotary evaporator under vacuum and  
142 the concentrate was lyophilized. Finally, total phenolic compounds were  
143 determined by the Folin–Ciocalteu reagent <sup>24</sup>.

144

### 145 **RNA isolation and cDNA synthesis**

146

147 Mycelia were collected from 48 h-old cultures, frozen in liquid nitrogen and  
148 stored at  $-80^{\circ}\text{C}$  before nucleic acid extraction. RNA was isolated from 1 g of  
149 mycelium previously ground to a fine powder with a mortar and pestle with liquid  
150 nitrogen. Pulverized mycelium was added to a pre-heated mixture of 10 mL of  
151 extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, 1% (w/v)  
152 sodium-n-laurylsarcosine (SDS), 1% (w/v) polyvinyl-pyrrolidone 40, 1%  $\beta$ -  
153 mercaptoethanol) and 5 mL of Tris-equilibrated phenol. After homogenization with  
154 a Polytron PT 45/80 for 1 min, the extract was incubated at  $65^{\circ}\text{C}$  for 15 min and  
155 cooled before adding 5 mL of chloroform:isoamyl alcohol (24:1, v/v). The  
156 homogenate was centrifuged at  $3900 \times g$  for 20 min at  $4^{\circ}\text{C}$  and the aqueous  
157 phase was re-extracted with 10 mL of phenol:chloroform:isoamyl alcohol (25:24:1,  
158 v/v/v). Nucleic acids were precipitated by adding 2 volumes of cold ethanol and  
159 centrifuged immediately at  $27,200 \times g$  for 15 min. The resulting pellet was  
160 dissolved in 900  $\mu\text{L}$  of TES (10 mM Tris-HCl, 5 mM EDTA, 0.1% SDS, pH 7.5)  
161 and RNA was precipitated overnight at  $-20^{\circ}\text{C}$  by adding 300  $\mu\text{L}$  of 12 M LiCl.  
162 After centrifugation at  $27,200 \times g$  for 60 min, the precipitated RNA was re-  
163 extracted with 250  $\mu\text{L}$  of 3 M sodium acetate (pH 6.0) to remove residual  
164 polysaccharides and, finally, dissolved in 200  $\mu\text{L}$  of water. RNA concentration was  
165 measured with a Multiskan Spectrum (Thermo, USA) and verified by agarose gel  
166 electrophoresis (1.2 %) and ethidium-bromide staining. Total RNA was treated  
167 with DNase (TURBO DNase, Ambion, USA) to remove contaminating genomic  
168 DNA. Single-strand cDNA was synthesized from 10  $\mu\text{g}$  of total RNA using  
169 SuperScript III reverse transcription kit and an oligo (dT) primer, according to the  
170 manufacturer's instruction (Invitrogen, USA).

171

## 172 **Quantification of relative gene expression by real-time RT-PCR**

173

174 Gene expression of two genes related to oxidative stress response, a  
175 superoxide dismutase (*sod*) and a peroxiredoxin (*prx*) from *A. carbonarius*, were  
176 analyzed using gene specific primer pairs, SODf//SODr (5'-  
177 CCCGGAAGTACTGACCCCTATGC-3' and 5'-AGGGCTTGAGGGCAATCTG-3') and  
178 PRXf//PRXr (5'-TCCTTCTTGAGGTTGGTGAAGC-3' and 5'-  
179 CTCAGAAGAAGTTCGGCGATG-3') respectively, designed in a previous work<sup>25</sup>.  
180 Real-time RT-PCR reactions were performed in a LightCycler 480 System (Roche,  
181 USA) using SYBR Green to monitor cDNA amplification. The ribosomal 18S RNA  
182 gene was used as a reference gene (forward 5'-  
183 GCAAATTACCCAATCCCGACAC-3' and reverse primer 5'-  
184 GAATTACCGCGGCTGCTG-3'). Amplifications were carried out in a final volume  
185 of 210  $\mu$ L containing 2  $\mu$ L cDNA template, 2  $\mu$ L LightCycler® 480 SYBR Green I  
186 Master (Roche, USA), 0.5  $\mu$ L of each primer (10  $\mu$ M), and 5  $\mu$ L H<sub>2</sub>O, following the  
187 manufacturer's instructions. PCR amplifications were performed in triplicate using  
188 the following conditions: 10 min at 94 °C, followed by 45 cycles of 10 s at 94 °C,  
189 10 s at 56 °C and 10 s at 72 °C. The corresponding real-time PCR efficiency (E)  
190 in the exponential phase was calculated according to the equation:  
191  $E = 10[-1/\text{slope}]$ . The relative expression of the target genes was calculated  
192 based on the E and the C<sub>p</sub> value of sample versus a control and expressed in  
193 comparison to the ribosomal 18S RNA (reference gene), according to the following  
194 equation<sup>26-27</sup>:  $\text{ratio} = (E_{\text{target}})^{\Delta C_p \text{target}(\text{control} - \text{sample})} / (E_{\text{ref}})^{\Delta C_p \text{ref}(\text{control} - \text{sample})}$ . Three  
195 technical replicates were done for each combination of cDNA and primer pair, and  
196 PCR reaction quality was checked by analyzing the dissociation and amplification



197 curves. To calculate the normalized relative gene expression levels (fold  
198 induction), data were analyzed using the Relative Expression Software Tool  
199 (REST) and the mathematical model based on mean threshold cycle differences  
200 between the sample and the control group<sup>28</sup>.

201

## 202 **Extraction and detection of OTA from culture**

203

204         OTA was extracted using a variation of a simple method described  
205 previously<sup>29</sup>. Briefly, three agar plugs (6 mm in diameter) were obtained from each  
206 *A. carbonarius* culture and placed in a vial containing 500 µL of methanol. After 60  
207 min, the extracts were shaken and filtered (Millex® SLHV 013NK, Millipore, USA)  
208 into another vial and stored at 4 °C until chromatographic analysis. Separation,  
209 detection and quantification of OTA were performed by injecting 20 µL of extract  
210 from each vial into an HPLC system consisting of a Dionex model P680A pump  
211 (Sunnyvale, USA) connected to a Dionex model RF-2000 programmable  
212 fluorescence detector and to a Dionex PDA-100 photodiode array detector. For  
213 determination of OTA, a C18 reversed-phase column (150×4.6 mm i.d., 5 µm  
214 particle size Kromasil C18 (Análisis Vínicos S.L., Spain), connected to a  
215 precolumn Kromasil C18 (10×4.6 mm i.d., 5 µm particle sizes, Análisis Vínicos  
216 S.L.) were used. For chromatographic separation of OTA, the mobile phase was  
217 acetonitrile: water: acetic acid, (57:41:2 v/v/v) under isocratic elution during 10  
218 min, at a flow rate of 1 mL min<sup>-1</sup>. OTA was determined by fluorescence detection  
219 at an excitation wavelength of 330 nm and an emission wavelength of 460 nm.  
220 The OTA standard was obtained from *A. ochraceus* (Sigma-Aldrich, USA). The  
221 assays were performed in triplicate.

222

## 223 **Measurement of ROS**

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225       Oxidation of the fluorogenic probe (2'-7'-dichloro-dihydrofluorescein diacetate,  
226 DCFDA) by ROS was monitored as described by Narasaiah *et al.*<sup>9</sup>. Mycelia (5  
227 mg) were collected from 48 h-old cultures on CYA and incubated with DCFDA (10  
228  $\mu$ M) for 30 min at 30 °C in the dark. Release of the fluorescent dichlorofluorescein  
229 ( $\lambda$  ex=490 nm,  $\lambda$  em=520 nm) was monitored using a Fluorescence Polarization  
230 microplate reader (PolarStar Omega, BMG LABTECH GmbH). The assays were  
231 performed in triplicate.

232

## 233 **Statistical analyses**

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235       All comparisons were analyzed by One way ANOVA followed by the Least  
236 significant different test (LSD), using Statgraphics Centurion Version XVI.  
237 Significance was defined as  $p < 0.05$ .

238

## 239 **RESULTS AND DISCUSSION**

240

### 241 **Effect of menadione on growth, OTA production level, ROS** 242 **generation, and gene expression of antioxidant enzymes**

243

244       As shown in Figure 1A, the growth rate decreased significantly as the  
245 concentration of menadione increased, reaching a maximum reduction of 52%  
246 when the concentration of menadione was 100  $\mu$ M. Interestingly, a statistically

247 significant positive correlation between menadione concentration, ROS  
248 generation, and OTA production level was found (Figure 1B). It is also worth  
249 noting that in spite of the reduction on fungal growth due to menadione, OTA  
250 production level significantly increased. All these data indicate that when *A.*  
251 *carbonarius* is under oxidative stress caused by superoxide radicals generated by  
252 menadione, OTA production level increases, highlighting the close relationship  
253 between oxidative stress and OTA production. These results disagree with those  
254 of Reverberi *et al.*<sup>30</sup>, who found that OTA production level by *A. ochraceus* was  
255 inhibited in the presence of 100  $\mu$ M menadione. These contradictory results could  
256 suggest that different OTA-producing species might regulate OTA production  
257 differently in response to oxidants/antioxidants, as pointed out by Palumbo *et al.*  
258<sup>17</sup>. However, our results are congruent with several previous studies in which  
259 oxidative stress was correlated with increased levels of mycotoxin biosynthesis in  
260 different mycotoxigenic fungi. These include the accumulation of deoxynivalenol  
261 by *F. graminearum*<sup>31-32</sup> and aflatoxins by *A. parasiticus* and *A. flavus*<sup>8, 33</sup>.

262 SOD and PRX are two enzymes involved in the antioxidant response.  
263 Expression of *sod* and *prx* genes was analysed in cultures with increasing  
264 quantities of menadione (0, 20 and 60  $\mu$ M). Surprisingly, although menadione  
265 increased ROS generation, it did not induce expression of *sod* and *prx* genes  
266 (Figure 1C). Indeed, the down regulation of the *sod* and *prx* genes was stronger  
267 when the menadione concentration was higher (60  $\mu$ M). These results suggest  
268 that oxidative stress produced by menadione might cause an inhibition of the  
269 fungal antioxidant response. However, based on our results it is difficult to  
270 speculate on the mechanisms by which menadione oxidative stress affects the  
271 expression of *sod* and *prx* genes.

272

273 **Effect of antioxidant agents on growth, OTA production level,**  
274 **ROS generation, and gene expression of antioxidant enzymes**

275

276 When CYA cultures were amended with BHT, the growth rate decreased  
277 with statistically significant differences for 5 and 10 mM, reaching a maximum  
278 reduction of 48% for 10 mM (Figure 2A). This result can be explained due to the  
279 BHT toxicity, which has been widely discussed<sup>34</sup>. It has been suggested that BHT  
280 toxicity is concentration-dependent as it can protect against osmotic fragility or, on  
281 the contrary, alter the physical properties of the cell wall increasing its permeability  
282<sup>35</sup>.

283 In the case of catechin and resveratrol, two natural antioxidants present in  
284 grapes, concentrations were selected to be representative of the levels of these  
285 compounds in grapes. In comparison to the effect of BHT, the growth of *A.*  
286 *carbonarius* was only barely affected by the catechin, resveratrol and polyphenol  
287 extract concentrations assayed in this study (Figures 2B, C and D). Nevertheless,  
288 it is worth noting that a statistically significant decrease of growth rate was  
289 detected for the highest resveratrol concentration tested (1500 µM) (Figure 2C).  
290 This is in agreement with previous studies where a clear reduction of *A.*  
291 *carbonarius* growth was shown using resveratrol<sup>36</sup>. Results also agree with the  
292 confirmed role played by resveratrol as a phytoalexin in grapes<sup>22</sup>. Furthermore,  
293 Galati *et al.*<sup>37</sup> observed that some polyphenols, among which resveratrol is  
294 included, may be metabolized by peroxidase to form prooxidant phenoxyl radicals  
295 that might generate ROS, H<sub>2</sub>O<sub>2</sub> and semiquinone or quinones, all of which are

296 potentially cytotoxic<sup>38-39</sup>. This prooxidant character would explain the growth  
297 inhibition effect of resveratrol found at the highest concentration tested (1500 µM).

298 The influence of the above mentioned antioxidants on OTA production was  
299 also tested (Figure 3). When BHT was added to culture media, a statistically  
300 significant increase in OTA production level was found for concentrations higher  
301 than 2.5 mM (Figure 3A). Nevertheless, as BHT concentrations increased, OTA  
302 production level decreased respect to the maximum obtained at 2.5 mM.  
303 Therefore, although BHT increased OTA production level, this increase is not  
304 directly proportional to BHT concentration. Figure 3A also shows the effect of BHT  
305 on ROS generation. Similarly to data from OTA production, an increase in ROS  
306 generation relative to the control was observed when BHT concentration was  
307 increased. This increase in ROS generation was not proportional to the increase in  
308 BHT concentration, as was observed in the OTA production level results. Thus, a  
309 correlation between OTA production and ROS generation was observed. These  
310 results are generally congruent with those reported by Barberis *et al.*<sup>18</sup> who  
311 observed a growth rate reduction and an increase of OTA production level by *A.*  
312 *carbonarius* when butylated hydroxyanisole (BHA), a chemical compound  
313 structurally similar to BHT, was added to the culture media. As it has previously  
314 been mentioned, these results can be explained because of the cytotoxic effect of  
315 BHT. In fact, a number of studies using different cell types have shown that BHA  
316 can be cytotoxic<sup>40</sup>. These studies also showed that BHA produced inhibition of  
317 mitochondrial activity and induction of ROS. As it was concluded for BHA by  
318 Barberis *et al.*<sup>18</sup>, these results suggest that BHT is not appropriate to control *A.*  
319 *carbonarius*.

320 Catechin, resveratrol and the polyphenol extract produced widely different  
321 effects on OTA production level depending on the concentrations assayed,  
322 ranging from a reduction of 22% (35  $\mu$ M of polyphenol extract ) to an increase of  
323 61% (1500  $\mu$ M of resveratrol) relative to the control treatment (Figures 3B, C and  
324 D). Both antioxidant and prooxidant capacity of a compound depend on several  
325 variables such as its chemical structure, concentration or mode of action. It must  
326 be noticed that phenolic antioxidants have been shown to undergo loss of activity  
327 and become prooxidants at high concentrations <sup>41</sup>. There is little information about  
328 the effect of catechin on OTA production by *A. carbonarius* (Figure 3B).  
329 Nevertheless, these results partially agree with those of Palumbo *et al.* <sup>17</sup>, where  
330 no OTA inhibition was observed in *A. carbonarius* when a higher amount of  
331 catechin (10 mM) was added to the culture medium. In the case of resveratrol  
332 (Figure 3C), several studies have been previously conducted to analyze its effect  
333 on OTA production by *A. carbonarius* yielding to contradictory results. Whereas  
334 Bavaresco *et al.* <sup>36</sup> reported that the presence of resveratrol triggered OTA  
335 production level in synthetic must medium, De Rossi *et al.* <sup>22</sup> found that when *A.*  
336 *carbonarius* was treated with resveratrol at 0.1 mM, a significant inhibition of OTA  
337 production level was evident under *in vitro* conditions. Resveratrol is a phytoalexin  
338 produced by grapes in response to fungal invasion. Therefore, a high level of  
339 infection would lead to a greater production of resveratrol, which in turn, according  
340 to our results, could induce a greater biosynthesis of OTA. The positive correlation  
341 between high levels of OTA and the content of resveratrol in the wine has been  
342 reported previously by Perrone *et al.* <sup>42</sup>. In contrast to catechin and resveratrol, the  
343 polyphenol extract was able to reduce significantly the content of OTA (22%) at  
344 the highest concentration assayed (35 ppm) (Figure 3D). These data may suggest

345 that other phenolic antioxidants present in the polyphenolic extract, and different  
346 from those used in this study could be more effective in reducing OTA production  
347 level by *A. carbonarius*. Additional studies to accurately determine the phenolic  
348 antioxidant compounds responsible for the significative reduction in OTA  
349 production would be needed. Moreover, further investigations regarding  
350 bioavailability and fungal response to these phenolic antioxidants under ecological  
351 conditions would be of interest.

352 The inhibitory effect of the antioxidant compounds on mycotoxin production  
353 was quite small in comparison with data obtained in previous studies <sup>15, 17</sup>.  
354 However, the antioxidant concentrations used in those studies (10-12 mM) were  
355 meaningfully bigger than the ones used in the present work. Gallic and tannic  
356 acids almost completely reduced aflatoxin production by *A. flavus* at 12 mM and 2  
357 mM, respectively <sup>43</sup>. Similarly, to inhibit aflatoxin biosynthesis by *A. parasiticus*,  
358 anthocyanidin treatments with concentrations higher than 4 mM were needed,  
359 while natural concentrations occur on the order of micromolar <sup>44</sup>. Catechin and  
360 resveratrol concentrations assayed in this study were chosen based on the levels  
361 normally present in grapes. We intended to analyze whether they could have  
362 some effect on OTA production at natural concentrations.

363 Regarding ROS production, when catechin and resveratrol were used, none  
364 of the antioxidant concentrations tested significantly reduced ROS generation,  
365 which correlates with the fact that no reduction of OTA was observed (Figure 3B,  
366 C). However, the polyphenol extract showed a statistically significant antioxidant  
367 effect when the culture medium was supplemented with 20 and 35 ppm of  
368 polyphenol extract, as ROS generated were lower compared to control (Figure  
369 3D). Nevertheless, due to the OTA production data at 20 ppm, it was not possible

370 to establish a total correlation between OTA production level and ROS generation.  
371 It would be interesting to carry out a deeper study to determine whether the OTA  
372 reduction observed at 35 ppm is a consequence of the antioxidant effect of the  
373 polyphenolic extract.

374 Figure 4 shows the effect of the antioxidant compounds on the expression  
375 of *sod* and *prx*. Analyses were carried out using two different concentrations, low  
376 and high, of each antioxidant. Additionally, data from OTA production level were  
377 also considered to choose the working concentration. In the case of BHT,  
378 expression of the *sod* and *prx* was down regulated and this down regulation was  
379 higher at 10 mM than at 2.5 mM of BHT. Within both genes studied, down  
380 regulation was higher in the case of *sod*, which changed from 2.2 to 5.4 fold when  
381 the BHT concentration was increased from 2.5 to 10 mM, whereas the expression  
382 of *prx* changed from 2.6 to 3.4 fold. Additionally, when catechin was added to  
383 culture media an important change on the expression of *sod* and *prx* was  
384 observed depending on the catechin concentration used (Figure 4B). Whereas  
385 expression was barely affected at 25  $\mu$ M, it was strongly down regulated at 1500  
386  $\mu$ M (10.7 and 8.5 fold for *sod* and *prx*, respectively). In contrast, the effect of  
387 resveratrol on the expression of *sod* and *prx* was quite different (Figure 4C).  
388 Interestingly, the expression of *sod* and *prx* was up regulated when cultures were  
389 supplemented with a resveratrol concentration that increases OTA production level  
390 (1500  $\mu$ M). This result indicates that these oxidative enzymes and especially SOD,  
391 which was particularly up regulated, participate in the fungal response to oxidative  
392 stress induced by high resveratrol conditions. Since a direct correlation between  
393 higher resveratrol concentrations and higher OTA contamination in the wine has  
394 been reported <sup>42</sup>, this observation suggest an special importance of SOD for the



395 survival of OTA-producing strains of *A. carbonarius* in the grape stressed  
396 environment. Finally, gene expression of *sod* and *prx* was studied on *A.*  
397 *carbonarius* cultures supplemented with 20 and 35 ppm of polyphenol extract and  
398 incubated for 48 hours (Figure 4D). Both concentrations led to a reduction of the  
399 expression of *sod* and *prx*, but this down regulation was greater at 20 ppm, in  
400 parallel with an increase of OTA production level. If we compare data from OTA  
401 production and gene expression, two different patterns in the expression of *sod*  
402 and *prx* are apparent, suggesting that *A. carbonarius* may have two different  
403 mechanisms of oxidative stress response. When resveratrol exhibited a prooxidant  
404 behavior that increased OTA production level, the expression of *sod* and *prx* was  
405 up regulated, suggesting that SOD and PRX enzymes participate in the cellular  
406 response to oxidative stress. In contrast, prooxidant concentrations of catechin  
407 and the polyphenol extract behaved like menadione. When these compounds had  
408 a prooxidant effect which enhanced OTA production level, the expression of genes  
409 was down regulated.

410

## 411 **CONCLUSION**

412 *A. carbonarius* responds to oxidative stress caused by high concentrations  
413 of menadione or BHT, increasing OTA production. Naturally occurring  
414 concentrations of grape phenolic compounds did not reduce OTA production in our  
415 study. Therefore, the use of naturally occurring concentrations of these  
416 compounds to reduce oxidative stress and thereby reduce ochratoxigenesis is not  
417 adequate as a sole tool for OTA contamination control in grapes.

418

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561

## 562 **Figure captions**

563

564 **Figure 1:** Effect of menadione on *A. carbonarius* W04-40. (A) Growth rate (cm  
565 day<sup>-1</sup>) of *A. carbonarius* W04-40 grown in presence of different concentrations of  
566 menadione at 28 °C. Letters indicate homogeneous groups (ANOVA, p < 0.05).  
567 (B) OTA production by *A. carbonarius* grown in CYA plates with increasing

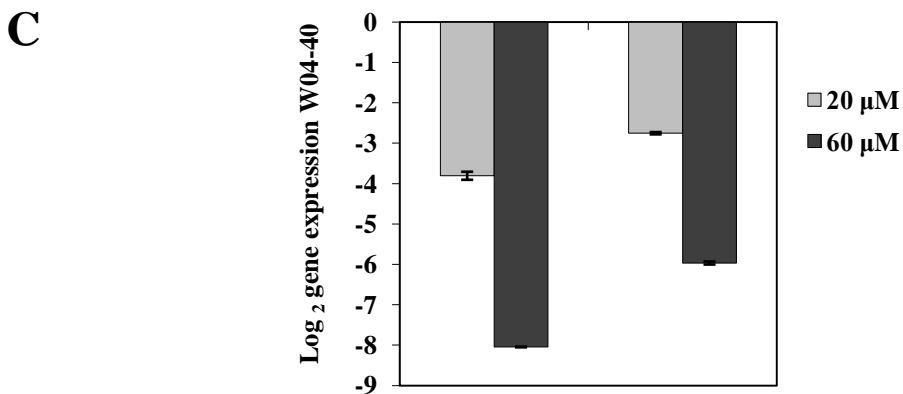
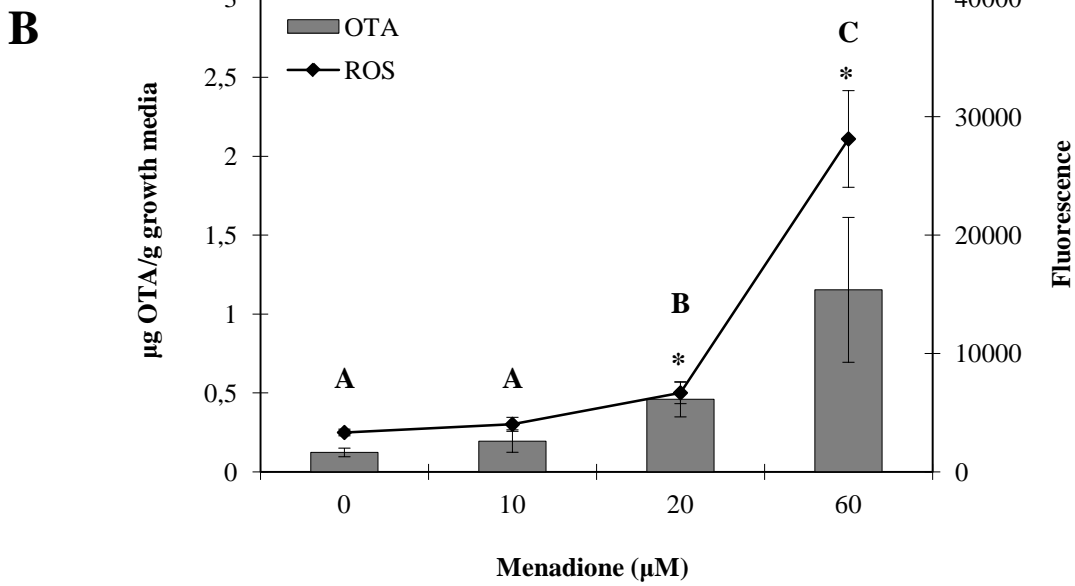
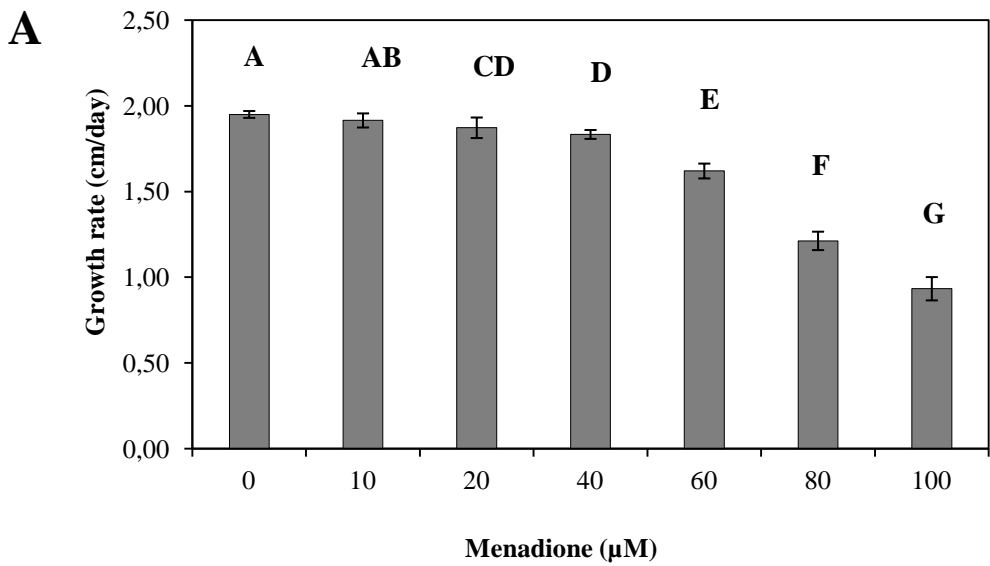
568 concentrations of menadione (left y-axis). Fluorescence values, proportional to  
569 ROS, monitored by the probe DCFDA (right y-axis) after 48 h. Letters indicate  
570 homogeneous groups for fluorescence and (\*) indicates statistically significant  
571 difference compared to the control condition for OTA production (ANOVA,  $p <$   
572 0.05). (C) Relative expression of *sod* and *prx* in *A. carbonarius* W04-40 grown for  
573 48 h in the presence of different concentrations of menadione with respect to  
574 expression level in the same medium without menadione. Error bars indicate  
575 standard errors.

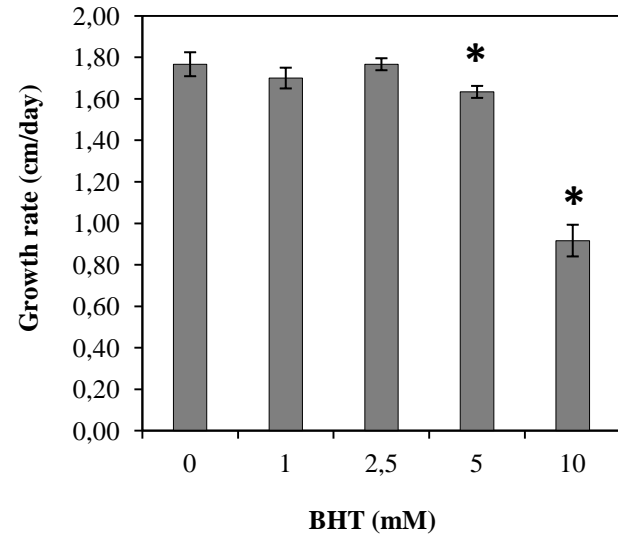
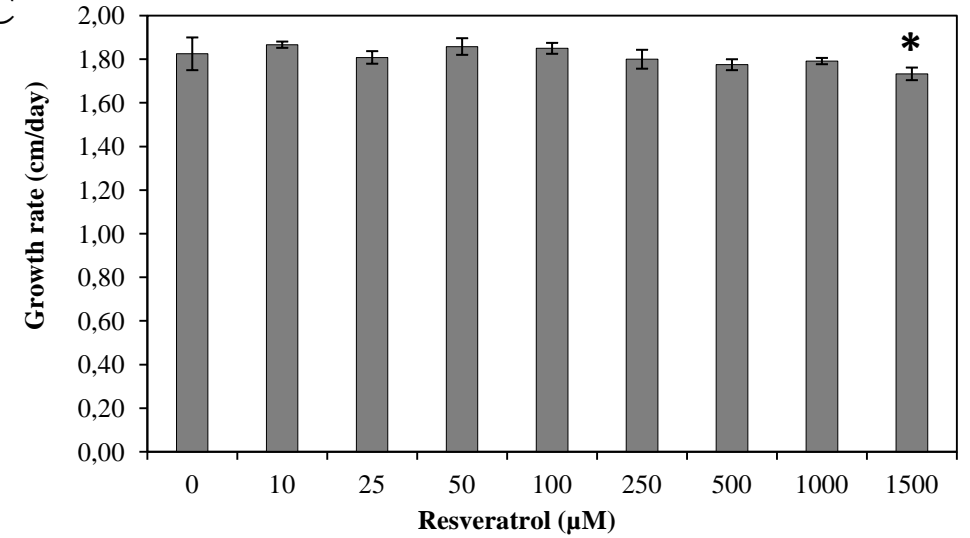
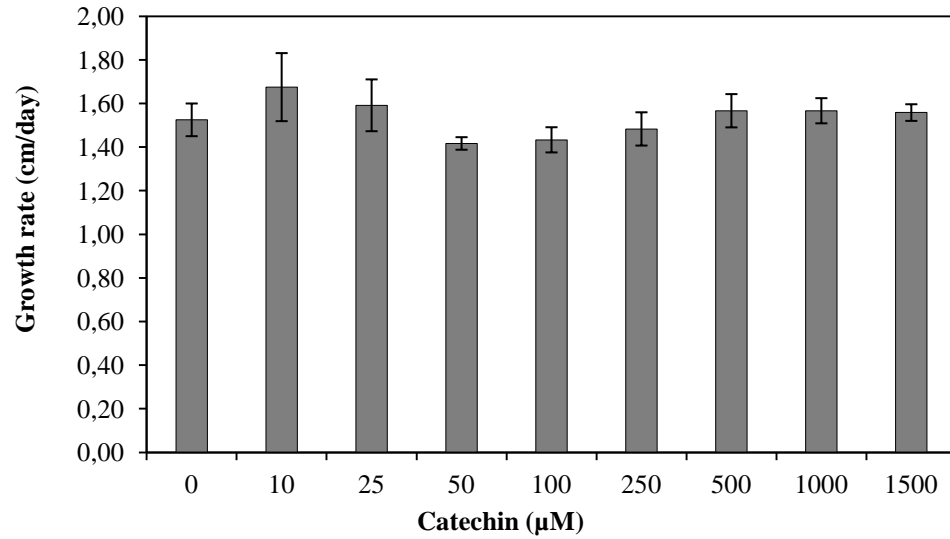
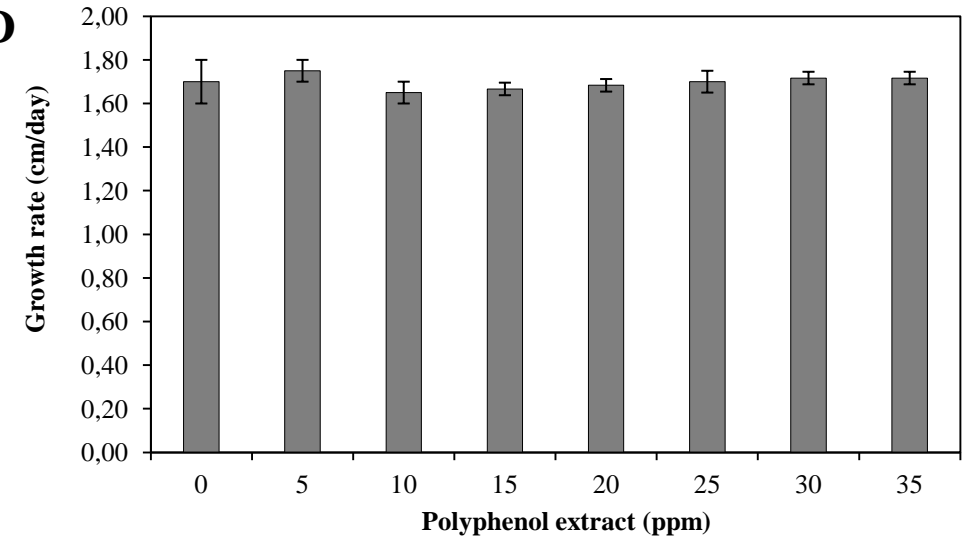
576 **Figure 2:** Growth rate ( $\text{cm day}^{-1}$ ) of *A. carbonarius* W04-40 grown in increasing  
577 concentrations of BHT (A), catechin (B), resveratrol (C) and polyphenol extract (D)  
578 at 30 °C. (\*) indicates statistically significant difference (ANOVA,  $p <$  0.05).

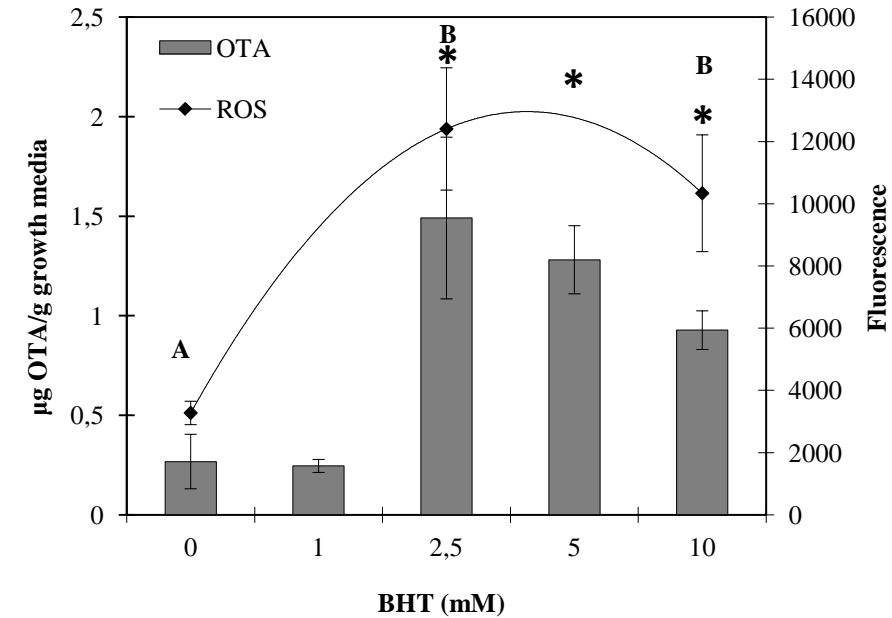
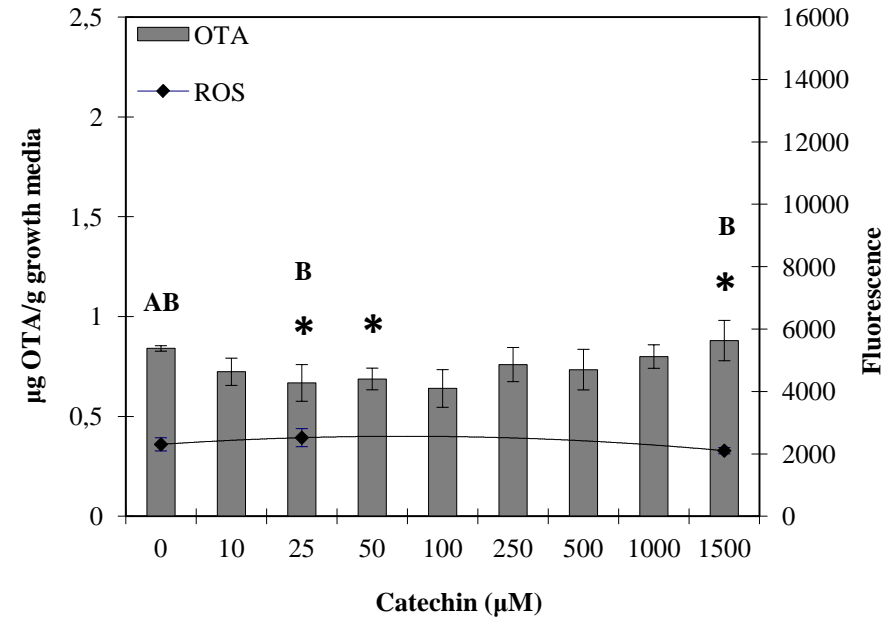
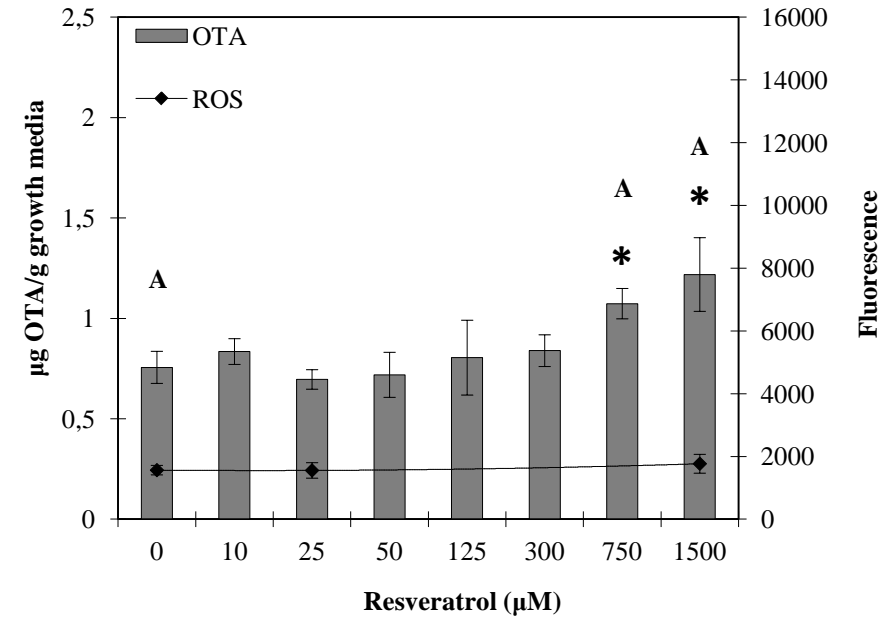
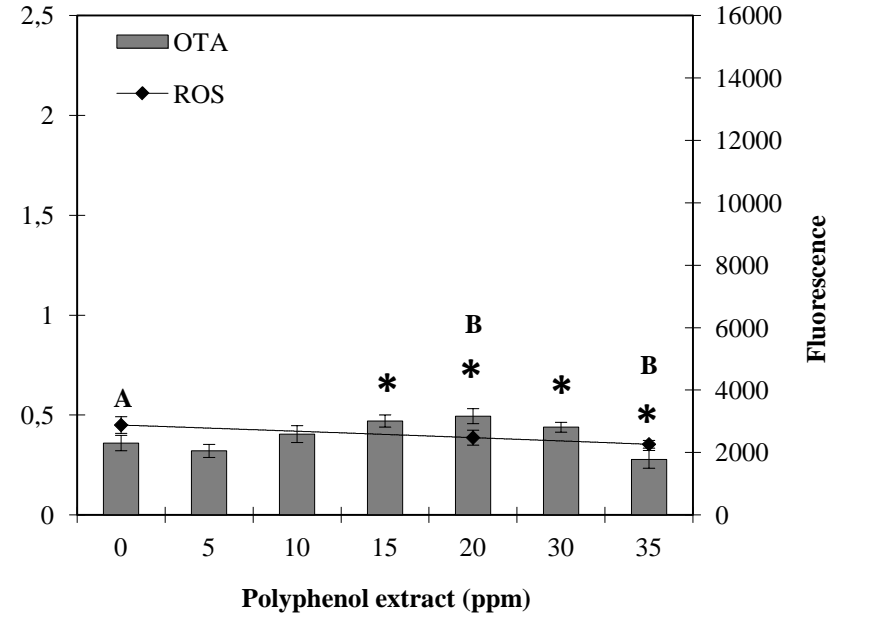
579 **Figure 3:** Effect of antioxidant agents on *A. carbonarius* W04-40. OTA production  
580 by *A. carbonarius* grown in CYA plates with increasing concentrations of  
581 antioxidant agents (left y-axis). Fluorescence values, proportional to ROS,  
582 monitored by the probe DCFDA (right y-axis) after 48 h. Letters indicate  
583 homogeneous groups for fluorescence and (\*) indicates statistically significant  
584 difference compared to the control condition for OTA production (ANOVA,  $p <$   
585 0.05). BHT (A), catechin (B), resveratrol (C) and polyphenol extract (D)

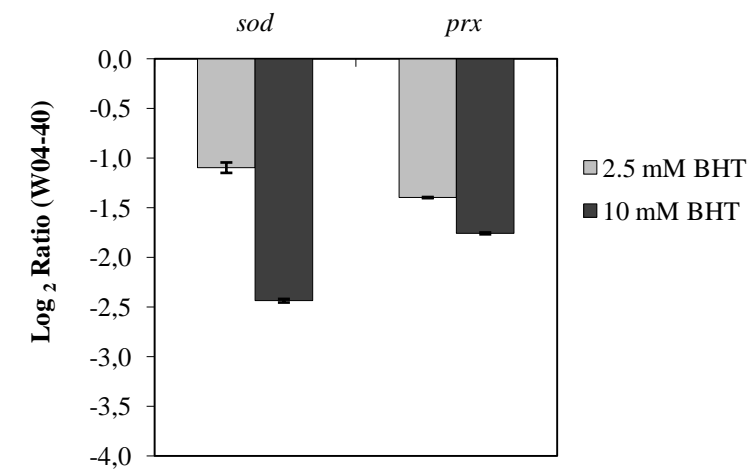
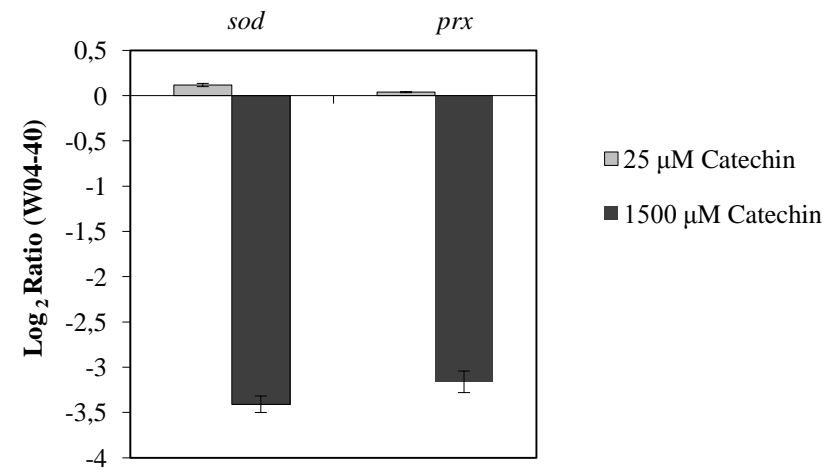
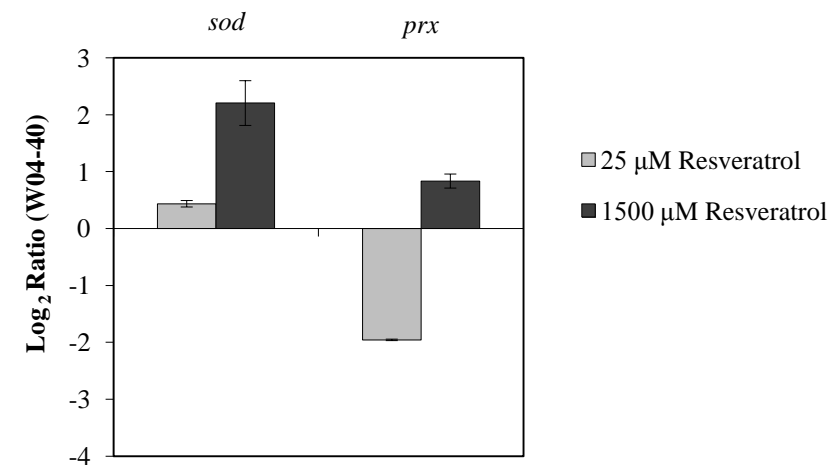
586 **Figure 4:** Relative expression of *sod* and *prx* in *A. carbonarius* W04-40 grown for  
587 48h in the presence of BHT (A), catechin (B), resveratrol (C) and polyphenol  
588 extract (D) with respect to the expression level in the same medium without the  
589 antioxidants. Error bars indicate standard errors.





**A****C****B****D**

**A****B****C****D**

**A****B****C****D**