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

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Abstract

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

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

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

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Fungi and culture conditions

The OTA-producing *A. carbonarius* strain W04-40 was isolated from a Spanish vineyard by Martínez-Culebras and Ramón [23] and deposited in the Institute of Agrochemistry and Food Technology of the Spanish National Research Council (IATA-CSIC).

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

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

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

Extraction of phenolic compounds from grapes

The extraction of phenolic compounds from red grapes (Bobal variety) was performed just from the grape skins. Grapes were peeled and skins washed with distilled water. Then, skins were dried at 80ºC for three hours. Eighty grams of dried skins were frozen and ground with liquid nitrogen, and 600 mL of methanol-water (80:20) were added for the extraction of phenolic compounds. After homogenization with a polytron PT 45/80 (Kinematica AG, Switzerland)) for 30 seconds, four times, the extract was incubated at 45°C for 10 min. Then, the homogenate was centrifuged at 5000 ×*g* for 30 min. The supernatant was recovered and the residue was re-extracted with another 600 mL of methanol-water (80:20). Methanol was evaporated in a rotary evaporator under vacuum and the concentrate was lyophilized. Finally, total phenolic compounds were determined by the Folin−Ciocalteu reagent [24].

RNA isolation and cDNA synthesis

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

Quantification of relative gene expression by real-time RT-PCR

Gene expression of two genes related to oxidative stress response, a superoxide dismutase (*sod*) and a peroxiredoxin (*prx*) from *A. carbonarius*, were analyzed using gene specific primer pairs, SODf//SODr (5'-CCCGGAACTGACCCTATGC-3' and 5'-AGGGCTTGAGGGCAATCTG-3') and PRXf/PRXr (5'-TCCTTCTTGAGGTTGGTGAAGC-3' and 5'-CTCAGAAGAAGTTCGGCGATG-3') respectively, designed in a previous work [25]. Real-time RT-PCR reactions were performed in a LightCycler 480 System (Roche, USA) using SYBR Green to monitor cDNA amplification. The ribosomal 18S RNA gene was used as a reference gene (forward 5'-GCAAATTACCCAATCCCGACAC-3' and reverse primer 5'-GAATTACCGCGGCTGCTG-3'). Amplifications were carried out in a final volume of 210 µL containing 2 µL cDNA template, 2 µL LightCycler® 480 SYBR Green I Master (Roche, USA), 0.5 µL of each primer (10 µM), and 5 µL H2O, following the manufacturer's instructions. PCR amplifications were performed in triplicate using the following conditions: 10 min at 94 °C, followed by 45 cycles of 10 s at 94 °C, 10 s at 56 °C and 10 s at 72 °C. The corresponding real-time PCR efficiency (E) in the exponential phase was calculated according to the equation: $E = 10[-1/slope]$. The relative expression of the target genes was calculated based on the E and the Cp value of sample versus a control and expressed in comparison to the ribosomal 18S RNA (reference gene), according to the following equation [26-27]: $ratio = (E_{target})^{\Delta CPtarget(control - sample)} / (E_{ref})^{\Delta CPref(control - sample)}$. Three technical replicates were done for each combination of cDNA and primer pair, and PCR reaction quality was checked by analyzing the dissociation and amplification

curves. To calculate the normalized relative gene expression levels (fold induction), data were analyzed using the Relative Expression Software Tool (REST) and the mathematical model based on mean threshold cycle differences between the sample and the control group [28].

Extraction and detection of OTA from culture

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

Measurement of ROS

Oxidation of the fluorogenic probe (2'-7'-dichlo-dihydrofluorescein diacetate, DCFDA) by ROS was monitored as described by Narasaiah *et al*. [9]. Mycelia (5 mg) were collected from 48 h-old cultures on CYA and incubated with DCFDA (10 µM) for 30 min at 30 ºC in the dark. Release of the fluorescent dichlorofluorescein (λ ex=490 nm, λ em=520 nm) was monitored using a Fluorescence Polarization microplate reader (PolarStar Omega, BMG LABTECH GmbH). The assays were performed in triplicate.

Statistical analyses

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

RESULTS AND DISCUSSION

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

As shown in Figure 1A, the growth rate decreased significantly as the concentration of menadione increased, reaching a maximum reduction of 52% when the concentration of menadione was 100 µM. 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*. [30], who found that OTA production level by *A. ochraceus* was
255 inhibited in the presence of 100 µM 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 [17]. 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* [31-32] and aflatoxins by *A. parasiticus* and *A. flavus* [8, 33].

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 µM). 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 µM). 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.

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

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

In the case of catechin and resveratrol, two natural antioxidants present in grapes, concentrations were selected to be representative of the levels of these compounds in grapes. In comparison to the effect of BHT, the growth of *A. carbonarius* was only barely affected by the catechin, resveratrol and polyphenol extract concentrations assayed in this study (Figures 2B, C and D). Nevertheless, it is worth noting that a statistically significant decrease of growth rate was detected for the highest resveratrol concentration tested (1500 µM) (Figure 2C). This is in agreement with previous studies where a clear reduction of *A. carbonarius* growth was shown using resveratrol [36]. Results also agree with the confirmed role played by resveratrol as a phytoalexin in grapes [22]. Furthermore, Galati *et al*. [37] observed that some polyphenols, among which resveratrol is included, may be metabolized by peroxidase to form prooxidant phenoxyl radicals that might generate ROS, H_2O_2 and semiquinone or quinones, all of which are

potentially cytotoxic [38-39]. This prooxidant character would explain the growth inhibition effect of resveratrol found at the highest concentration tested (1500 µM).

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

Catechin, resveratrol and the polyphenol extract produced widely different effects on OTA production level depending on the concentrations assayed, ranging from a reduction of 22% (35 µM of polyphenol extract) to an increase of 61% (1500 µM of resveratrol) relative to the control treatment (Figures 3B, C and D). Both antioxidant and prooxidant capacity of a compound depend on several variables such as its chemical structure, concentration or mode of action. It must be noticed that phenolic antioxidants have been shown to undergo loss of activity and become prooxidants at high concentrations [41]. There is little information about the effect of catechin on OTA production by *A. carbonarius* (Figure 3B). Nevertheless, these results partially agree with those of Palumbo *et al.* [17], where no OTA inhibition was observed in *A. carbonarius* when a higher amount of catechin (10 mM) was added to the culture medium. In the case of resveratrol (Figure 3C), several studies have been previously conducted to analyze its effect on OTA production by *A. carbonarius* yielding to contradictory results. Whereas Bavaresco *et al*. [36] reported that the presence of resveratrol triggered OTA production level in synthetic must medium, De Rossi *et al*. [22] found that when *A. carbonarius* was treated with resveratrol at 0.1 mM, a significant inhibition of OTA production level was evident under *in vitro* conditions. Resveratrol is a phytoalexin produced by grapes in response to fungal invasion. Therefore, a high level of infection would lead to a greater production of resveratrol, which in turn, according to our results, could induce a greater biosynthesis of OTA. The positive correlation between high levels of OTA and the content of resveratrol in the wine has been reported previously by Perrone *et al*. [42]. In contrast to catechin and resveratrol, the polyphenol extract was able to reduce significantly the content of OTA (22%) at 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 [15, 17].
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 [43]. 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 [44] . 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 µM, it was strongly down regulated at 1500
386 µM (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 µM). 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 [42], this observation suggest an special importance of SOD for the

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

CONCLUSION

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

ACKNOWLEDGEMENTS

A. Crespo-Sempere was recipient of a FPI fellowship from the Spanish Government. This research was supported by grants AGL2005-00707 and AGL2008-04828-C03-02 from the Spanish Government.

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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^{-1}) of *A. carbonarius* W04-40 grown in presence of different concentrations of
566 menadione at 28 ºC. Letters indicate homogeneous groups (ANOVA, $p < 0.05$).
567 (B) OTA production by *A. carbonarius* grown in CYA plates with increasing

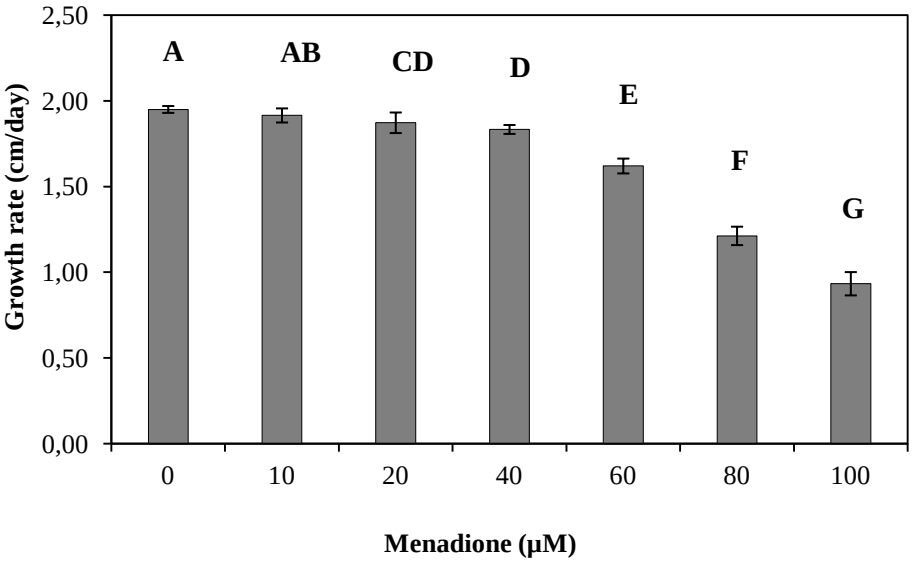
concentrations of menadione (left y-axis). Fluorescence values, proportional to ROS, monitored by the probe DCFDA (right y-axis) after 48 h. Letters indicate homogeneous groups for fluorescence and (*) indicates statistically significant difference compared to the control condition for OTA production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

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

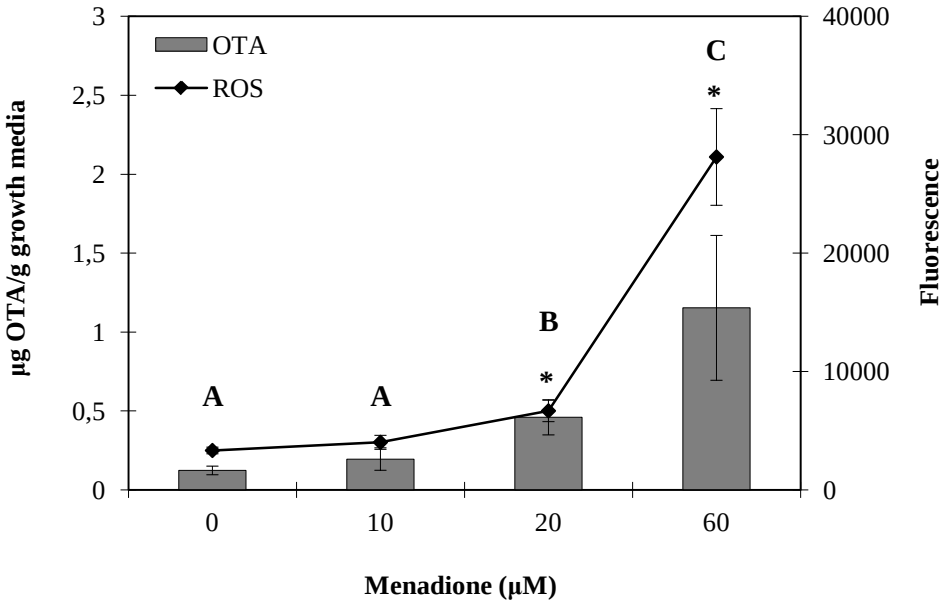
Figure 3: Effect of antioxidant agents on *A. carbonarius* W04-40. OTA production by *A. carbonarius* grown in CYA plates with increasing concentrations of antioxidant agents (left y-axis). Fluorescence values, proportional to ROS, monitored by the probe DCFDA (right y-axis) after 48 h. Letters indicate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

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

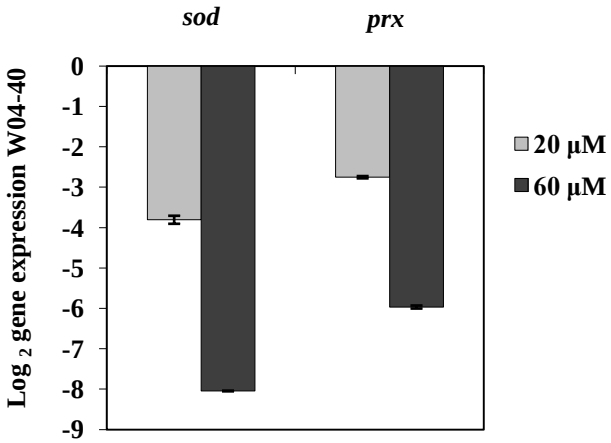
A


2,50
2,00
1,50
1,00
0,50
0,00
Growth rate (cm/day)
A
AB
CD
D
E
F
G
0
10
20
40
60
80
100
Menadione (µM)

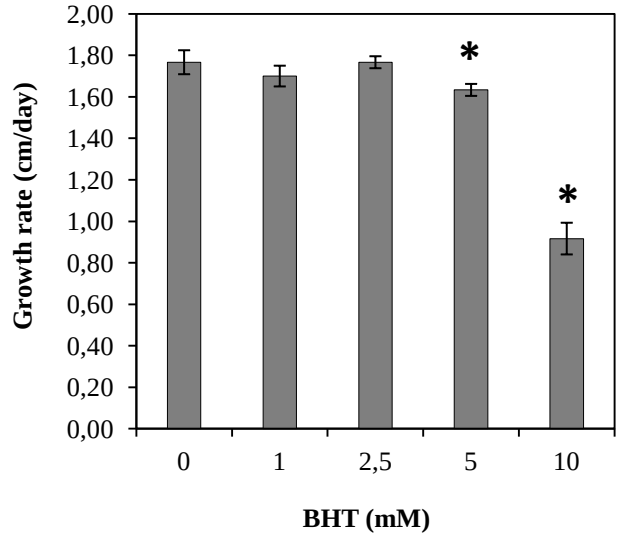
B


3
2,5
2
1,5
1
0,5
0
µg OTA/g growth media
OTA
ROS
40000
30000
20000
10000
0
Fluorescence
A
A
B
C
*
*
0
10
20
60
Menadione (µM)

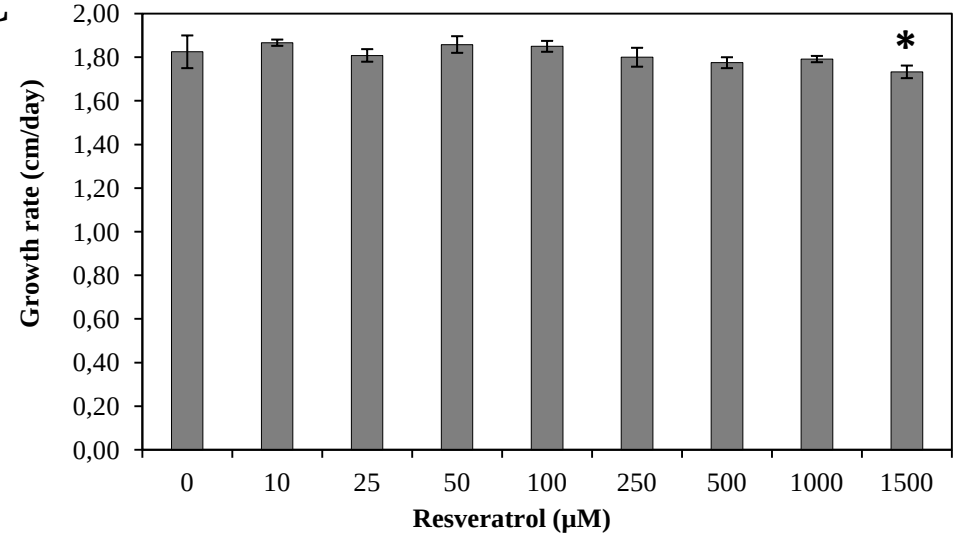
C


sod
prx
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
Log 2 gene expression W04-40
20 µM
60 µM

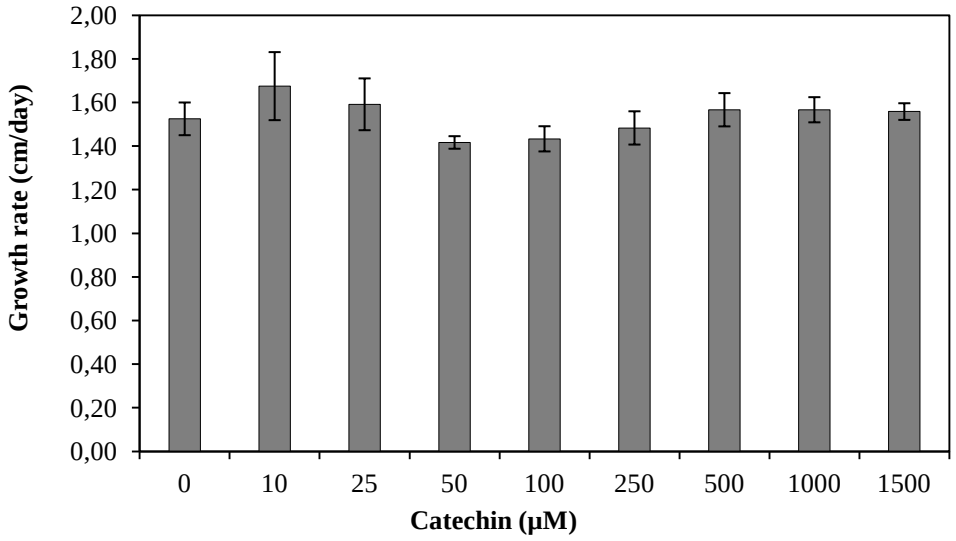
A


Growth rate (cm/day)
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
*
*
0
1
2,5
5
10
BHT (mM)

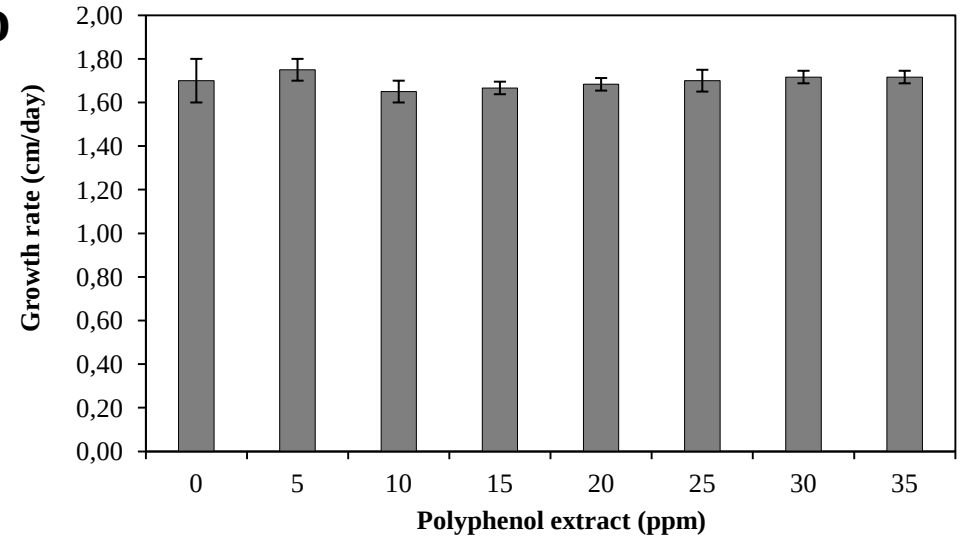
C

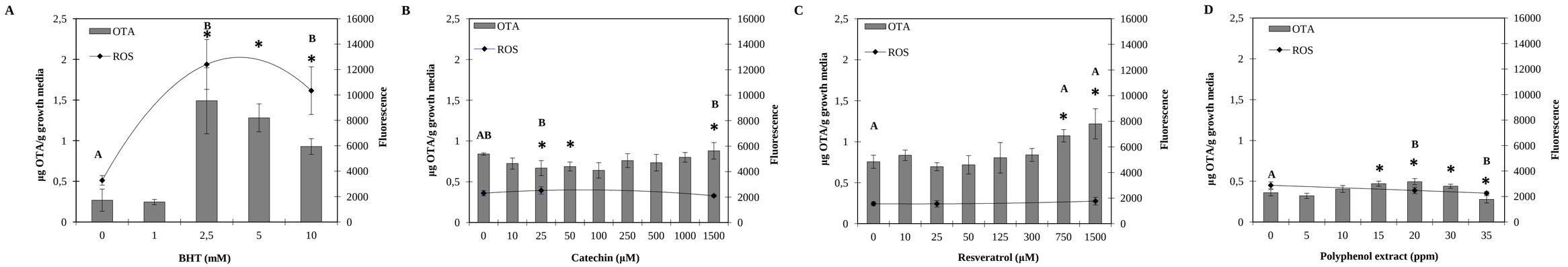

Growth rate (cm/day)
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
*
0
10
25
50
100
250
500
1000
1500
Resveratrol (µM)

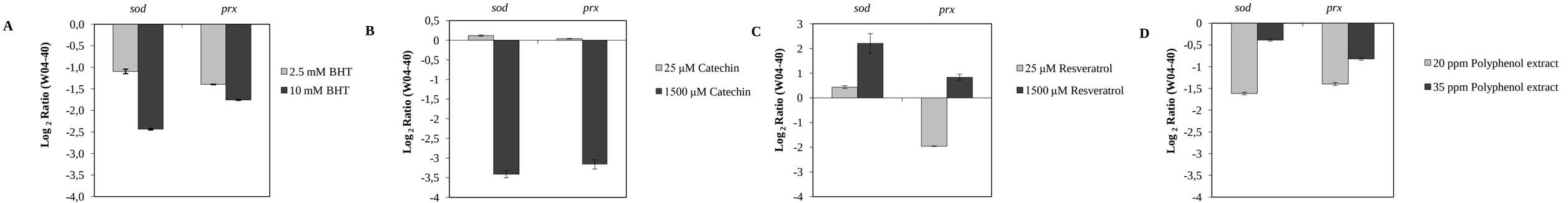
B


Growth rate (cm/day)
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0
10
25
50
100
250
500
1000
1500
Catechin (µM)

D


Growth rate (cm/day)
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0
5
10
15
20
25
30
35
Polyphenol extract (ppm)


A
B
C
D
OTA
ROS
µg OTA/g growth media
Fluorescence
BHT (mM)
Catechin (µM)
Resveratrol (µM)
Polyphenol extract (ppm)


A
sod
prx
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
-3,0
-3,5
-4,0
Log 2 Ratio (W04-40)
2.5 mM BHT
10 mM BHT
B
sod
prx
0,5
0
-0,5
-1
-1,5
-2
-2,5
-3
-3,5
-4
Log 2 Ratio (W04-40)
25 µM Catechin
1500 µM Catechin
C
sod
prx
3
2
1
0
-1
-2
-3
-4
Log2 Ratio (W04-40)
25 µM Resveratrol
1500 µM Resveratrol
D
sod
prx
0
-0,5
-1
-1,5
-2
-2,5
-3
-3,5
-4
Log 2 Ratio (W04-40)
20 ppm Polyphenol extract
35 ppm Polyphenol extract